Pilot Study of Neoadjuvant Treatment with Erlotinib in Nonmetastatic Head and Neck Squamous Cell Carcinoma

Fabienne Thomas,1 Philippe Rochaix,1,2 Adil Benlyazid,2 Jérôme Sarini,2 Michel Rives,2 Jean Louis Lefebvre,3 Ben C. Allal,1 Frédéric Courbon,2 Etienne Chatelut,1 and Jean-Pierre Delord1,2

Abstract

Purpose: To determine the safety and efficacy of erlotinib given as neoadjuvant treatment in patients with head and neck squamous cell carcinoma (HNSCC). Further objectives were to identify markers of response to erlotinib and to assess the pharmacodynamic effects of erlotinib in tumor cells.

Experimental Design: Patients with locally advanced nonmetastatic HNSCC were treated with erlotinib 150 mg daily pending surgical management. Tumor samples were collected before and after erlotinib treatment and were analyzed using immunohistochemistry. Epidermal growth factor receptor copy number was determined in tumors using CISH analysis.

Results: Between November 2003 and December 2005, 35 patients were included in the study. Neoadjuvant treatment with erlotinib in HNSCC patients was well tolerated and did not necessitate modification to routine surgical procedures. Among 31 evaluable patients, erlotinib was given for a median of 20 days. At the time of surgery, tumor shrinkage was observed in nine patients (29%). Immunohistochemistry analyses were done for 31 patients and showed a decrease in phosphorylated tyrosine residues and phosphorylated erk immunostaining after erlotinib treatment. In a retrospective analysis, baseline p21\textsuperscript{waf} expression in the basal-like cell layer was statistically positively correlated with clinical response to treatment. Epidermal growth factor receptor copy number did not correlate with response to erlotinib.

Conclusion: Neoadjuvant treatment of HNSCC with erlotinib was well tolerated. Baseline p21\textsuperscript{waf} expression was associated with response to erlotinib and so might be useful as a tool to select patients for erlotinib therapy in this setting.

The human epidermal growth factor receptor (EGFR) is a tyrosine kinase receptor that plays an important role in several cellular signaling pathways, including those involved in proliferation and survival. EGFR has a well-established role in several solid tumor types and constitutes a clinically validated target for anticancer therapies. Erlotinib (OSI-774, Tarceva) is a potent, orally available EGFR tyrosine kinase inhibitor that blocks EGFR-mediated intracellular signaling and induces tumor cell cycle arrest. Erlotinib is approved by the U.S. Food and Drug Administration and the European Medicines Agency for treatment of patients with locally advanced or metastatic non–small cell lung cancer (NSCLC) after failure of at least one prior chemotherapy regimen (1). It is also approved by the U.S. Food and Drug Administration for treatment, in combination with gemcitabine, of locally advanced unresectable or metastatic pancreatic cancer. Several studies in NSCLC have shown that erlotinib and gefitinib (Iressa; another EGFR tyrosine kinase inhibitor) produce radiographic responses in ~10% of patients treated in the second line or third line setting (2–4). Clinical characteristics associated with tumor response have been extensively described (5–8) and include female gender, never-smoking status, adenocarcinoma histology, Asian ethnic origin, EGFR gene amplification, and the presence of specific mutations in the EGFR tyrosine kinase domain. The study of molecular biomarkers of erlotinib response (7) showed that the incidence of EGFR mutations in lung cancer was 22%.

Erlotinib has been implicated in the tumorigenesis of head and neck squamous cell carcinoma (HNSCC; ref. 9). The antitumor activity of erlotinib, alone or in combination with cisplatin, has been shown in vivo using murine xenografts of a human HNSCC cell line (10). Furthermore, in a phase I study, erlotinib produced a stable disease lasting for 15 months in one patient with HNSCC (11). In a subsequent phase II study, erlotinib was well tolerated in a heavily pretreated population of patients with HNSCC and produced disease stabilization in 38% of cases, with a median duration of 16.1 weeks (12). Recently,
Patients

Patients were eligible if they had nonmetastatic, histologically confirmed HNSCC (stage ≥ T2N × M0) and were candidates for first line curative surgical treatment or had been scheduled for surgery by necessity (neck nodes dissection for bulky lymphadenopathies before radiotherapy). Other eligibility criteria included WHO performance status of 0-1, ability to swallow food, age of >18 years, and provision of written informed consent.

Neoadjuvant Treatment with Erlotinib in HNSCC

Erlotinib (Tarceva), an epidermal growth factor receptor (EGFR)-targeting agent, showed encouraging results in combination with radiotherapy for treatment of locally advanced HNSCCs (13) that led to its approval in this indication. A phase III study published by Burtness et al. (14) showed that the combination of cisplatin and cetuximab was active in the first line treatment of recurrent HNSCC. EGFR-targeted molecules are likely to become a therapeutic option in HNSCC; however, there is a clear medical need to identify which patients are most likely to benefit from therapy with EGFR inhibitors. Contrary to NSCLC, few factors predictive of response have been identified in HNSCC. Numerous teams have assessed the existence of EGFR tyrosine kinase mutations in this disease (15, 16) but they seem to be rare at least in Caucasian patients (17, 18). Development and intensity of skin rash caused by anti-EGFR therapies have been correlated with improved survival (4, 19). Recently, Agulnik et al. (20) investigated tumor and skin tissue samples to identify biomarkers correlated with response to treatment with erlotinib and cisplatin. Their results suggest that HNSCC patients with high gene copy number of EGFR gene may have higher response rate. Among the EGFR signaling proteins investigated before and after treatment, the decrease of phosphorylated EGFR in both normal and tumor tissue was linked with increased overall survival, indicating that decrease in phosphorylated EGFR may represent a potential surrogate marker for outcome. However, the results were obtained for erlotinib in combination with cisplatin in patients participating to phases I and II who were already heavily pretreated. Such biomarkers should be examined in patients treated with erlotinib as a single agent to characterize the clinical response to this particular therapy.

Therefore, we designed a clinical pilot trial to firstly assess if neoadjuvant treatment with erlotinib in patients awaiting surgery for HNSCC is feasible and, secondly, to identify any molecular factors predictive of response and to assess the pharmacodynamic effects of treatment.

Materials and Methods

Patients

Patients were eligible if they had had recent massive gastrointestinal hemorrhage, the presence of a closely contraindication in the form of a major impairment of general condition, or an on-going unmanaged serious infectious disease or major metabolic disorder. Other exclusion criteria included neutrophil count of <1 × 10³ per liter or platelet count of <75 × 10³ per liter at study entry, bilirubin at >1.5-fold above the upper limit of normal, and kidney failure (glomerular filtration rate of <40 mL/min, calculated using Cockcroft's formula). Pregnant women and women of childbearing potential were also excluded.

Treatment plan

After diagnosis, patients underwent routine pan-endoscopy. Treatment with oral erlotinib (150 mg/day; F. Hoffmann-La Roche) was started the following day. Patients were treated for a variable period (range, 18-30 days), corresponding to the time between pan-endoscopy and surgical resection. In the event of grade 2 diarrhea or skin rash that was symptomatically unacceptable to the patient, treatment was withheld until resolution to grade 1, and then erlotinib was restarted at a dose of 100 mg/day. If toxicity recurred, erlotinib was stopped.

Clinical evaluation

Collection of a full medical history, physical examination, electrocardiogram, and laboratory tests were done at baseline. Computed tomography imaging of the involved site and systematic radiologic chest evaluation were done within 1 month of enrollment to ensure that no lung metastases were present. Toxicities were evaluated at each visit and graded using the National Cancer Institute Common Toxicity Criteria, version 2.0. Tumor response was assessed using CT scans taken before and after treatment (on the day before surgery). CT scans to confirm response were not possible as the patients were operated upon. Due to the very short treatment period, patients with tumor shrinkage of >25% were arbitrarily considered as responders.

Tissue biopsies

Tumor tissue biopsies were collected both before and after treatment. A portion of each sample was snap frozen in liquid nitrogen; the remainder was formalin-fixed for 24 h and then paraffin embedded for histologic diagnosis and immunohistochemistry evaluations.

Immunohistochemistry

Immunohistochemistry analyses were done on 4-μm-thick formalin-fixed paraffin sections. Details of the antibodies and antigen retrieval methods used are summarized in Table 1. Slides were processed using an Autostainer slide processor (Dako). Antibodies were incubated with samples for 1 h, and antigen-antibody complexes were visualized using a two-step peroxidase-conjugated polymer backbone system (EnVision, Dako) according to the manufacturer’s instructions. The substrate used was 3,3’-diaminobenzidine. Negative controls were done by omitting the primary antibody. Immunohistochemistry analyses were evaluated using the immunoreactive score (IRS) according to Remmele et al. (21). The IRS (range, 0-12) is the product of the “staining intensity score” (0-3 scale) and “percentage of cells stained score” (0-4 scale). The staining intensity scores 0, 1, 2, and 3 correspond respectively to no staining, weak, moderate, and high intensity of staining.

Table 1. Antibodies and protocols used for immunohistochemistry analyses

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody</th>
<th>Dilution</th>
<th>Retrieval method</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR</td>
<td>PharmDX (Dako)</td>
<td>1:1,000</td>
<td>Microwave heat, * 1 mmol/L EDTA buffer (pH 9)</td>
</tr>
<tr>
<td>Phosphorylated EGFR Tyr 1086</td>
<td>36-9700 (Zymed)</td>
<td>1:1,000</td>
<td>Microwave heat, * 10 mmol/L citrate buffer (pH 6)</td>
</tr>
<tr>
<td>Phosphorylated Tyr</td>
<td>4G10 (Upstate)</td>
<td>1:100</td>
<td>Microwave heat, * 1 mmol/L EDTA buffer (pH 9)</td>
</tr>
<tr>
<td>Phosphorylated Akt</td>
<td>CR473 (Upstate)</td>
<td>1:100</td>
<td>95°C, 40 min, target retrieval solution (pH 9.9, Dako)*</td>
</tr>
<tr>
<td>STAT3</td>
<td>SC8019 (Santa Cruz)</td>
<td>1:250</td>
<td>Microwave heat, * 10 mmol/L citrate buffer (pH 6)</td>
</tr>
<tr>
<td>STAT3b</td>
<td>SC1656 (Santa Cruz)</td>
<td>1:100</td>
<td>95°C, 40 min, target retrieval solution (pH 9.9, Dako)*</td>
</tr>
<tr>
<td>Phosphorylated erk 1/2</td>
<td>SC7383s (Santa Cruz)</td>
<td>1:100</td>
<td>95°C, 40 min, target retrieval solution (pH 9.9, Dako)*</td>
</tr>
<tr>
<td>p21[539]</td>
<td>SX118 (Dako)</td>
<td>1:25</td>
<td>95°C, 40 min, target retrieval solution (pH 9.9, Dako)*</td>
</tr>
<tr>
<td>p27</td>
<td>SX53G8 (Dako)</td>
<td>1:50</td>
<td>95°C, 40 min, target retrieval solution (pH 9.9, Dako)*</td>
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</tbody>
</table>

*3 × 5 min at 750 W.
strong staining. The percentage of cells stained scores 0, 1, 2, 3, and 4 correspond respectively to 0, <10%, from 10% to 50%, from 51% to 80%, and >80% of stained cells. For this study, samples with IRS of >3 were classified as positive for the antigen under investigation. Scores are presented for staining of the peripheral layer of cells of the tumor nests, which is referred to here as the basal-like cell layer. Compared with assessments of the whole tumor, staining of the basal-like cell layer was more reproducible and independent of tumor differentiation (Fig. 1).

CISH. CISH analyses were done on 4-μm-thick formalin-fixed paraffin sections. Briefly, tissue sections were deparaffinized in two changes of xylene, rinsed in three washes of ethanol (100%, 80%, and 50%) and washed in distilled running water. The slides were placed in heated (95°C) CISH pretreatment buffer (ZytoVision) for 15 min and washed in distilled running water. The tissue was digested for 7 min with pepsin digestion solution (ZytoVision) at room temperature, washed in distilled water, dehydrated with alcohol, and then dried in a 37°C oven. Ten to twenty microliters of EGFR amplification probe (ZytoVision) were applied to the designated area, and a coverslip was sealed with rubber cement. The slide was dried, followed by probe denaturation at 95°C for 5 min and hybridization at 37°C overnight in a Hybridizer oven (Dako). The slides were washed in 0.5% SSC for 5 min at room temperature, followed by 0.5% SSC for 5 min at 75°C and water for 5 min at room temperature. For immunodetection, slides were washed in PBS with 0.025% Tween 20 twice for 2 min each. Slides were incubated with nonspecific blocking solution (ZytoVision) for 10 min at room temperature, then incubated with mouse antidigoxigenin antibody for 30 min at room temperature, washed in PBS with 0.025% Tween 20 twice for 2 min each, incubated with horseradish peroxidase–conjugated goat anti-mouse antibody for 15 min, and finally washed again with PBS with 0.025% Tween 20 twice for 2 min each. The slides were then incubated with 3,3′-diaminobenzidine (ZytoVision) for 30 min at room temperature and washed in distilled water twice for 2 min each. Slides were counterstained with hematoxylin. The CISH-prepared slides were examined at 1,000× by bright-field microscopy. Two hundred tumor nuclei were examined in each case, and the mean of numbers of spot per tumor nuclei was reported as the CISH result.

Pharmacokinetic sampling and analysis. Blood samples were collected 2 h after erlotinib administration on day 1 (C2h) and on the day of surgery, −24 h after the last administration of erlotinib. Blood samples were centrifuged (1,500 g, 15 min) within an hour, and plasma was frozen at -20°C. Determination of erlotinib and OSI-420 (its major metabolite) concentrations was done by MDS PharmaMontreal using a coupled liquid chromatography–mass spectrometry technique.

DNA extraction and molecular study. Normal DNA was extracted from whole blood using the DNA QIAamp midi kit (Qiagen). Tumor

Fig. 1. p21^\text{wdm} immunostaining in whole tumor and basal-like cell layer. First example: percentage of labeled cells is evaluated at 40% in whole tumor (A) but <1% in basal-like cell layer (B). Second example: percentage of labeled cells is evaluated at 60% in both whole tumor (C) and basal-like cell layer (D).
cells were isolated from biopsies using a PixCell laser capture microdissection system (Arcturus), and DNA was extracted using the DNA QIAamp micro kit (Qiagen).

Exons 18 to 21 (inclusive) of the EGFR gene were amplified by PCR and sequenced from both normal and tumor DNA. Forward and reverse PCR primers were 5’-ATGTTAGGGCGTGAGGTGAC-3’ and 5’-GiGTITGGAAAACTTCACTGTIT-3’ for exon 18, 5’-TAACATCCACCGAGATCA-TG-3’ and 5’-TCTGCTCTAGACCCTGCTCAT-3’ for exon 19, 5’-ATCGATCATGTGGTCTCAGTCC-3’ and 5’-AGGAGCCAGGATCTCCCTCA- CAT-3’ for exon 20, and 5’-ATAAATCGCCAGTCCGAGA-3’ and 5’-TTTTCCTGACACCAGGGACCA-3’ for exon 21. A M13 primer sequence was incorporated at the 5’ end of all PCR primers to facilitate subsequent DNA sequencing. Each 100-µl PCR reaction contained template DNA (25 ng from normal tissue; 5 µl (not quantified) of sample extracted from tumor tissue), 0.2 mmol/L of deoxynucleotide triphosphate, 0.5 µmol/L of each primer, and 2.5 units of Taq DNA polymerase (HotStarTaq, 5 units/μl; Qiagen) in 1 × reaction buffer. The PCR conditions were as follows: initial incubation at 95°C for 15 min followed by 36 amplification cycles (45 s at 94°C, 45 s at 60°C, and 1 min at 72°C) and a final elongation at 72°C for 10 min. PCR products were sequenced using the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems) with forward or reverse M13 primers. Each reaction contained 6 µl of purified PCR product, 8 µl of sequencing mix, 4 µl of 1 µmol/L M13 reverse or forward primer, and 2 µl of water. Sequencing reactions were carried out as follows: 5 min at 96°C followed by 25 cycles of amplification (30 s at 96°C, 30 s at 50°C, and 4 min at 60°C). Products were analyzed using an ABI Prism 3100 genetic analyzer (Applied Biosystems).

Statistical analysis. Immunohistochemistry results (i.e., IRS score) before and after treatment were compared using repeated measures ANOVA. Correlations between clinical response and toxicity grade versus pharmacokinetic data were also assessed using ANOVA. Statistical relationships between EGFR genotype or categorical immunohistochemistry results (i.e., IRS ≤3 or >3) or toxicity grade and response to treatment were assessed using a χ² test. EGFR gene copy number was compared in responders versus nonresponders using a Mann-Whitney test because of heteroscedasticity.

Results

Patient characteristics. Between November 2003 and December 2005, 35 patients were included in the study, four of whom subsequently withdrew consent. The baseline characteristics of these patients are shown in Table 2. The median treatment duration among the 31 evaluable patients was 20 days (range, 8-29 days). Excluding nine patients who discontinued treatment for toxicity (6) or noncompliance reasons (3), the median time on study was 23 days (range, 18-29 days). Of the nine patients who discontinued, four restarted erlotinib at 100 mg/day after interruption for toxicity (3) or noncompliance (1).

Toxicity. Among 31 patients assessable for toxicity, grade 3 pruritus associated with grade 2 rash (most often maculopapular) was observed in six patients (19%), leading to treatment cessation. Three patients agreed to restart erlotinib at 100 mg/day after several days, when rash had diminished to grade 1. There were no other grade 3 to grade 4 adverse events. Other frequent toxicities included grade 1 asthenia (26%) and grade 1 diarrhea in a further two patients. There were no treatment-related deaths.

Efficacy. Efficacy was assessed for 31 patients. Clinical and radiologic tumor shrinkage was observed in nine patients: five partial responses and four cases of 25% to 50% tumor shrinkage. Because of the short treatment duration, all nine patients (29%) were considered as responders, all of whom received continuous erlotinib treatment from pan-endoscopy to the day of surgery. Among the remaining patients considered as nonresponders, two (6%) had progression (25% and 28% increases in tumor volume) and the other 20 (65%) had stable disease. Figure 2 illustrates the tumor size modification observed in the 31 patients. They all underwent surgery after treatment without any delay in the routine procedure. No additional postoperative morbidity was observed.

There was no correlation between toxicity and response to treatment. Among the nine responder patients, only three underwent toxicity with a grade 2, whereas 12 of the 22 nonresponder patients experienced grade 2 or grade 3 skin rash.

Pathologic examination and immunohistochemistry. Immunohistochemistry results were available for the 31 patients who were evaluable for efficacy (Table 3). All tumors tested expressed EGFR at a moderate to strong level before erlotinib treatment. Changes in tumor immunohistochemistry profiles after erlotinib treatment were reported in Table 3. Substantial decreases in phosphorylated Tyr and phosphorylated erk 1/2 IRS were observed after erlotinib treatment (significant for phosphorylated erk). It is worth noting that no specific changes in immunohistochemistry profiles were observed in responders versus nonresponders (data not shown).

To identify potential predictive markers, various pre-erlotinib variables were compared with clinical outcome. There was no significant correlation between clinical outcome and either morphologic criteria, level of tumor differentiation, proliferation index, or anatomic sublocalisation (data not shown). However, the preerlotinib IRS for p21val was significantly (P = 0.0003; power, 0.9753; z = 0.05), positively correlated with clinical response to treatment. Using a receiver operating characteristic curve, we determined a cutoff of three for the IRS, which produced a statistically significant test (χ² = 17.559; P = 0.00003) that predicted tumor response to treatment with a sensitivity of 1, a specificity of 0.81, a positive predictive value of 0.69, and a negative predictive value of 1 (Table 4). None of the other immunohistochemistry data were predictive of tumor response.

CISH. Thirty patients of 31 were assessable for CISH analysis. The mean copy number of EGFR gene was 2.6 per cell (range, 1.6-6.9). Patients who respond to treatment (n = 9)

Table 2. Patient characteristics at baseline

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enrolled patients</td>
<td>35</td>
</tr>
<tr>
<td>Age (y), range/median</td>
<td>40-81/54</td>
</tr>
<tr>
<td>Male/female</td>
<td>32/3</td>
</tr>
<tr>
<td>Eastern Cooperative Oncology Group performance status</td>
<td>0 15</td>
</tr>
<tr>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>Site of primary tumor</td>
<td>Oral cavity 17</td>
</tr>
<tr>
<td>Oropharynx 7</td>
<td></td>
</tr>
<tr>
<td>Hypopharynx 5</td>
<td></td>
</tr>
<tr>
<td>Larynx 6</td>
<td></td>
</tr>
<tr>
<td>Smoking history</td>
<td>Never smokers 5</td>
</tr>
<tr>
<td>Former smokers 6</td>
<td></td>
</tr>
<tr>
<td>Current smokers 20</td>
<td></td>
</tr>
<tr>
<td>Unknown 4</td>
<td></td>
</tr>
<tr>
<td>Use of anticadics</td>
<td>Treated/not treated/unknown 3/31/1</td>
</tr>
</tbody>
</table>
were likely to have a slightly but not significant higher number of copies than nonresponders (2.98 copies versus 2.42 copies; \( P = 0.87 \)).

There was no correlation between EGFR gene copy number and IRS score for EGFR expression.

According to the classification previously proposed (22), 21 patients were nonamplified (EGFR copy number, <2.8), six presented a low polysomy (copy number, between 2.8 and 4.5), and three had a high polysomy (copy number, between 4.5 and 7.1). There was no difference in drug response or EGFR expression between these three groups. Two of the three patients with high polysomy were responders; however, this trend was not significant.

**Pharmacokinetic study.** Two hours after erlotinib administration on day 1, the mean (SD) plasma concentrations \((C_{2h})\) of erlotinib and its major metabolite OSI-420 were 966.8 (409.0) ng/mL and 74.3 (25.4) ng/mL, respectively \((n = 31)\). On the day of surgery, erlotinib and OSI-420 trough plasma concentrations were 614.2 (607.7) ng/mL and 49.6 (57.5) ng/mL, respectively \((n = 21)\). There was no significant relationship between erlotinib or OSI-420 plasma concentrations and either grade of toxicity or clinical outcome (data not shown). A trend of higher plasma erlotinib concentrations in responder versus nonresponder patients was observed for \(C_{2h}\) (1162.9 ng/mL versus 886.5 ng/mL, \( P = 0.09 \), respectively), but not for trough concentrations (545.1 ng/mL versus 656.7 ng/mL, respectively). There was no significant relationship between either \(C_{2h}\) or trough erlotinib concentration and toxicity grade.

Treatment with antacids had no effect in the levels of erlotinib exposure, but the two nonsmoker patients had higher trough erlotinib plasma concentrations (i.e., 728 and 1,920 ng/mL) significantly higher than smokers (mean value, 418 ng/mL).

**EGFR gene sequencing.** No mutations in exons 18 to 21 of the EGFR gene were detected in either normal or tumoral DNA from 31 patients. A common single-nucleotide polymorphism in exon 20, position 2,607 (NM_005228.3) was identified in 26 patients. Five patients were homozygous wild-type (G-G), nine were heterozygous (G-A), and fourteen were homozygous.

### Table 3. IRS scores from immunohistochemistry analyses before and after erlotinib treatment \((n = 31)\)

<table>
<thead>
<tr>
<th>Antigen</th>
<th>No. positive samples (%)</th>
<th>IRS results before erlotinib treatment</th>
<th>IRS results after erlotinib treatment</th>
<th>Change in IRS after erlotinib treatment (R M ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean ± SD</td>
<td>Range</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>EGFR</td>
<td>31 (100)</td>
<td>9.87 ± 3.21</td>
<td>3-12</td>
<td>9.90 ± 3.34</td>
</tr>
<tr>
<td>Phosphorylated EGFR</td>
<td>27 (87)</td>
<td>4.25 ± 3.25</td>
<td>0-12</td>
<td>3.48 ± 2.93</td>
</tr>
<tr>
<td>Phosphorylated Tyr</td>
<td>29 (94)</td>
<td>5.74 ± 3.78</td>
<td>0-12</td>
<td>3.80 ± 3.23</td>
</tr>
<tr>
<td>Phosphorylated Akt</td>
<td>25 (81)</td>
<td>2.87 ± 2.88</td>
<td>0-12</td>
<td>2.48 ± 2.09</td>
</tr>
<tr>
<td>STAT3</td>
<td>30 (97)</td>
<td>5.73 ± 3.23</td>
<td>0-12</td>
<td>5.83 ± 3.80</td>
</tr>
<tr>
<td>STAT5b</td>
<td>29 (94)</td>
<td>5.03 ± 3.74</td>
<td>0-12</td>
<td>4.87 ± 3.74</td>
</tr>
<tr>
<td>Phosphorylated erk 1/2</td>
<td>23 (74)</td>
<td>2.48 ± 2.69</td>
<td>0-9</td>
<td>1.12 ± 1.52</td>
</tr>
<tr>
<td>p21/WAF</td>
<td>29 (94)</td>
<td>3.25 ± 2.71</td>
<td>0-12</td>
<td>3.74 ± 2.95</td>
</tr>
<tr>
<td>p27</td>
<td>29 (94)</td>
<td>2.38 ± 1.56</td>
<td>0-6</td>
<td>2.93 ± 2.44</td>
</tr>
</tbody>
</table>

**Fig. 2.** Percentage of tumor size modification observed for each patient.

Abbreviations: R M ANOVA, repeated measures ANOVA; ns, not significant.
variant (A-A) in both normal and tumor DNA. For three patients, the polymorphism was present only in tumor DNA (heterozygous). This frequent polymorphism does not lead to a change in the protein sequence. There was no correlation between the presence of this polymorphism in either normal or tumor DNA and response to treatment.

**Discussion**

One of the major challenges in the field of EGFR-targeted therapy is the identification of those patients most likely to derive clinical benefit. A great deal of research has been done to investigate potential predictive factors for EGFR tyrosine kinase inhibitors in advanced NSCLC. However, to date, little information is available regarding predictive factors for these drugs in HNSCC. Neoadjuvant treatment of patients with HNSCC provides a convenient setting for assessing the pharmacokinetic-pharmacodynamic relationships of targeted therapies.

In this study, a short period (18-29 days) of neoadjuvant treatment with erlotinib was well tolerated in patients with nonmetastatic HNSCC. Importantly, neoadjuvant erlotinib did not necessitate any modifications to routine surgical procedures. Of 31 patients who were assessable for efficacy, nine (29%) had tumor shrinkage. A further 65% of patients had stable disease at the time of surgery; because of the relatively short treatment period, it is uncertain whether any of these patients would have obtained a tumor response with prolonged therapy. Improvements in symptoms and general health were experienced by 7 of 10 patients with symptomatic tumor involvement.

An important secondary objective of this trial was to obtain pretreatment and posttreatment samples for pharmacokinetic and molecular analysis and to study pharmacokinetic-pharmacodynamic relationships and potential factors predictive of response to erlotinib. There was no significant correlation between clinical response or grade of toxicity and the levels of concentrations of erlotinib or its major metabolite (OSI-420). In a previous study of erlotinib in patients with recurrent or metastatic HNSCC, there was also no correlation between pharmacokinetic variables and toxicity, although the median plasma concentrations at 5 to 10 h postdose for erlotinib and OSI-420 did seem to predict for improved survival (12). Pharmacokinetic-pharmacodynamic relationships will be further studied using a population approach to account for patient characteristics; this might explain the interindividual pharmacokinetic variability observed.

Several studies have shown that mutations in the tyrosine kinase domain of EGFR are associated with response to gefitinib or erlotinib in patients with advanced NSCLC (5, 23, 24). However, any possible relationship between mutation status and survival with EGFR tyrosine kinase inhibitor is less clear. In the present study, we did not identify any tyrosine kinase domain mutations, either in normal or tumor tissue. Previous reports have shown that EGFR tyrosine kinase mutations occur more commonly in patients who have never smoked and in tumors of adenocarcinoma histology and therefore may be associated with a particular route of oncogenesis in NSCLC. These mutations may correspond to a particular molecular phenotype that is less common in HNSCC tumors, as shown by Loeffler-Ragg et al. (17). Moreover, Cohen et al. (16) did not identify any EGFR mutations in HNSCC patients responsive to erlotinib or gefitinib.

Our results did not show that EGFR gene copy number was correlated with clinical response contrary to previous reports in NSCLC (7, 25). However, in our cohort of HNSCC patients, only three patients had a high polysomy and no patients presented a gene amplification. Similar percentages of EGFR amplification were recently described in HNSCC by Agulnik et al. (20), and they suggest that high EGFR gene copy number may predict for a better response to erlotinib, although this trend was not significant. Interestingly, two of our three patients with high polysomy experienced clinical response. One can hypothesize that EGFR amplification and/or a high polysomy may predict clinical response to erlotinib. However, the number of patients with this gene amplification and/or high polysomy in our study is too weak, and this hypothesis needs to be tested in larger cohorts of HNSCC patients. On the other hand, seven patients experienced clinical response without any EGFR gene amplification, which suggests that other biomarkers should be taken into account.

For immunohistochemistry analyses, we evaluated immunostaining in the whole tumor and in the basal-like cell layer of the tumor. Results are only presented here for staining in the basal-like cell layer because staining of this layer was more reproducible and independent of tumor differentiation (data not shown). By comparing the immunohistochemistry profiles of tumor tissue samples taken before and after treatment, we showed that erlotinib effectively inhibits EGFR signaling in the tumor, with substantial reductions observed in both phosphorylated Tyr and phosphorylated erk 1/2 after treatment. These data confirm that the dose of erlotinib used in this study was sufficient to achieve effective inhibition of the target. We also showed that tumor p21waf immunostaining at baseline was correlated with clinical response to neoadjuvant erlotinib. In squamous cell carcinoma cell lines, the cytostatic effect of erlotinib is mediated by p21waf (26). Furthermore, it has been reported that loss of p21waf expression is correlated with poor prognosis in squamous cell carcinoma (27). Our data suggest that in HNSCC, p21waf expression is an important factor in mediating the inhibition of EGFR signaling by erlotinib as it has been shown previously with gefitinib in vitro (28). Interestingly, the correlation between p21waf immunostaining and clinical outcome was strongest when considering staining only in the basal-like cell layer of the tumor, compared with staining in the whole tumor (data not shown). It may be hypothesized that this cell layer contains the proliferative and invasive power of the tumor and thus represents the major target of EGFR tyrosine kinase inhibitor in HNSCC. Further study of p21waf as a potential predictive marker in patients with HNSCC is warranted. In particular, it will be important to

<table>
<thead>
<tr>
<th>p21waf IRS</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-3</td>
<td>18</td>
</tr>
<tr>
<td>4-12</td>
<td>13</td>
</tr>
<tr>
<td>Total</td>
<td>31</td>
</tr>
</tbody>
</table>

Table 4. Clinical outcome on erlotinib therapy according to the preerlotinib IRS for p21waf in the basal-like cell layer, using 3 as a cutoff.
determine whether p21\textsuperscript{raf} immunostaining correlates with improved survival, because studies in both HNSCC and NSCLC have shown that tumor response is not a good surrogate of survival (6, 29).

We believe that, as suggested previously (30), the design of this study is particularly suited to the investigation of targeted agents, such as erlotinib. The collection of large pretreatment and posttreatment tumor samples provides the opportunity to test many hypotheses that can be further investigated by additional retrospective analyses and ultimately validated in prospective studies. Important questions that may be addressed using the “pilot” neoadjuvant study approach include determination of a biologically effective dose, preliminary assessment of antitumor activity, and the identification of potential factors predictive of response. The present study confirmed the feasibility of this approach and identified a potential factor (p21\textsuperscript{raf} immunostaining) that could assist oncologists and radiotherapists in decisions regarding adjuvant treatment in HNSCC. Because patients in this study had at least stage T2 tumors, all received adjuvant radiotherapy. An interesting future study would be to compare long-term adjuvant erlotinib with observation in patients who are most likely to respond to erlotinib.

In conclusion, the use of neoadjuvant erlotinib was well tolerated in patients with nonmetastatic HNSCC. Further study is necessary to determine whether the encouraging antitumor activity noted in this study produces clinical benefits for patients, such as improved survival or improved resection rates.

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Fabienne Thomas, Philippe Rochaix, Adil Benlyazid, et al.


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