Assessment of erlotinib pharmacodynamics in tumors and skin of patients with head and neck cancer

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Background: The purpose of the study was to evaluate the effects of erlotinib on epidermal growth factor receptor (EGFR)-related signaling elements in tumor and skin from patients with advanced squamous cell carcinoma of the head and neck (HNSCC) and seek relationships between relevant clinical, biological, and pharmacokinetic parameters.

Patients and methods: Immunostaining for EGFR, p-EGFR, p-ERK, p-Akt, and p27 were analyzed semiquantitatively in serial tumor and skin samples from participating patients. Steady-state trough concentrations of erlotinib and its metabolite OSI-420 were also determined.

Results: Of 25 patients enrolled, 20 (80%) paired pre- and posttreatment skin biopsies and seven (28%) paired tumor biopsies were evaluable for at least one immunohistochemical parameter. The severity of skin toxicity related to time to progression (TTP) (P = 0.048) and overall survival (P < 0.001). Cmin values for erlotinib and OSI-420 also related to TTP (P = 0.042 and 0.036, respectively). Erlotinib treatment was associated with decreased p-EGFR expression in 66% of evaluable tumor samples, which seemed related to increased TTP and survival, and p27 was up-regulated in 59% of evaluable skin biopsy samples following treatment.

Conclusions: The feasibility of obtaining serial evaluable biopsies of HNSCC was suboptimal. Nevertheless, erlotinib inhibited p-EGFR in HNSCC tumors, which appeared associated to clinical benefit, and induced p27 in biopsies of normal skin.

Key words: EGFR, erlotinib, head and neck cancer, pharmacodynamics, translational introduction

Despite the availability of many nonspecific cytotoxic chemotherapeutics to treat patients with recurrent or metastatic squamous cell carcinoma of the head and neck (HNSCC), the impact of these agents on other indices indicative of clinical benefit has been negligible, and effective rationally designed target-based therapies may portend greater therapeutic indices [1]. Erlotinib, an oral low molecular weight quinazoline and highly specific and potent inhibitor of the epidermal growth factor receptor (EGFR) tyrosine kinase (TK), has demonstrated notable antiproliferative activity in a wide range of malignancies in preclinical and clinical studies [2–5]. The profound inhibitory effects of erlotinib on EGFR TK and cell proliferation have been associated with accumulation of the cell cycle regulator p27, cell cycle arrest, and apoptosis in preclinical studies [6, 7]. Preclinical studies conducted with erlotinib have demonstrated maximum in vivo inhibition of EGFR autophosphorylation in mice bearing HN5 human HNSCC xenografts 1 h after a single dose of 100 mg/kg, which remains significantly inhibited at 24 h. Furthermore, the tumor growth inhibition is strongly linked to inhibition of EGFR TK, and both effects have been related to the magnitude of erlotinib plasma concentrations [6, 7].

The results of a phase 1 study of erlotinib indicated that diarrhea and an acneiform rash were the principal toxic effects of erlotinib and a daily oral dose of 150 mg was determined to be the maximum tolerated and recommended dose for disease-directed studies [8]. This was further confirmed in a multicenter phase 2 study of erlotinib in 115 heavily pretreated HNSCC patients, in which erlotinib produced partial response (PR) in 4.3% [95% confidence interval (CI), 1.4% to 9.9%] and stabilization of disease in an additional 38.3% (median duration, 16.1 weeks) of patients [9]. An exploratory subgroup analysis indicated that survival related to the severity of skin toxicity. The median survival of patients who had at least grade 2 skin toxicity was 7.4 months compared with 4.0 months for patients without skin toxicity (P = 0.045). The results of previous studies also related skin toxicity to the exposure of...
erlotinib [8]. Furthermore, the results of translational studies carried out in the context of the phase 1 evaluation of erlotinib, in which 56 skin biopsies were collected from 28 patients treated with erlotinib at doses ranging from 25 to 200 mg/day, demonstrated biological effects in the skin of 40% of patients, consisting of up-regulated expression of p27 and reduced phosphorylation of EGFR.

On the basis of the results of preclinical and exploratory translational and clinical studies, the present evaluation was carried out to obtain more robust biological and pharmacodynamic information on the effects of erlotinib 150 mg/day in a homogenous group of patients. This supplemental study, in which serial tumor and skin biopsies and plasma sampling for pharmacokinetics were obtained, was carried out in conjunction with a multicenter phase 2 study of erlotinib in patients with HNSCC [9]. The principal objectives were to evaluate the biological effects of erlotinib on EGFR activation and signaling in skin and tumor tissues of patients with HNSCC, the concordance of serial effects in skin and tumor, and to seek relationships between relevant biological and pharmacokinetic parameters, toxicity, and clinical benefit.

patients and methods

study

This study was carried out at two institutions that participated in a multicenter phase 2 study of erlotinib (“Tarceva”® OSIPharma Inc., Melville, NY) in patients with recurrent or metastatic HNSCC. The clinical results of the study have been reported previously, but neither the clinical nor the biological results of this supplemental translational study, which was conducted after the clinical study, have been reported [9].

patients

The candidates for this study were patients with advanced HNSCC who were enrolled and treated in the referred multicenter phase 2 trial of erlotinib, provided that their neoplasms had demonstrated EGFR-positive immunostaining, as defined previously, and were amenable to serial tissue sampling [9]. The precise eligibility criteria, design of the principal clinical trial from which this study was derived, and both the clinical and pharmacological results of the principal study have been reported [9]. Briefly, patients might have had prior induction or concurrent chemotherapy and up to one palliative regimen before entry into the study, in addition to adequate Eastern Cooperative Oncology Group (ECOG) performance status ranging from zero to two and adequate hematopoietic, hepatic, and renal functions. Patients could not have had chemotherapy within 28 days before the start of erlotinib treatment. Bidimensionally measurable disease was also an eligibility requirement. Eligible patients were treated with erlotinib at a dose of 150 mg daily. Each 28-day period was arbitrarily defined as one cycle of treatment.

A complete history and physical examination, including a skin assessment and complete standard blood profile and urination, were carried out at screening, and these assessments were repeated weekly from weeks 1 to 5, and every 4 weeks thereafter. Immunostaining for EGFR was carried out on either the original paraffin-embedded or a newly biopsied tumor specimen by a central laboratory (IMPATH Inc., Los Angeles, CA). Tumor evaluations were carried out within 28 days before treatment and every 8 weeks during treatment. Patients were able to continue treatment if they did not develop progressive disease. A complete response (CR) was defined as the disappearance of all active disease on two measurements separated by a minimum period of 4 weeks. A PR was defined as at least a 50% reduction in the sum of the bidimensional product of all measurable disease documented by measurements separated by at least 4 weeks without the appearance of new lesions. Progressive disease was defined as a 25% increase in the sum of the product of the bidimensional measurements of all measurable disease. All patients gave written informed consent before entry into the study, in accordance with federal and institutional guidelines.

acquisition and immunohistochemistry analysis of the skin and tumor tissues

Tru-cut skin and tumor biopsies were carried out before treatment with erlotinib and after the last dose of the first 28-day cycle using standard techniques. Biopsies were obtained using an 8-mm (depth) × 4 mm (width) punch biopsy device. Specimens were processed as described previously [10]. Samples were stored at −80°C until analysis. Tissues were fixed in 10% neutral buffered formalin for 24 h and processed into paraffin blocks. Total EGFR, ERK, and Akt, as well as phosphorylated EGFR (Tyr1173), ERK (Thr202/Tyr204), and Akt (Ser 473), were assessed on 4-µm sections of skin and tumor biopsies. In addition, p27 immunostaining was assessed in the skin and tumor biopsies as described previously [10].

Sections were heated to 60°C and dehydrated in xylene and graded alcohols. Antigen retrieval was carried out with 0.01 M citrate buffer (pH 6.0) at 95°C for phospho-EGFR (Upstate Biotechnology, Lake Placid, NY), p27 (Zymed, San Francisco, CA), ERK (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-ERK (Cell Signaling Technology, Danvers, MA), Akt (Santa Cruz Biotechnology, Santa Cruz, CA) and phospho-Akt (Cell Signaling Technology, Danvers, MA); proteinase K for 5 min for EGFR (Zymed). Sections were incubated in primary antibody diluted in 50 nM Tris–HCl (pH 7.6), 150 mM NaCl, Tween 20 (0.1%) (TBS-T) containing 1% ovalbumin and 1 mg/ml sodium azide (12 h), followed by incubations with biotinylated secondary antibody for 15 min, peroxidase-labeled streptavidin for 15 min (LSAB-2 System Dako Corp, Carpenteria, CA) and diaminobenzidine and hydrogen peroxide chromogen substrate (Dako Corp, Carpenteria, CA) along with DAB enhancer (Signet, Dedham, MA) for 10 min. Slides were counter stained with hematoxylin and mounted. The negative controls were incubated with immunoglobulin fraction (normal mouse immunoglobulin). The intensity of staining in each sample was assessed semiquantitatively for every staining by scoring 0 (no staining), + (10%–20% staining of skin or tumor cells), 2+ (20%–50% staining of cells), and 3+ (>50% of cells stained). The two pathologist coauthors (JK and SM) conducted scoring in a blinded fashion.

pharmacological analysis

Plasma concentrations of erlotinib and its metabolite OSI-420 were determined at various times relative to drug administration, following the schema described in the principal study [9]. Briefly, blood was sampled randomly using one of three possible sampling windows including trough (20–25 h after treatment or before treatment), maximum (C_{max}) (2–5 h after treatment) and 5–10 h after treatment. A randomization scheme was provided to specify the drug administration times relative to clinic visits for each individual patient; thus, the pharmacokinetic sampling scheme should have covered all three windows for each patient during the study. Blood samples were centrifuged immediately after collection, and the plasma was stored at −80°C until analysis.

The plasma samples were analyzed by MDS Pharma Services (St Laurent, Quebec, Canada) for erlotinib and OSI-420 using validated liquid chromatography/mass spectrometry/mass spectrometry assays as described previously [8]. The trough plasma concentration at steady state (C_{min}) for each individual was calculated as the average of all pretreatment (i.e. trough sampling window) concentrations from day 8 until the discontinuation of treatment.
statistical analysis

Pretreatment and on-therapy scores for the quantitative variables EGFR, p-EGFR, ERK, p-ERK, Akt, p-Akt, and p27 in tumor and skin biopsy samples were assessed using descriptive distributional frequencies. Hypothesis testing and formal comparative analyses were not carried out on these parameters due to the limited sample size. Furthermore, the requirement for repetitive multiple testing carried out was felt from the outset to limit formal statistical comparisons and interpretation of such results.

Median time to progression (TTP) and overall survival (OS) proportions were calculated using the Kaplan–Meier method. Differences in TTP and OS between groups of patients with regard to biopsy parameters or intensity of skin rash were assessed using the log-rank test. The Cox proportional hazards model was then employed to examine the relationships between TTP and OS and relevant pharmacokinetic parameters relating to erlotinib exposure.

All statistical analyses were conducted using the SAS System for Windows, version 8.0 (SAS/STAT User’s Guide, Version 8.0; SAS Institute, Cary, NC, 1990).

results

general

Twenty-five patients with advanced HNSCC were enrolled onto this biological and pharmacodynamic study. Patient characteristics did not differ significantly from those of patients who participated in the parental phase 2 study [9]. The median ECOG performance status was one (range 0–2); the median age was 54 years (range 15–88); and the ratio of male to female patients was 17:8. The ratio of male to female patients was significant (95% CI 1% to 26%).

Of the 25 patients enrolled in the study, two patients experienced complete and PRs respectively [objective response rate, 8% (95% CI 1% to 26%)]. Nine (36%) patients had stable disease as their best response. The median TTP for all patients was 11.3 weeks (95% CI 7.4–15). The OS was 21.1 weeks (95% CI 11.4–38.3).

Rash and diarrhea, which occurred in 19 (76%) and 5 (20%) patients, respectively, were the principal drug-related toxic effects. The severity of these erlotinib-related toxic effects was, however, mild or moderate in most cases. With regard to skin toxicity, 12 (38%) and 6 (24%) patients experienced National Cancer Institute Common Toxicity Criteria (NCI CTC) (Version 2.0) grades 1 and 2 skin toxicity, respectively, whereas one patient (4%) developed grade 3 toxicity.

Table 1. Feasibility of immunohistochemical assessments in tumor and skin

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Relationship to treatment</th>
<th>EGFR Total number of evaluable samples (number of evaluable paired samples for assay)</th>
<th>p-EGFR</th>
<th>ERK Total number of evaluable samples (number of evaluable paired samples for assay)</th>
<th>p-ERK</th>
<th>Akt Total number of evaluable samples (number of evaluable paired samples for assay)</th>
<th>p-Akt</th>
<th>p27</th>
<th>Fully evaluable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin</td>
<td>Before treatment</td>
<td>24 (20)</td>
<td>24 (20)</td>
<td>24 (20)</td>
<td>24 (18)</td>
<td>24 (20)</td>
<td>23 (19)</td>
<td>23 (19)</td>
<td>22 (17)</td>
</tr>
<tr>
<td></td>
<td>After treatment</td>
<td>20 (20)</td>
<td>20 (20)</td>
<td>20 (20)</td>
<td>18 (18)</td>
<td>20 (20)</td>
<td>20 (19)</td>
<td>20 (19)</td>
<td>18 (17)</td>
</tr>
<tr>
<td>Tumor</td>
<td>Before treatment</td>
<td>13 (7)</td>
<td>13 (6)</td>
<td>11 (3)</td>
<td>11 (4)</td>
<td>10 (3)</td>
<td>11 (3)</td>
<td>6 (2)</td>
<td>6 (2)</td>
</tr>
<tr>
<td></td>
<td>After treatment</td>
<td>11 (7)</td>
<td>9 (6)</td>
<td>7 (3)</td>
<td>8 (4)</td>
<td>7 (3)</td>
<td>7 (3)</td>
<td>4 (2)</td>
<td>4 (2)</td>
</tr>
<tr>
<td></td>
<td>Fully evaluable</td>
<td>6</td>
<td>5</td>
<td>2</td>
<td>2</td>
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<td>1</td>
<td>1</td>
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EGFR, epidermal growth factor receptor.

The feasibility of obtaining evaluable biopsy samples from skin and tumor tissues is reflected in the data presented in Table 1. Although the principal objective of the study was to assess the biological and pharmacodynamic effects of erlotinib in serially sampled skin and tumor tissues obtained before and after treatment, paired skin and tumor biopsies were assessable for at least one immunohistochemical determinant in 20 (80%) and seven (28%) patients, respectively. Of these, 17 (68%) paired skin biopsies and two (8%) paired tumor biopsies were assessable for all designated studies, and fully evaluable paired samples from both cutaneous and tumor biopsies were available from only a single subject. At least most (89%) of the samples were obtained and collected as planned, insufficient valid tissue for immunohistochemistry analysis precluded adequate evaluation of many of the molecular end points.

Pretreatment skin and tumor biopsies without evaluable posttreatment samples were obtained from four (16%) and six (24%) additional patients, respectively, while posttreatment tumor biopsies without evaluable pretreatment samples were available in four (16%) additional patients. Of those 10 patients with unpaired tumor biopsies, posttherapy tumor biopsies were not carried out in three (12%) patients who were taken off study before day 28 due to disease progression, and seven (28%) patients had insufficient, if any, tumor tissue in their paired pre-or posttreatment biopsy samples for the designated analyses.

effects of erlotinib on signaling elements

The results of immunohistochemistry studies to assess the effects of erlotinib on the EGFR, as well as phosphorylation of the EGFR (p-EGFR), ERK (p-ERK), and Akt (p-Akt), and induction of p27 expression in both skin and tumor samples are shown in Figures 1 and 2. The intensity of EGFR immunostaining in skin and tumor tissues was apparently not affected by erlotinib treatment. Decrements in the intensity of p-EGFR immunostaining were, however, evident in seven (35%) of 20 successively sampled skin biopsies and, as shown in Figure 3, in four (66%) of six serially sampled, evaluable head and neck tumors during erlotinib treatment.

The effects of erlotinib treatment on activated (phosphorylated) signaling pathway elements downstream of the EGFR were assessed by the intensity of p-ERK and p-Akt immunostaining during erlotinib treatment. The intensity of p-ERK immunostaining decreased during erlotinib treatment in seven (38.8%) of 18 successively sampled skin tissues, whereas decrements in p-Akt immunostaining were evident in five (26.3%) of 19 paired skin samples. These analyses were much
more limited in tumor tissues because of the small number of paired pre- and posttreatment tumor samples that were evaluable. The effects of erlotinib on the cyclin-dependent kinase inhibitor p27 were assessed. p27 expression was up-regulated following erlotinib treatment in 11 (59%) of the 19 paired skin samples. Only two tumor biopsies were evaluable for paired comparisons of drug effects on p27.

clinical benefit
Both TTP and OS were related to the severity of skin toxicity as measured by NCI CTC grade (0, 1, or ≥2). Kaplan–Meier curves depicting TTP and OS as a function of skin rash intensity for the 25 patients who participated in the study are shown in Figure 4. For patients whose skin rash intensity was graded as NCI CTC 0, 1, and ≥2, median TTP values were 1.6, 2.6, and 4 months, respectively (P = 0.048). Respective values for OS were 1.9, 6.7, and 10.3 months (P < 0.0001). There was also a trend for patients with more severe skin toxicity to have more pronounced objective antitumor activity (N = 22, P = 0.061). The severest NCI grade of skin toxicity of the two patients who had CR or PRs was two, whereas the median skin rash intensities of patients who had stable disease and progressive disease as their best response were one (range 1–2) and one (range 0–3), respectively.

TTP was also related to the magnitude of $C_{ss,min}$ for both erlotinib and OSI-420 (N = 18, P = 0.042 and 0.036, respectively). However, although OSI-420 $C_{ss,min}$ values were also related to OS (N = 18, P = 0.019), no relationship between erlotinib $C_{ss,min}$ values and OS was apparent (N = 18, P = 0.35). Additionally, categorical response was not related to $C_{ss,min}$ values for either erlotinib or OSI-420 (N = 16, P = 0.283 and 0.663, respectively), but the small numbers of patients in each objective response category limit the power of this analysis. $C_{ss,min}$ values for neither erlotinib nor OSI-420 correlated with the severity of skin toxicity as assessed by NCI CTC grade (N = 18, P = 0.736 and 0.542, respectively).

Relationships between clinical benefit and treatment-related changes in the activated signaling elements p-EGFR, p-ERK, p-Akt, and p27 expression in both skin and tumors were evaluated. Both TTP and OS were longer in the four patients in whom p-EGFR immunostaining in tumor tissues decreased following a 28-day course of erlotinib treatment, when compared with the other two assessable patients without
p-EGFR decrements (9.8 versus 4 weeks and 25.7 versus 6.6 weeks, respectively), as shown in Figure 5. There was also an apparent prolonged TTP in patients in whom p27 expression in skin had increased following treatment with erlotinib [18.5 versus 10.3 weeks, \( P = 0.091, N = 19 \)]. No other relationships between clinical benefit end points and immunohistochemical results were apparent, but the small numbers of paired tumor samples and repetitive testing limited the statistical power and interpretation of these analyses.

**Discussion**

Despite the study’s principal objective of obtaining serial biopsies of HNSCC, a tumor in which repeated sampling is generally considered feasible, the proportion of patients with paired pre- and posttreatment samples that were definitely evaluable for immunostaining of activated EGFR and downstream signaling elements was surprisingly low. For example, the effects of erlotinib on EGFR phosphorylation could be evaluated in only 80% and 24% of paired skin and tumor tissues, respectively. Since serial tumor sampling has been proposed as being critical in the efficient and optimal development of a wide range of rationally designed target-based anticancer therapeutics, it is important to note that the feasibility of evaluating such studies in this trial, as well as in other carefully conducted translational evaluations with end points mandating serial tumor sampling has been disappointing [11, 12]. One potential reason for this low yield of evaluable tissues is that some of the samples were obtained by fine needle biopsy, due to safety reasons, and without image guidance (data not available). Image-guided core needle biopsies may maximize the feasibility of those pharmacodynamic studies that are on the basis of tumor tissue sampling, as recently reported [13]. Furthermore, the high proportion of patients treated with radiation, as well as those who had demonstrable tumor regression following treatment with erlotinib and other treatments, increases the likelihood of fibrosis in peritumoral tissues, which was indeed found in biopsies assessed in this study.

The findings of the present study that relate to both antitumor activity and toxicity, particularly the typical acneiform skin rash, do not differ from those reported in the larger parental phase 2 study of erlotinib in patients with HNSCC from which this biological study was derived [9]. As observed in previous studies of erlotinib, as well as other small molecules and mAbs targeting EGFR in patients with non-small-cell lung and colon cancers, clinical benefit related to the severity of cutaneous toxicity in the
present study [9, 11, 14, 15]. Similar to other trials, relationships between pharmacological parameters have not clearly related to the intensity of the skin rash [8, 9]. In the present study, although no clear and interpretable cutaneous pharmacodynamic relationships were evident, the \( C_{\text{ss,min}} \) values of both erlotinib and its principal metabolite OSI-420 related to TTP, and, in the case of OSI-420, to OS, but not to skin toxicity.

These observations indicate that drug exposure is a principal determinant of neither rash severity nor clinical benefit, and there are likely alternate explanations to account for the relationship between clinical benefit and skin toxicity [9, 16].

Any apparent relationships between biological evaluations in tumor tissues and clinical benefit in this study must be interpreted with caution due to the small numbers of patients with sufficient tumor tissue evaluable for biological assessments. The study, however, provides some evidence that erlotinib does inhibit phosphorylation of its putative EGFR target in the tumors of patients with HNSCC. These results complement and expand other small pharmacodynamic studies of both EGFR-targeting small molecules and mAbs in several disease settings [12, 17–19]. In addition, the results of this study are the first to indicate that drug-induced inhibition of EGFR phosphorylation in tumors might relate to clinical benefit and provide a foundation for future evaluations, with greater sample size and, perhaps, in disease settings in which the magnitude of clinical benefit is greater than HNSCC.

Another treatment-related biological effect indicated in this study was the induction of p27 in 59% of serially sampled skin biopsies. Of all the subcellular effects of EGFR-targeted therapeutics, including effects on signaling and cell cycle elements, assessed in clinical studies to date, it appears that up-regulation of p27 may be the most consistent and prominent biological effect noted in both tumors and normal tissues following treatment [10, 15, 20–23]. For example, p27 up-regulation was noted in the serially sampled skin biopsies of 56% of patients who participated in a phase 1 study of erlotinib, and this effect was dose related [25]. Furthermore, the induction of p27 in skin tissue in preclinical models appears to relate to both EGFR pathway inhibition and tumor growth arrest [24, 25].

acknowledgements

circles (•) progressed or expired].

Figure 5. Kaplan–Meier curves depicting: (A) Time to progression (TTP) and (B) Overall survival (OS) as a function of whether or not there was a decrement in p-epidermal growth factor receptor (EGFR) immunostaining in squamous cell carcinoma of the head and neck (HNSSC) tumors during erlotinib treatment. Both TTP (9.8 versus 4 weeks) and OS (25.7 versus 6.6 weeks) seemed related to decrements in p-EGFR immunostaining in tumors [Unfilled circles (○), censored; Filled circles (●) progressed or expired].

references
19. Tabernero J, Rojo F, Jimenez E et al. A phase I PK and serial tumor and skin pharmacodynamic (PD) study of weekly (q1w), every 2-week (q2w) or every 3-week (q3w) 1-hour (h) infusion ZD1839, a humanized monoclonal anti-epidermal growth factor receptor (EGFR) antibody, in patients (pt) with advanced tumors. Proc Am Soc Clin Oncol 2003; 22: 191.