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## Buccal Mucosa Cells as *In vivo* Model to Evaluate Gefitinib Activity in Patients with Advanced Non – Small Cell Lung Cancer

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**Abstract Purpose:** To evaluate the role of pretreatment and posttreatment expression in buccal mucosa cells of signal transduction proteins activated by epidermal growth factor receptor, including phosphorylated epidermal growth factor receptor (p-EGFR), phosphorylated mitogen-activated protein kinase (p-MAPK), and phosphorylated AKT (p-AKT), in predicting gefitinib activity in advanced non – small cell lung cancer patients. Expression of the same proteins was also assessed on corresponding tissue samples for comparison. Moreover, *EGFR* gene mutations and copy number were analyzed.

**Experimental Design:** Protein expression was evaluated by standard immunocytochemistry in buccal smears, obtained by scraping immediately before and after 2 weeks of gefitinib treatment, and in the available archival tumor specimens. *EGFR* gene mutations were evaluated by direct sequencing and gene copy number was determined by fluorescence *in situ* hybridization. Data were correlated with gefitinib toxicity and objective response.

**Results:** Fifty-eight patients with pretreated advanced non – small cell lung cancer were enrolled and nine of these patients (15%) showed an objective response to gefitinib (including two complete responses). Toxicity ( $P = 0.025$ ) and baseline p-AKT expression in buccal mucosa cells ( $P = 0.061$ ) showed a potential predictive role. On the contrary, the probability of achieving an objective response was not affected by pretreatment expression of EGFR, p-EGFR, and p-MAPK, either in buccal mucosa or in tumor tissue. Responders showed a nonstatistically significant trend toward a more pronounced reduction in the expression of p-EGFR, p-MAPK, and p-AKT after gefitinib treatment. Among responders, five of six (83%) tumors showed *EGFR* gene mutation, whereas none of the tumors from patients with stable or progressive disease did ( $P < 0.001$ ).

**Conclusions:** Epithelial cells obtained from buccal mucosa may be used to assess the pharmacodynamic effect of EGFR-targeted agents, and pretreatment p-AKT expression may be a possible predictive biomarker of *in vivo* gefitinib activity.

Epidermal growth factor receptor (EGFR) is frequently over-expressed in non – small cell lung cancer (NSCLC). Its activation plays an important role in the cascade of intracellular signaling events that regulate tumor cell proliferation, migration, resistance to apoptosis, and angiogenesis. EGFR phosphorylation is associated with activation of Ras – mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) – AKT pathways (1).

Inhibition of EGFR phosphorylation with small molecules that reversibly bind the ATP pocket in the EGFR intracellular tyrosine kinase domain, as gefitinib and erlotinib, represents an effective new pharmacologic strategy for the treatment of advanced NSCLC (2). These drugs produced tumor regression in 10% to 20% of patients with heavily pretreated NSCLC, with mild to moderate toxicity, consisting mainly of acne-like rash and diarrhea (3 – 5).

Clinical experience thus far suggests that certain patient subgroups only may benefit most from EGFR tyrosine kinase inhibitors (TKI). A higher probability of response seem to associate with female sex, never smoking, Asian ethnicity, and adenocarcinoma histotype, particularly with bronchiolo-alveolar features (2). Although clinical characteristics can be useful for the identification of candidate to EGFR TKIs, ideal patient selection should relay mostly on target expression profile (6). The discovery that specific mutations in the tyrosine kinase domain (exons 18-21) of *EGFR* gene account for an increased NSCLC sensitivity to gefitinib or erlotinib has opened a new avenue to patient selection and novel therapeutic strategies (7). Other trials have suggested that *EGFR* gene copy number,

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assessed by fluorescence *in situ* hybridization (FISH), and EGFR protein expression, assessed by immunohistochemistry, may predict responders to EGFR TKIs (8). Phosphorylation status as measure of activation of the two major downstream signaling pathways, Ras-MAPK and PI3K-AKT cascades, has also undergone investigation as a potential molecular marker of tumor responsiveness to gefitinib or erlotinib. p-AKT overexpression correlated with a better outcome, whereas MAPK overexpression did not (9, 10).

The role of tissue biomarkers in predicting response to EGFR TKIs is still under examination because of conflicting results in different studies thus far done. Additionally, in the clinical practice, the use of tissue biomarkers is hampered by the lack of sufficient tumor tissue in the majority of lung cancer patients. For this reason, surrogate tissue markers would be of great value for selecting treatment candidates and for monitoring gefitinib or erlotinib effect. Albanell et al. (11) evaluated the response of normal skin to inhibition of EGFR-mediated signaling as a surrogate marker of pharmacodynamic gefitinib properties. Adjei et al. (12, 13) showed the inhibition of prelamin A farnesylation in buccal mucosa cells of NSCLC patients treated with farnesyl transferase inhibitors, confirming that these agents inhibit protein farnesylation *in vivo*. The possible predictive role of female gender, skin toxicity (14), and certain *EGFR* gene polymorphisms (15) may lead to hypothesize a differential inherited sensitivity to EGFR TKIs supporting the use of normal tissue as a surrogate of the neoplastic one.

In this study, we evaluated the possible predictive role of both pretreatment and posttreatment expression of different signal transduction proteins activated by EGFR, including p-EGFR, p-MAPK, and p-AKT, in buccal mucosa cells of NSCLC patients treated with gefitinib within an Expanded Access Program.

## Patients and Methods

### Patients

Patients were enrolled consecutively in this study, as part of a gefitinib Expanded Access Program, conducted at the University Hospital of Parma and the National Institute for Cancer Research of Genova. The study was approved by the institutional ethical review boards and written informed consent was obtained from each patient before enrollment. Eligibility criteria included histologically or cytologically confirmed NSCLC with measurable, locally advanced, or metastatic disease progressing or relapsing after at least one line of standard chemotherapy. Patients had to have a performance status ranging from 0 to 2, according to Eastern Cooperative Oncology Group. Patients received oral gefitinib at the daily dose of 250 mg and were evaluated for response according to the Response Evaluation Criteria in Solid Tumors (RECIST; ref. 16). Tumor response was assessed by computed tomography scan after 2 months. Patients obtaining a complete response, partial response, or stable disease continued the therapy, with response assessment every 2 months, until progression. Toxicity was assessed monthly by clinical examination, blood counts, and chemistry, according to National Cancer Institute of Canada Common Toxicity Criteria version 2.0. Survival was calculated from the date of therapy initiation to the date of death or last contact.

### Methods

**EGFR signaling immunocytochemistry and immunohistochemistry.** Buccal smears were obtained by brushing from all patients before therapy and after 2 weeks of treatment. Samples were fixed on slides with citofix and air-dried. Protein expression was evaluated by

immunocytochemistry. Smears were incubated with primary antibodies at room temperature for 1 h at the following dilutions: mouse monoclonal antibody clone H11 to EGFR (DAKO), 1:100; mouse monoclonal antibody to p-EGFR (Chemicon), 1:50; rabbit polyclonal p-p44/42 MAPK (Thr<sup>202</sup>/Tyr<sup>204</sup>) antibody to phosphorylated MAPK extracellular signal-regulated kinase 1/2 (Cell Signaling Technology), 1:50; rabbit polyclonal p-AKT (Ser<sup>473</sup>) antibody to p-AKT (Cell Signaling Technology), 1:50. Smears were rinsed in PBS (pH 7.4 and incubated 20 min with secondary antibodies). Supersensitive kit streptavidin-biotin peroxidase conjugate (A. MENARINI Diagnostics) was applied for 20 min and, finally, smears were rinsed with PBS, developed with 3,3'-diaminobenzidine (Cromogen) for 4 min, and counterstained with H&E for 1 min.

Paraffin-embedded tissue sections from tumor specimens obtained at the time of primary diagnosis, when available, were analyzed for EGFR, p-EGFR, p-MAPK, and p-AKT expression. Briefly, 4- $\mu$ m tissue sections were deparaffinized in xylene and hydrated in graded alcohol, and endogenous peroxidase activity was blocked by 30 min treatment with 3% hydrogen peroxide in absolute methanol at room temperature. Sections were then processed as indicated for buccal smears.

The immunostaining for EGFR, p-EGFR, p-MAPK, and p-AKT expression was classified in two categories: "negative" [including negative samples and samples with >10% positive cells, but with weak staining (1+)] and "positive" [if >10% of the tumor cells stained moderately (2+), and if >10% of the cells stained strongly (3+); ref. 17]. p-AKT expression was considered positive when nuclear staining was observed, because AKT activation results in its translocation from the cytoplasm to the nucleus (9). Protein expression variation after gefitinib treatment was defined when the intensity of staining changed and/or the percentage of positive cells was modified of almost 10%. The immunocytochemical and immunohistochemical evaluations were assessed by two observers (M.L. and M.C.), blinded to clinical and other biological results. If discrepancies occurred, a consensus score was reached after discussion.

**Flow cytometry.** Buccal mucosa cells were harvested in 5 mL HBSS, spun down at 200  $\times$  g, and permeabilized by the Fix & Perm Cell Permeabilization kit (Caltag). Cells (50  $\mu$ L at 10  $\times$  10<sup>6</sup>/mL) were dispensed in Falcon tubes and incubated with 100  $\mu$ L reagent A for 15 min at room temperature. Cells were washed once in 3 mL PBS + 0.1% NaN<sub>3</sub> + 5% FCS (PBS-A) and centrifuged, and the pellet was incubated with 100  $\mu$ L reagent B for 5 min. Isotype control (20  $\mu$ L) was added in the control tube and the specific antibodies (the same used in immunocytochemistry), at 1:100 final dilution, were added in the test tubes. After 15 to 30 min, cells were washed once in 3 mL PBS-A, incubated with 20  $\mu$ L anti-mouse Ig-FITC (Becton Dickinson) for 15 min, washed again, and resuspended in 0.5 mL PBS-A. Ten thousand stained cells, gated on physical parameters, were acquired with a FACSCalibur flow cytometer (Becton Dickinson) equipped with a standard filter setting. Single histograms relative to FITC fluorescence intensity were evaluated. Overlay histograms were used to visually inspect the increase in staining due to the specific binding of anti-phosphorylated antibodies, compared with the negative control. Results were expressed as percentage of FITC-positive cells in the test tubes by setting in the control tube a statistical region M1 to include only 1% to 2% FITC-positive cells.

**EGFR and K-ras mutational analysis.** For DNA extraction, serial 4- $\mu$ m-thick sections of formalin-fixed, paraffin-embedded tissue were stained with hematoxylin and examined under a stereomicroscope, and normal and tumor areas were manually microdissected using sterile scalpels. At least 80% neoplastic cell enrichment was obtained for each tumor sample. DNA extraction and purification were done with a commercial kit according to the manufacturer's protocol (Dneasy Tissue kit, Qiagen, Inc.). Amplifications of exons 18 to 21 of *EGFR* and exon 2 of *K-ras* were done using nested primers as published (18–21). PCR fragments were sequenced and analyzed in both forward and reverse directions, and heterozygous variants (mutations) were confirmed by two independent amplifications.

**Table 1.** Patient characteristics

|                           | No. patients (N = 58) |
|---------------------------|-----------------------|
| Gender, n (%)             |                       |
| Males                     | 39 (67)               |
| Females                   | 19 (33)               |
| Age (y)                   |                       |
| Median (range)            | 67 (44-82)            |
| Histology, n (%)          |                       |
| Adenocarcinoma            | 43 (74)               |
| Nonadenocarcinoma         | 15 (26)               |
| Stage, n (%)              |                       |
| IV                        | 58 (100)              |
| PS ECOG, n (%)            |                       |
| 0                         | 14 (24)               |
| 1                         | 36 (62)               |
| 2                         | 8 (14)                |
| Smoking status, n (%)     |                       |
| Current or former smokers | 47 (81)               |
| No smokers                | 11 (19)               |

Abbreviation: PS ECOG, performance status, Eastern Cooperative Oncology Group.

**EGFR amplification by FISH.** Formalin-fixed, paraffin-embedded tissue were cut into 4- $\mu$ m-thick sections and incubated overnight at 56°C. Deparaffinization, pretreatment, enzyme digestion, and fixation of slides were done using the Vysis Paraffin Pretreatment Kit (Vysis) according to the manufacturer's recommended protocol. Denaturation and hybridization were carried out in a HYBrite Denaturation/Hybridization System for FISH (Vysis). EGFR FISH was done using LSI EGFR Spectrum Orange/CEP 7 Spectrum Green probe (Vysis). Tissue sections were denatured at 85°C for 5 min and hybridized overnight at 37°C. The slides were then washed in wash buffer at 72°C for 2 min and counterstained with 4',6-diamidino-2-phenylindole.

For each specimen, at least 200 cells were scored for EGFR evaluation by image analysis. FISH images were processed at  $\times 1,000$  magnification using an Olympus BX41 fluorescence microscope with a 100 W mercury lamp. Separate narrow band pass filters were used for the detection of Spectrum Orange, Spectrum Green, and 4',6-diamidino-2-phenylindole. Images were processed using the Software Cytovision (Applied Imaging; Olympus distributor). Patients were classified into two strata according to the frequency of tumor cells with a specific number of copies of the EGFR gene and chromosome 7 centromere: FISH negative, with no or low genomic gain (less than four copies of the gene in  $\geq 40\%$  of cells), and FISH positive, with a high level of polysomy (four or more copies of the gene in  $\geq 40\%$  of cells) or gene amplification, defined by the presence of tight gene clusters and a ratio of gene/chromosome per cell of 2, or 15 copies of the genes per cell in 10% of analyzed cells.

**Statistical analysis.** The objective clinical response rate in various subgroups of patients was compared using a two-tailed Fisher's exact test. A logistic regression analysis, including age; gender; performance status; EGFR; p-EGFR, p-MAPK, and p-AKT in buccal mucosa and in tumor specimens; EGFR gene mutations; and EGFR gene amplification, was used to identify independent predictive variables influencing the clinical response. The log-rank test was used to evaluate binary prognostic factors in univariate analysis.

## Results

A total of 58 previously treated patients with advanced NSCLC were prospectively enrolled in this study from May 2003 to July 2005. The main patient characteristics are reported in Table 1. Baseline patient characteristics were as follows:

39 males (67%), median age 67 years (range 44-82 years), adenocarcinoma histology in 43 (74%) and stage IV in all patients, and Eastern Cooperative Oncology Group performance status 0 to 1 in 50 (86%) patients. The majority of patients were current or former smokers (81%).

After 2 months of gefitinib treatment, nine patients (15%) had an objective response [seven partial responses (PR) and two complete responses (CR)], 13 patients (22%) had stable disease (SD), and 36 (63%) patients had progressive disease (PD). For the entire group, the median survival was 6 months (Fig. 1). Responding patients (CR + PR) had a median survival of 18.5 months versus 5.1 months of nonresponding ones (SD + PD).

**Clinical characteristics and toxicity.** Female sex, never smoking status, and adenocarcinoma histology were associated with a higher probability of response to gefitinib treatment (Table 2).

During treatment, toxicity of any grade and type was observed in 29 patients (50%), represented by skin rash and gastrointestinal toxicity; in particular, 24 patients (41%) developed an acne-like rash. A statistically significant correlation between general toxicity and objective response was found ( $P = 0.025$ ); in fact, toxicity of any grade and type was observed in 89% of responding patients (CR + PR) and 43% of nonresponding ones (SD + PD). We did not find the same result for skin toxicity ( $P = 0.142$ ; Table 2). Considering disease control (disease control rate = CR + PR + SD), both general toxicity ( $P = 0.003$ ) and skin toxicity ( $P = 0.013$ ) evidenced a predictive significance. Moreover, patients who developed toxicity during gefitinib therapy had a longer survival (Table 2).

**EGFR, p-EGFR, p-MAPK, and p-AKT expression on buccal mucosa.** Baseline buccal mucosa scraping were obtained from 58 patients; however, only 45, 48, 44, and 48 cases for EGFR, p-EGFR, p-MAPK, and p-AKT analysis, respectively, had adequate antigen preservation allowing reliable immunocytochemistry. After 2 weeks of gefitinib treatment, 45, 45, 44, and 43 scraping samples for EGFR, p-EGFR, p-MAPK, and p-AKT, respectively, were available.

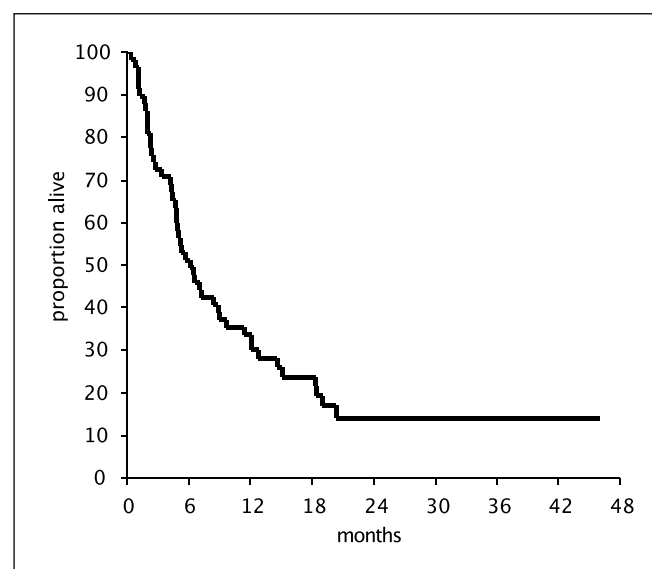


**Fig. 1.** Kaplan-Meier plot of survival time of overall population.

**Table 2.** General characteristics in gefitinib responder and nonresponder patients

| Characteristics   | CR + PR,<br>n (%) | SD + PD,<br>n (%) | P     | DCR, n (%) | PD, n (%) | P     | Median<br>survival (mo) | P      |
|-------------------|-------------------|-------------------|-------|------------|-----------|-------|-------------------------|--------|
| Sex               |                   |                   |       |            |           |       |                         |        |
| Female            | 6 (32)            | 13 (68)           | 0.047 | 10 (53)    | 9 (47)    | 0.151 | 8.4                     | 0.245  |
| Male              | 3 (8)             | 36 (92)           |       | 12 (31)    | 27 (69)   |       | 5.3                     |        |
| Smoking status    |                   |                   |       |            |           |       |                         |        |
| Nonsmokers        | 4 (36)            | 7 (64)            | 0.056 | 6 (55)     | 5 (45)    | 0.302 | 12.7                    | 0.064  |
| Current or former | 5 (11)            | 42 (89)           |       | 16 (34)    | 31 (66)   |       | 5.3                     |        |
| Histology         |                   |                   |       |            |           |       |                         |        |
| Adenocarcinoma    | 8 (19)            | 35 (81)           | 0.422 | 19 (44)    | 24 (56)   | 0.128 | 8.4                     | <0.001 |
| Nonadenocarcinoma | 1 (7)             | 14 (93)           |       | 3 (20)     | 12 (80)   |       | 2.8                     |        |
| Toxicity          |                   |                   |       |            |           |       |                         |        |
| Yes               | 8 (28)            | 21 (72)           | 0.025 | 17 (59)    | 12 (41)   | 0.003 | 12                      | 0.002  |
| No                | 1 (3)             | 28 (97)           |       | 5 (17)     | 24 (83)   |       | 2.8                     |        |
| Skin toxicity     |                   |                   |       |            |           |       |                         |        |
| Yes               | 6 (25)            | 18 (75)           | 0.142 | 14 (58)    | 10 (42)   | 0.013 | 11.4                    | 0.026  |
| No                | 3 (9)             | 31 (91)           |       | 8 (23)     | 26 (77)   |       | 4.8                     |        |

NOTE: Disease control rate is computed as CR + PR + SD.

Abbreviation: DCR, disease control rate.

EGFR was positive in 53%, p-EGFR in 37%, p-MAPK in 66%, and p-AKT in 46% of patients before gefitinib treatment. We evaluated the association between the baseline status of EGFR, p-EGFR, p-MAPK, and p-AKT and the response to gefitinib (Table 3). p-AKT overexpression was observed in 78% of responder patients and 38% of nonresponders ( $P = 0.061$ ). p-EGFR expression showed a nonstatistically significant correlation trend with objective response (overexpression in 56% of responders and in 36% of nonresponders). On the contrary, response to gefitinib did not differ according to baseline EGFR and p-MAPK status. Concerning survival, no difference was evidenced according to baseline protein expression (Table 3).

The predictive role of early variation of protein expression in buccal mucosa cells after 2-week gefitinib treatment was also evaluated. Among responding patients (CR + PR), 67% showed a reduction of expression of p-EGFR and p-MAPK and 57% showed a reduction of p-AKT expression. Patients with stable disease reported a variable pattern of marker expression changes, whereas patients with progressive disease reported a trend toward an increase in protein expression (Fig. 2). However, no statistically significant difference between responders and nonresponders in terms of protein expression changes was found. Figures 3 and 4 show two examples. Flow cytometry approach to detect marker expression showed a high degree of concordance with immunocytochemistry results (data not shown).

**EGFR, p-EGFR, p-MAPK, and p-AKT expression on tumor tissue.** The expression of EGFR, p-EGFR, p-MAPK, and p-AKT in corresponding tumor tissue was evaluated, when available. The analysis was possible in tumor specimens of 21 patients. EGFR was positive in 84%, p-EGFR in 28%, p-MAPK in 44%, and p-AKT in 23% of patients. We evaluated the association between the EGFR, p-EGFR, p-MAPK, and p-AKT status and response to gefitinib. Clinical response to gefitinib and survival did not differ according to tissue protein expression (Table 3).

**Correlation between protein expression on buccal mucosa versus tumor tissue.** The correlation between protein expression on buccal mucosa and on corresponding tumor tissue was obtained for EGFR, p-EGFR, p-MAPK, and p-AKT in 17, 15,

16, and 19 patients, respectively. No statistically significant correlation was found; in particular, in 10, 11, 9, and 8 cases for EGFR, p-EGFR, p-MAPK, and p-AKT, respectively, a protein expression discrepancy between buccal mucosa and tumor tissue was observed (negative in buccal mucosa and positive in tumor tissue or vice versa).

**Correlation between toxicity and protein expression on buccal mucosa.** The correlation between toxicity and protein expression on buccal mucosa, both as baseline status and as early variation, was evaluated. As for baseline status of EGFR, p-EGFR, p-MAPK, and p-AKT, and gefitinib toxicity, we did not observe any correlation ( $P = 0.550$ ,  $P = 0.383$ ,  $P = 1$ ,  $P = 0.573$  for EGFR, p-EGFR, p-MAPK, and p-AKT, respectively). Moreover, no correlation was found as for early variation of protein expression ( $P = 0.202$ ,  $P = 1$ ,  $P = 1$ ,  $P = 0.542$  for EGFR, p-EGFR, p-MAPK, and p-AKT, respectively).

**EGFR and K-ras gene mutational analysis.** EGFR mutational analysis was done on 21 patients for which paraffin-embedded blocks were available. Five heterozygous mutations were observed; all these mutations have previously been described (18–20). We found four exon 19 deletions (two 2235del15, one 2240del12, and one 2240del18) and one exon 21 missense mutation (L858R). The presence of EGFR mutation was significantly associated only with female sex ( $P = 0.012$ ).

All EGFR-mutated patients had an objective response (Table 3). Among responders, five of six (83%) tumors showed EGFR gene mutation, as opposed to none of the tumors from patients with SD or PD ( $P < 0.001$ ).

About K-ras mutation, we found only one K-ras mutation in a nonresponder patient.

**EGFR gene copy number.** EGFR FISH analysis was done on 18 tumor specimens, with EGFR FISH positive in seven patients (38.9%). FISH analysis showed high polysomy or amplification of EGFR gene in 60% of responding patients (CR + PR). The differences between responders and nonresponders were not statistically significant and, moreover, no difference was observed in median survival according to FISH status (Table 3). No correlation was observed between EGFR gene mutations and EGFR gene copy number ( $P = 0.280$ ).

**Clinical and biological characteristics of responder patients.** Table 4 shows the clinical and biological characteristics of the nine responding patients (two CR and seven PR). Responding patients were mostly females (six of nine), with adenocarcinoma (eight of nine), who developed toxicity during the treatment (eight of nine) and with p-AKT overexpression in buccal mucosa cells (seven of nine).

## Discussion

The purpose of this study was to assess whether buccal mucosa epithelial cells can be useful for assessing biological markers to predict treatment outcome in NSCLC patients receiving EGFR TKIs. Thus, we evaluated the correlation between objective response to gefitinib and both pretreatment and posttreatment expression of different signal transduction markers activated by EGFR, including p-EGFR, p-MAPK, and p-AKT, in buccal mucosa cells.

Normal epithelial cells of the skin and buccal mucosa are expected to react to agents interfering with epithelial growth factors similarly to neoplastic ones and have already been proven useful to study the pharmacodynamic effect of certain

molecular targeted agents (11–13). Our hypothesis was that early down-regulation of EGFR signaling could anticipate clinical response induced by gefitinib treatment.

The results of our study show that patients with an objective response to gefitinib had a trend toward a reduction in expression of p-EGFR, p-MAPK, and p-AKT after gefitinib treatment, whereas those with stable disease reported a variable pattern of marker expression changes and patients with progressive disease had a trend toward an increased protein expression. Although statistical significance was not reached, this observation suggests that the study of buccal mucosa cells may represent an easy way to monitor the pharmacodynamic effect of EGFR TKIs correlating with clinical effect.

Our results are in agreement with those of Spector et al. (22) obtained with lapatinib therapy that showed, in tumor biopsies from different malignancies, p-EGFR, p-AKT, and p-MAPK inhibition in responding patients after 21 days of treatment. However, the limited sample size and the variability of the biological results of both studies do not allow to draw any definitive conclusion.

The variable pharmacodynamic effects seen in our study are similar to that obtained with gefitinib and erlotinib in normal

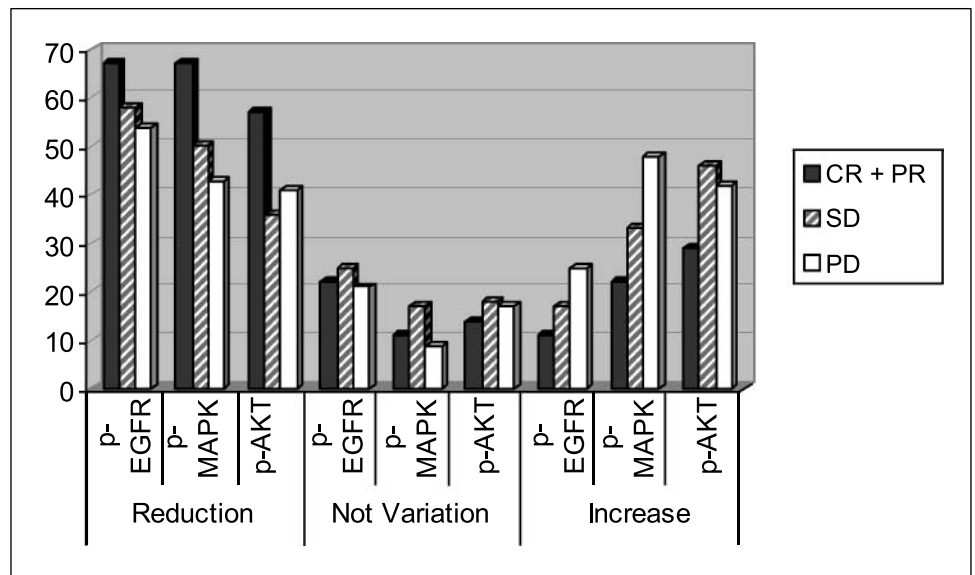
**Table 3.** Protein expression in buccal mucosa cells and in the tumor tissue, *EGFR* gene mutations, and *EGFR* gene copy number in gefitinib responder and nonresponder patients

| Characteristics        | CR + PR,<br>n (%) | SD + PD,<br>n (%) | P      | DCR, n (%) | PD, n (%) | P     | Median<br>survival (mo) | P     |
|------------------------|-------------------|-------------------|--------|------------|-----------|-------|-------------------------|-------|
| EGFR buccal mucosa*    |                   |                   |        |            |           |       |                         |       |
| Positive               | 3 (12)            | 21 (88)           | 0.267  | 9 (37)     | 15 (63)   | 0.238 | 7                       | 0.606 |
| Negative               | 6 (29)            | 15 (71)           |        | 12 (57)    | 9 (43)    |       | 6.6                     |       |
| p-EGFR buccal mucosa*  |                   |                   |        |            |           |       |                         |       |
| Positive               | 5 (28)            | 13 (72)           | 0.265  | 10 (56)    | 8 (44)    | 0.240 | 8.8                     | 0.187 |
| Negative               | 4 (13)            | 26 (87)           |        | 11 (37)    | 19 (63)   |       | 5.3                     |       |
| p-MAPK buccal mucosa*  |                   |                   |        |            |           |       |                         |       |
| Positive               | 4 (14)            | 25 (86)           | 0.235  | 13 (45)    | 16 (55)   | 0.752 | 6.6                     | 0.869 |
| Negative               | 5 (33)            | 10 (67)           |        | 8 (53)     | 7 (47)    |       | 9                       |       |
| p-AKT buccal mucosa*   |                   |                   |        |            |           |       |                         |       |
| Positive               | 7 (32)            | 15 (68)           | 0.061  | 13 (59)    | 9 (41)    | 0.079 | 7                       | 0.158 |
| Negative               | 2 (8)             | 24 (92)           |        | 8 (31)     | 18 (69)   |       | 6                       |       |
| EGFR tumor tissue*     |                   |                   |        |            |           |       |                         |       |
| Positive               | 5 (31)            | 11 (69)           | 0.530  | 7 (44)     | 9 (56)    | 0.582 | 5.7                     | 0.436 |
| Negative               | 0 (0)             | 3 (100)           |        | 2 (67)     | 1 (33)    |       | 17.3                    |       |
| p-EGFR tumor tissue*   |                   |                   |        |            |           |       |                         |       |
| Positive               | 0 (0)             | 5 (100)           | 0.278  | 1 (20)     | 4 (80)    | 0.314 | 5.1                     | 0.221 |
| Negative               | 4 (31)            | 9 (69)            |        | 7 (54)     | 6 (46)    |       | 18.5                    |       |
| p-MAPK tumor tissue*   |                   |                   |        |            |           |       |                         |       |
| Positive               | 3 (37)            | 5 (63)            | 0.608  | 6 (75)     | 2 (25)    | 0.188 | 8.8                     | 0.892 |
| Negative               | 2 (20)            | 8 (80)            |        | 4 (40)     | 6 (60)    |       | 5.1                     |       |
| p-AKT tumor tissue*    |                   |                   |        |            |           |       |                         |       |
| Positive               | 2 (40)            | 3 (60)            | 0.548  | 3 (60)     | 2 (40)    | 0.624 | 12.9                    | 0.863 |
| Negative               | 3 (18)            | 13 (82)           |        | 6 (41)     | 10 (59)   |       | 8.8                     |       |
| EGFR gene mutations    |                   |                   |        |            |           |       |                         |       |
| Mutated                | 5 (100)           | 0 (0)             | <0.001 | 5 (100)    | 0 (0)     | 0.035 | 18.5                    | 0.544 |
| Wild-type              | 1 (6)             | 15 (94)           |        | 6 (37)     | 10 (63)   |       | 8.8                     |       |
| EGFR FISH <sup>†</sup> |                   |                   |        |            |           |       |                         |       |
| Positive               | 3 (43)            | 4 (57)            | 0.326  | 4 (57)     | 3 (43)    | 0.630 | 8.8                     | 0.179 |
| Negative               | 2 (18)            | 9 (82)            |        | 4 (36)     | 7 (64)    |       | 18.5                    |       |

\*The immunostaining for EGFR, p-EGFR, p-MAPK, and p-AKT expression was classified in two categories: "negative" [including negative samples and samples with >10% positive cells, but with weak staining (1+)] and "positive" [if >10% of the tumor cells stained moderately (2+), and if >10% of the cells stained strongly (3+)].

<sup>†</sup> EGFR FISH negative: no or low genomic gain (<four copies of the gene in ≥40% of cells); FISH positive: high level of polysomy (four or more copies of the gene in ≥40% of cells) or gene amplification, defined by the presence of tight gene clusters and a ratio of gene/chromosome per cell of ≥2, or ≥15 copies of the genes per cell in ≥10% of analyzed cells.

**Fig. 2.** Variation of p-EGFR, p-MAPK, and p-AKT expression in buccal mucosa cells after 2 wk of gefitinib treatment, in patients with CR + PR, SD, and PD. "Reduction" or "increase" of protein expression variation were defined when the intensity of staining changed and/or the percentage of positive cells was modified of almost 10%, as decrease or increase, respectively; otherwise, the variation was classified as "not variation".



EGFR-expressing tissues, as skin (11, 23). The results from skin biopsy analysis showed an inhibition of most EGFR downstream signaling molecules, such as p-EGFR and p-MAPK, after gefitinib treatment. On the contrary, as in our study, EGFR expression was not modified (11). These biological effects in the skin did not necessarily correlate with clinical response.

Similarly, in our study, some nonresponders still had evidence of EGFR downstream signaling inhibition with gefitinib, suggesting that redundant signaling pathways might regulate the growth and/or survival of NSCLC.

Other pharmacodynamic studies of gefitinib have been done in tumor biopsies from patients with advanced breast (24),

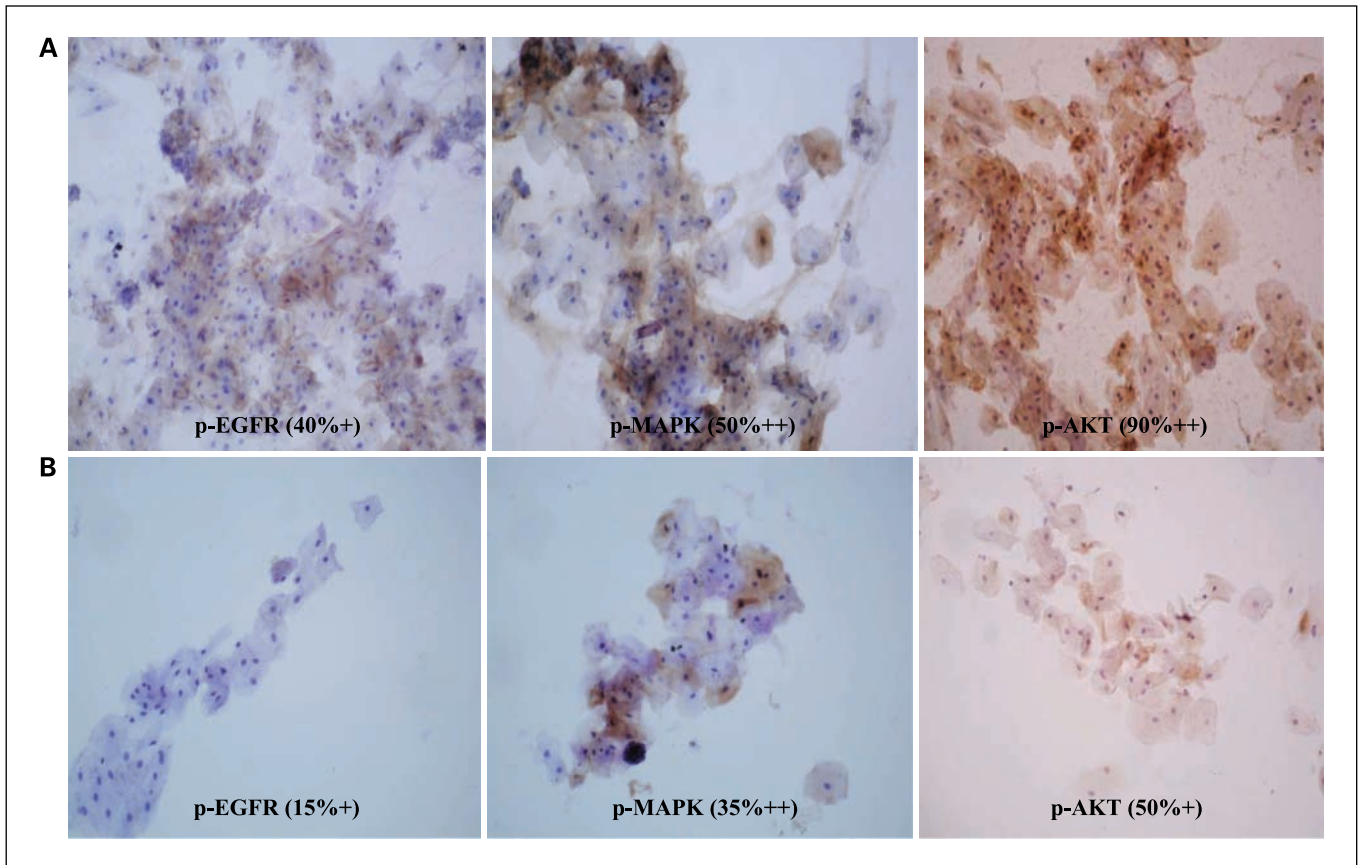


**Fig. 3.** Immunocytochemical staining of p-EGFR, p-MAPK, and p-AKT at baseline (A) and at day 15 (B) on buccal mucosa cells in responder patient [combination of percentage of positive cells and staining intensity (negative 0, weak 1+, moderate 2+, and strong 3+)]. Original magnification,  $\times 200$ .

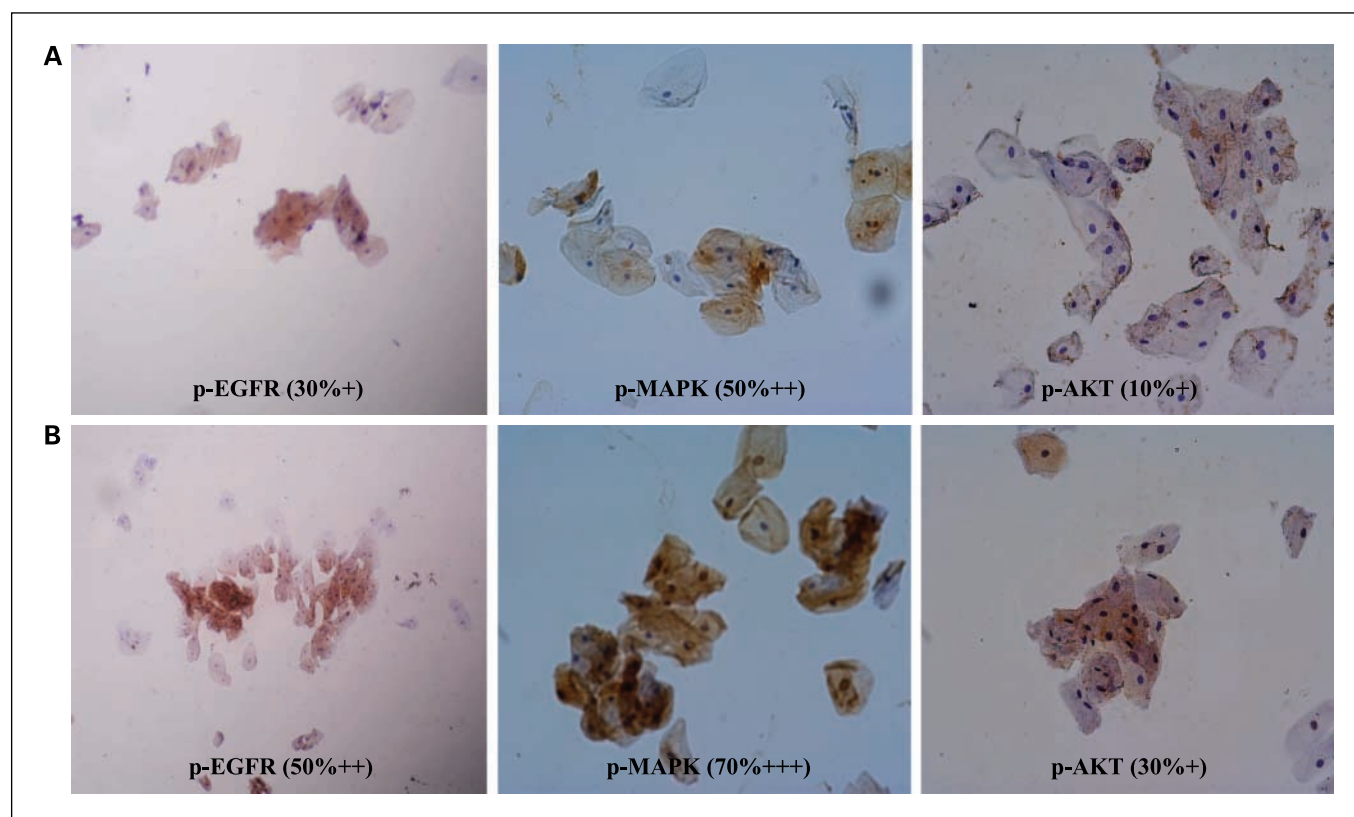


**Fig. 4.** Immunocytochemical staining of p-EGFR, p-MAPK, and p-AKT at baseline (A) and at day 15 (B) on buccal mucosa cells in nonresponder patient [combination of percentage of positive cells and staining intensity (negative 0, weak 1+, moderate 2+, and strong 3+)]. Original magnification,  $\times 200$ .

colorectal (25), and gastric cancer (26), with various results. In breast cancer, a significant decrease in p-EGFR and p-MAPK, but not in p-AKT, expression after gefitinib treatment was shown (24). In patients with previously treated colorectal cancer, gefitinib is inactive as single agent, and expression of p-EGFR and p-MAPK did not decrease after 1 week of gefitinib. However, a trend toward decreased posttreatment levels of p-AKT

was observed in patients with a progression-free survival higher than the median, although in the absence of statistical significance (25). Finally, in advanced gastric carcinoma, levels of p-EGFR, but not of p-MAPK and p-AKT, were significantly reduced after gefitinib treatment although this did not translate into clinical benefit (26). In some cases, however, gefitinib inhibited p-AKT and these tumors had enhanced apoptosis.

**Table 4.** Clinical and biological characteristics in nine responder patients to gefitinib

| Age | Sex | Histology | Smoking status | Response | Toxicity | p-AKT buccal mucosa* | EGFR mutation | EGFR FISH <sup>†</sup> | Survival (mo) |
|-----|-----|-----------|----------------|----------|----------|----------------------|---------------|------------------------|---------------|
| 66  | M   | ADK       | Yes            | PR       | Yes      | Positive             | NA            | NA                     | 18.4          |
| 59  | F   | ADK       | Yes            | PR       | Yes      | Positive             | Mutated       | Negative               | 18.5          |
| 73  | F   | ADK       | Yes            | CR       | Yes      | Positive             | Mutated       | Negative               | 21.9+         |
| 52  | F   | Non-ADK   | No             | PR       | No       | Negative             | Mutated       | Positive               | 2.3           |
| 55  | F   | ADK       | Yes            | CR       | Yes      | Negative             | Mutated       | Positive               | 46.1+         |
| 69  | M   | ADK       | Yes            | PR       | Yes      | Positive             | NA            | NA                     | 3.1           |
| 62  | F   | ADK       | No             | PR       | Yes      | Positive             | NA            | NA                     | 20.3          |
| 44  | M   | ADK       | No             | PR       | Yes      | Positive             | Wild-type     | NA                     | 18.5+         |
| 62  | F   | ADK       | No             | PR       | Yes      | Positive             | Mutated       | Positive               | 8.2           |

NOTE: "Yes" smoking status means current or former smoker.

Abbreviations: ADK, adenocarcinoma; Non-ADK, nonadenocarcinoma; NA, not available.

\*p-AKT buccal mucosa expression was classified in two categories: negative [including negative samples and samples with  $>10\%$  positive cells, but with weak staining (1+)] and positive [if  $>10\%$  of the tumor cells stained moderately (2+), and if  $>10\%$  of the cells stained strongly (3+)].

<sup>†</sup>EGFR FISH negative: no or low genomic gain (less than four copies of the gene in  $\geq 40\%$  of cells); FISH positive: high level of polysomy (four or more copies of the gene in  $\geq 40\%$  of cells) or gene amplification, defined by presence of tight gene clusters and a ratio of gene/chromosome per cell of  $\geq 2$ , or  $\geq 15$  copies of the genes per cell in  $\geq 10\%$  of analyzed cells.

We also aimed to assess whether buccal mucosa could be used as surrogate tissue for baseline expression of EGFR signaling proteins predicting gefitinib treatment outcome. The rationale behind this hypothesis was based on the observation that response to EGFR TKIs seems to correlate with female gender, toxicity, and *EGFR* gene polymorphisms, suggesting the possible existence of some inherited genetic factor influencing sensitivity to this class of compounds. In addition, given the difficulty in obtaining adequate tumor specimens in lung cancer, the availability of a reliable surrogate tissue for biological studies would be of great value. We thus analyzed the association between the EGFR, p-EGFR, p-MAPK, and p-AKT baseline status, in buccal mucosa cells and in the available corresponding tumor tissue, and objective response to gefitinib. Among these biomarkers, only p-AKT pretreatment expression in buccal mucosa cells, but not in the tumor tissue, evidenced a borderline potential predictive role ( $P = 0.061$ ). In addition, buccal mucosa p-EGFR overexpression showed a nonstatistically significant trend of correlation with objective response. These results are consistent with those of Cappuzzo et al. (9), who reported a correlation between tumor tissue p-AKT, but not p-MAPK, overexpression and response to gefitinib. Similarly, Han et al. (10) reported that activation of AKT predicted gefitinib sensitivity, whereas overexpression of p-MAPK was associated with poor response. Thus, the antiapoptotic signaling pathway (AKT) seems to be more important than the proliferative pathway (MAPK) as a determinant of response to gefitinib. Our study is the first to support a potential role of p-AKT expression in buccal mucosa cells in predicting activity of EGFR-TKI treatment.

Interestingly, as reported in other studies, in our series EGFR tumor expression was not correlated to gefitinib activity (27–29). However, a significant correlation between EGFR expression and sensitivity to gefitinib was observed using a different antibody, a different scoring system, and a higher amount of diagnostic material (30). The lack of standardization in staining procedures and guidelines for interpretation of the EGFR assessment may be the major reason for the conflicting results across studies.

Many trials documented a relationship among female gender, adenocarcinoma histology, never smoking status, and

higher response rates to EGFR TKIs (3–5). In our study, we confirm the predictive role of female sex ( $P = 0.047$ ), but not for smoking status and histology, likely due to the limited sample size, because 89% and 93% of current/former smokers and nonadenocarcinoma patients, respectively, were nonresponders.

We also found that overall and skin toxicity were associated with response ( $P = 0.025$ ) and survival ( $P = 0.002$ ) in patients treated with gefitinib. Gefitinib is quite well tolerated, with generally mild, dose-dependent adverse effects mostly related to skin disorders and diarrhea. Skin rash typically resolves during treatment or after temporary or permanent drug withdrawal and it seemed to be, thus far, a potential predictive factor for activity of erlotinib, but not of gefitinib (14). In the IDEAL-2 study, although a skin rash was observed in all responders, this adverse event showed only a weak positive predictive value, occurring also in 65% of patients who did not respond (4). In our study, considering disease control rate, skin toxicity was observed only in 28% of nonresponding patients versus 64% in responding ones ( $P = 0.013$ ).

Concerning tissue biomarkers, EGFR mutational status and FISH analyses were done in 21 tumor available specimens. We found five already previously described (18–20) *EGFR* gene mutations (24%), which resulted to highly predictive of gefitinib activity, consistent with most reports (31). *EGFR* FISH analysis was shown to be a good predictive marker for gefitinib sensitivity in NSCLC patients (8, 30). In our study, we reported increased *EGFR* gene copy number in 60% of responding patients, but the difference between responders and nonresponders was not statistically significant, again likely due to the limited sample size (18 tumor specimens).

Our study has some possible limitations. First, the sample size is rather small, particularly for cases with available tumor tissue. Second, the study has a retrospective nature and we lack a prospective validation of our findings. Finally, there are no precise guidelines for criteria and cutoffs in immunochemistry studies. However, despite these limitations, the observation that buccal mucosa p-AKT may predict gefitinib treatment outcome is original and, in our opinion, deserves attention and further confirmatory studies.

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