Preclinical and Clinical Evidence that Deoxy-2-[^18F]fluoro-d-glucose Positron Emission Tomography with Computed Tomography Is a Reliable Tool for the Detection of Early Molecular Responses to Erlotinib in Head and Neck Cancer

Sébastien Vergez, Jean-Pierre Delord, Fabienne Thomas, Philippe Rochaix, Olivier Caselles, Thomas Filleron, Séverine Brillouet, Pierre Canal, Frédéric Courbon, and Ben C. Allal

Abstract

Purpose: There is a clinical need to identify predictive markers of the responses to epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKI). Deoxy-2-[^18F]fluoro-d-glucose positron emission tomography with computed tomography (18FDG-PET/CT) could be a tool of choice for monitoring the early effects of this class of agent on tumor activity.

Experimental Design: Using models of human head and neck carcinoma (CAL33 and CAL166 cell lines), we first tested in vitro and in vivo whether the in vivo changes in 18FDG-PET/CT uptake were associated with the molecular and cellular effects of the EGFR-TKI erlotinib. Then, the pathologic and morphologic changes and the 18FDG-PET/CT uptake before and after erlotinib exposure in patients were analyzed.

Results: Erlotinib strongly inhibited extracellular signal-regulated kinase-1/2 (ERK-1/2) phosphorylation both in the preclinical models and in patients. Western blotting, immunofluorescence, and immunohistochemistry showed that erlotinib did not modify Glut-1 expression at the protein level either in cell line models or in tumor tissue from mouse xenografts or in patients. Phospho-ERK-1/2 inhibition was associated with a reduction in 18FDG uptake in animal and human tumors. The biological volume was more accurate than the standardized uptake value for the evaluation of the molecular responses.

Conclusion: These results show that the 18FDG-PET/CT response is a reliable surrogate marker of the effects of erlotinib in head and neck carcinoma.

Epithelial growth factor receptor (EGFR) inhibitors have clinical activity in various tumor types, including head and neck squamous cell carcinoma (HNSCC), used alone or in combination with cytotoxic agents. Unfortunately, only a subgroup of patients benefit from the use of these targeted agents.

Consequently, there is a clear medical need for the early identification of those patients most likely to benefit from this targeted treatment. The development of tools to select these patients could facilitate their therapeutic cure and the determination of their biologically effective dose and, subsequently, lead to individualization of treatment.

Deoxy-2-[^18F]fluoro-d-glucose positron emission tomography with computed tomography (18FDG-PET/CT) has become an important noninvasive technique for examining cancer staging and detecting recurrent neoplasms. Many clinical trials have shown that 18FDG-PET imaging could provide an early indication of therapeutic responses that are well correlated with clinical outcomes. 18FDG-PET could be particularly useful for the evaluation of the proportion of active tumor cells during treatment with EGFR tyrosine kinase inhibitors (EGFR-TKI). Some changes in tumor volume are observed late or not at all; for example, when intratumoral necrosis and fibrosis prevent tumor shrinkage and could actually cause a paradoxical expansion of some tumors due to intratumoral bleeding or edema. This is not the case for the changes in metabolic activity of the tumors, which could be highlighted early. Indeed, some studies have evaluated the predictive value of 18FDG-PET as a consequence of the correlation of early metabolic responses with the clinical
outcome, as has been described for the response to imatinib in gastrointestinal stromal tumors (3).

Therefore, although some results indicating that molecular imaging with \(^{18}\)FDG-PET could be a valuable tool for drug development and use (3), it remains to be shown that the changes in \(^{18}\)FDG uptake correlate with the molecular responses (MR) induced by erlotinib.

Our goal was to establish the rationale of the use of \(^{18}\)FDG-PET/CT as a surrogate marker for the early evaluation of EGFR-TKI. For this purpose, we developed preclinical models to validate \(^{18}\)FDG-PET/CT imaging for the early evaluation of the molecular effects of erlotinib in head and neck squamous cell carcinoma cell lines. We then cross-validated this tool during a clinical trial designed to assess the pharmacodynamic effects of erlotinib in patients with head and neck squamous cell carcinoma who received erlotinib as neoadjuvant treatment for a short time period before surgery. This is the first study providing, from preclinical models to patients, a reliable proof of efficiency of this method.

**Materials and Methods**

**Animals and agents**

Female Swiss athymic nude mice, ages 4 to 5 weeks (Charles River Laboratories), were maintained in accordance with the standards of the Federation of European Laboratory Animal Science Associations and were included in protocols following a 2-week quarantine. Erlotinib (OSI-774, Tarceva) was kindly provided by F. Hoffmann-La Roche, Inc. \(^{18}\)F-FDG (Glucotep) was from Cyclopharma.

**Antibodies**

The antibodies used for Western blotting were as follows: anti-phospho-EGFR/HER-1 (Tyr1173; Euromedex); anti-total EGFR/HER-1 (Ab-12) and anti-tubulin \(\beta\) (Neo-Markers, Interchim); anti–Glut-1 and anti–phospho-ERK-1/2 pAb (Cell Signaling); anti–ERK-1/2 (c-16; Santa Cruz Biotech, Tebu-Bio SA); anti-p27\(^{kip1}\) (DAKO); and peroxidase-conjugated secondary mouse or rabbit antibodies (Bio-Rad).

**Cell culture**

CAL33 and CAL166 cells (human head and neck carcinoma, Centre Antoine Lacassagne Nice, France; ref. 8) were cultured in DMEM containing 10% fetal bovine serum supplemented with 2 mmol/L L-glutamine (culture medium; Cambrex Biosciences) at 37°C in a humidified atmosphere and 5% CO\(_2\).

**Western blot analysis**

On day 1, cells (1.5 \(\times\) 10\(^6\)) were plated in culture medium in 100-mm culture dishes. On day 2, for the EGFR and ERK analyses cells, were either serum starved or not for 24 hours and then treated with either vehicle or erlotinib at concentrations of 3, 3.5, and 5 \(\mu\)mol/L for 24 to 72 hours. When necessary, cells were treated with 20 ng/mL EGF for the last 15 minutes of the experiment. The cells were harvested and lysed in lysis buffer [50 mmol/L Tris (pH 8), 150 mmol/L NaCl, 0.1% NP40, 5 mmol/L MgCl\(_2\), 50 mmol/L NaF, 2 mmol/L phenylmethylsulfonyl fluoride, 10 mmol/L DTT, 2 mmol/L orthovanadate, 5 mg/mL sodium deoxycholate, 6.4 mg/mL phosphatase substrate; Sigma 104]. For EGFR, ERK-1/2, Glut-1, p27\(^{kip1}\), or \(\beta\)-tubulin analysis, 70 \(\mu\)g of the cleared lysates were separated on a 7.5% or 12.5% SDS-PAGE gel, blotted onto polyvinylidene difluoride membranes (Amersham), and incubated with specific antibodies.

For the determination of the effects of erlotinib on Glut-1 expression, 24 hours after plating, cells were treated with either vehicle or erlotinib as previously described. Cells were harvested by trypsinization and counted. Three million cells were pelleted (820 \(\times\) g, 5 min), lysed, and analyzed by Western blot as described earlier (12.5% SDS-PAGE, Glut-1 antibody).

Detection was done using peroxidase-conjugated secondary antibodies (Bio-Rad) and an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech). The blots were scanned and analyzed with a Molecular Dynamics densitometer and ImageQuant software. Results are representative of three independent experiments.

**Translational Relevance**

We show that deoxy-2-[\(^{18}\)F]fluoro-d-glucose positron emission tomography with computed tomography (\(^{18}\)FDG-PET/CT) can be used as a surrogate marker for the early evaluation of epidermal growth factor receptor-tyrosine kinase inhibitor efficacy. For this purpose, we developed a preclinical models to validate \(^{18}\)FDG-PET/CT imaging for the early evaluation of the molecular effects of erlotinib in head and neck squamous cell carcinoma cell lines. We then cross-validated this tool during a clinical trial designed to assess the pharmacodynamic effects of erlotinib in patients who received erlotinib as neoadjuvant treatment for a short time period before surgery. This is the first study providing, from preclinical models to patients, a reliable proof of efficiency of this method.

Published OnlineFirst July 26, 2010; DOI: 10.1158/1078-0432.CCR-09-2795

Published OnlineFirst May 26, 2010; DOI: 10.1158/1078-0432.CCR-09-2795

OF2 Clin Cancer Res; 16(17) September 1, 2010 Clinical Cancer Research
Fluorescence

Immunofluorescence histology was done as described elsewhere (9). Cells were seeded on glass coverslips in six-well plates and were treated with either vehicle or erlotinib at 5 μmol/L for 48 hours. Membrane Glut-1 transporters were detected by incubation with antibody against Glut-1 plus a rhodamine-labeled secondary antibody, and images were recorded with a Princeton camera. Results are representative of three independent experiments in duplicate.

Determination of $^{18}$FDG uptake by CAL33 cells

On day 1, CAL33 cells ($1.5 \times 10^6$) were plated in 60-mm culture dishes in culture medium. Twenty-four hours later, cells were treated with either glucose (control cells) or 24.42 MBq/mL $^{18}$FDG (treated cells) for 30 minutes. The medium was collected; the cells were washed three times with 3 mL of PBS and each wash was collected in separated tubes. Cells were trypsinized (500 μL of trypsin) and collected in 2.5 mL of culture medium and were pelleted (850 × g, 5 min). Trypsin and supernatants were collected. The pelleted cells were either reserved (whole cells) or suspended in 500 μL of PBS, and lysed by three cycles of thermal shock (liquid nitrogen/37°C) followed by 15 minutes of centrifugation (15,000 × g) to obtain a lysate fraction. The supernatants, representing the cytosolic cell fraction, and the pellets, representing the membrane plus nuclear cell fraction, were collected. The results are expressed as the percentage of radioactivity (corrected for the physical decay of $^{18}$F) measured in each fraction versus the $^{18}$FDG activity at the time of treatment (100%) and are the mean ± SEM of two independent experiments in duplicate.

Effect of erlotinib on cell line xenografts in nude mice

CAL33 or CAL166 xenografts were established by s.c. injection of 1 × 10⁶ cells into both mouse flanks. When the tumor size reached ~200 mm³ (day 4 postimplantation), the mice were pooled and randomly assigned to two groups (control and treated with erlotinib) of six to eight animals.

For $^{18}$FDG-PET/CT imaging, mice were injected through the tail vein with 9.85 ± 1.5 MBq of $^{18}$FDG and then anesthetized by an i.p. injection of ketamin/xylazin solution (100/5 mg/kg). Anesthesia was maintained for 60 minutes and, if necessary, before the $^{18}$FDG-PET/CT imaging mice were rechallenged for anesthesia for the further 30 minutes of imaging [PET-CT Discovery ST, General Electric Health and Care (GEHC)], after which the mice were allowed to recover. Mice were maintained under a controlled temperature (around 22°C) during all the experiments. Mice were then treated orally with either saline (control group) or erlotinib at 100 mg/kg/d in saline (erlotinib group). Twenty-four hours later, the mice were imaged as described, and in some protocols, the mice were treated and scanned after 72 hours of treatment.

To perform the PET acquisition, mice were placed in a special box enabling four mice to be imaged at once (two controls and two treated). PET data handlings and reconstructions are shown in Supplementary Table S1. Tracer uptake was measured using the regions of interest (ROI) on cross-sectional images. ROIs with the same size were drawn around the tumor and a background region on transaxial slice images to determine the tumor-to-background ratio calculated by dividing the average pixel intensity within a tumor ROI by the average pixel intensity within the background ROI. Results are representative of at least two independent experiments with three mice per kinetic point.

At the end of the experiment, the mice were sacrificed; the tumors were removed and fixed in formalin for pathologic and immunohistochemical analysis.

Effect of neoadjuvant treatment with erlotinib in patients with HNSCC

Our team has published a pilot study of neoadjuvant treatment with erlotinib of nonmetastatic HNSCC (7). Patients were eligible if they were candidates for first-line curative surgical treatment or had been scheduled for surgery by necessity. After diagnosis, patients underwent routine pan-endoscopy and $^{18}$FDG-PET/CT. Treatment with 150 mg/d erlotinib orally started the following day. Patients were treated for 20 days on average (Table 1), corresponding to the time between pan-endoscopy and surgical resection. $^{18}$FDG-PET/CT examinations were repeated 48 hours before surgery at the latest. Pathologic examinations with immunostaining were done on biopsies before and after treatment.

$^{18}$F-FDG PET/CT acquisitions and interpretations

Serum glucose was measured before i.v. injection of 370 MBq of $^{18}$FDG (Glucotep Cyclopharma). A whole-body (from skull to pelvis) $^{18}$FDG-PET/CT acquisition was carried out (GEHC). Acquisitions were done in a two-dimensional mode (5 minutes per bed position). Two-dimensional sinograms were reconstructed in a 256 × 256 matrix, with a field of view of 50 cm and corrected for attenuation, random, and scatter. CT imaging was done for attenuation correction and anatomic correlation with a 200-mA tube current, a 140-kV tube voltage, a helical pitch of 0.75:1, and a reconstructed slice thickness of 3.75 mm with an interval of 3.27 mm between slices.

The two-dimensional $^{18}$FDG-PET/CT data corrected for attenuation were transferred to an Advantage Workstation 4.2 (GEHC). The maximum standardized uptake value ($\text{SUV}_{\text{max}}$ ref. 10) and the biological volume of the tumor (BV) were determined using commercial software (PET VCAR GEHC). $\text{SUV}_{\text{max}}$ was corrected for body weight ($\text{SUV}_{\text{bw}}$). BV was determined using a fixed threshold between the maximum pixel counts within the tumor and the background. We previously carried out a control study and determined that for the PET system and the acquisition protocol used, a threshold of 35% was the most accurate.

The metabolic response was expressed according to the change in either the SUV or the BV determined as
follows: \( \Delta \text{SUV} = (\text{SUV}_a - \text{SUV}_b)/\text{SUV}_b \times 100 \) and \( \delta \text{BV} = (\text{BV}_a - \text{BV}_b)/\text{BV}_b \times 100 \) ("b" stands for before treatment and "a" for after treatment).

**Immunohistochemistry**

Analyses were done on 4-μm-thick formalin-fixed paraffin sections of patient tumors and cell lines xenografts according to a procedure described elsewhere (7). For Glut-1 immunostaining, the antibody dilution was ready to use. Antigen retrieval was done using 10 mmol/L citrate buffer (pH 6) and 750-W microwave heat for 5 minutes × 3. Immunostaining intensity was assessed semiquantitatively using a four-point scale (i.e., 0, negative; +, weak; ++, moderate; +++, strong) and the percentage of labeled cells. Immunostaining analyses were evaluated using the immunoreactive score (IRS) according to Remmele et al. (11). The IRS (range, 0-12) is the product of the scores for staining intensity (0-3 scale) and percentage of cells stained (0-4 scale).

**Statistical analysis**

All results are expressed as mean ± SEM. Results were analyzed using Student's t test, and \( P < 0.05 \) was accepted as statistically significant. \( \text{SUV}_{\text{max}} \) and BV after and before treatment were compared using a paired nonparametric two-tailed test. Comparisons between IRS scores before and after treatment were done using Wilcoxon signed-rank test for paired data.

\( \Delta \text{SUV} \) and \( \delta \text{BV} \) were compared between two groups of patients defined by their MR (MR versus non-MR) using an unpaired nonparametric two-tailed test.

**Receiver operating characteristic analyses** were done to evaluate the overall performance of \( \text{SUV}_{\text{max}} \) and BV values and the relative remaining \( \Delta \text{SUV} \) and \( \delta \text{BV} \) as a prognostic test for the MRs.

**Results**

**Effects of erlotinib on cell line proliferation**

First, we checked the absence of somatic mutations in the tyrosine kinase domain of the EGFR in the CAL33 cell line (data not shown). We then studied the dose and time course effects of erlotinib on CAL33 proliferation and showed that erlotinib inhibited cell proliferation in a dose- and time-dependent manner with an IC\(_{50}\) of 4 ± 0.6 μmol/L (data not shown). This growth-inhibitory effect of erlotinib was paralleled by an inhibition of EGFR and ERK-1/2 phosphorylation associated with the

---

### Table 1. Patients’ characteristics

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Primary tumor</th>
<th>Cumulated dose of erlotinib (150 mg × no. days)</th>
<th>Cutaneous toxicity</th>
<th>Follow-up (mo)</th>
<th>Events/clinical status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Oral cavity</td>
<td>1,950</td>
<td>2</td>
<td>36</td>
<td>DF</td>
</tr>
<tr>
<td>2</td>
<td>Oral cavity</td>
<td>3,000</td>
<td>2</td>
<td>48</td>
<td>DF</td>
</tr>
<tr>
<td>3</td>
<td>Oral cavity</td>
<td>3,750</td>
<td>1</td>
<td>48</td>
<td>DF</td>
</tr>
<tr>
<td>4</td>
<td>Oral cavity</td>
<td>2,700</td>
<td>2</td>
<td>18</td>
<td>DRD</td>
</tr>
<tr>
<td>5</td>
<td>Hypopharynx</td>
<td>2,850</td>
<td>2</td>
<td>7</td>
<td>DRD</td>
</tr>
<tr>
<td>6</td>
<td>Oral cavity</td>
<td>2,700</td>
<td>0</td>
<td>48</td>
<td>DF</td>
</tr>
<tr>
<td>7</td>
<td>Oropharynx</td>
<td>3,300</td>
<td>2</td>
<td>35</td>
<td>DRD</td>
</tr>
<tr>
<td>8</td>
<td>Oral cavity</td>
<td>3,450</td>
<td>2</td>
<td>48</td>
<td>DF</td>
</tr>
<tr>
<td>9</td>
<td>Larynx</td>
<td>4,050</td>
<td>0</td>
<td>38</td>
<td>DF</td>
</tr>
<tr>
<td>10</td>
<td>Oropharynx</td>
<td>3,600</td>
<td>1</td>
<td>37</td>
<td>DuRD</td>
</tr>
<tr>
<td>11</td>
<td>Oral cavity</td>
<td>3,000</td>
<td>1</td>
<td>36</td>
<td>DF</td>
</tr>
<tr>
<td>12</td>
<td>Oral cavity</td>
<td>1,650</td>
<td>3</td>
<td>12</td>
<td>LR</td>
</tr>
<tr>
<td>13</td>
<td>Oropharynx</td>
<td>3,450</td>
<td>1</td>
<td>34</td>
<td>DF</td>
</tr>
<tr>
<td>14</td>
<td>Larynx</td>
<td>750</td>
<td>3</td>
<td>18</td>
<td>DF</td>
</tr>
<tr>
<td>15</td>
<td>Larynx</td>
<td>3,000</td>
<td>1</td>
<td>14</td>
<td>DRD</td>
</tr>
<tr>
<td>16</td>
<td>Larynx</td>
<td>3,600</td>
<td>1</td>
<td>16</td>
<td>DuRD</td>
</tr>
<tr>
<td>17</td>
<td>Oral cavity</td>
<td>3,900</td>
<td>1</td>
<td>27</td>
<td>DF</td>
</tr>
<tr>
<td>18</td>
<td>Oral cavity</td>
<td>4,950</td>
<td>1</td>
<td>6</td>
<td>DRD</td>
</tr>
</tbody>
</table>

**NOTE:** The tumor localization, cumulated dose of erlotinib, toxicity, and follow-up are detailed for 18 patients with nonmetastatic HNSCC who received neoadjuvant treatment with erlotinib between panendoscopy and surgical treatment.

Abbreviations: DF, disease-free; DRD, death related to the disease; DuRD, death unrelated to the disease; LR, local relapse.
upregulation of the cyclin-dependent kinase inhibitor p27kip1 (Fig. 1). These results showed that erlotinib inhibited its molecular target and regulated the proliferation pathway through inhibition of phospho-ERK-1/2, which we concentrated on as the marker of the molecular effects of erlotinib, translating its "biological response" which we also named "molecular response" for the following experiments and in the rest of the article.

We used a supplementary cell line, CAL166, described in the literature for overexpressing EGFR (12, 13). Nevertheless, we have quantified its EGFR expression versus that of CAL33 and found it to be two times higher (Supplementary Fig. S2). We then showed that erlotinib inhibited CAL166 proliferation in a dose- and time-dependent manner with an IC50 of 9.6 ± 0.2 μmol/L (data not shown), indicating a lower sensitivity to erlotinib compared with CAL33.

**Effect of erlotinib on CAL33 Glut-1 transporter expression**

18FDG is transported into cells by the Glut glucose transporter proteins, and thus we investigated their modification of expression under erlotinib treatment in a dose-dependent (3-5 μmol/L) and time-dependent (24-72 hours) manner. The levels of the Glut transporter proteins were determined by Western blotting. CAL33 and CAL166 cells expressed detectable and similar levels of Glut-1 (Supplementary Fig. S2) but not Glut-3 and Glut-4 transporters (data not shown). CAL33 treatment with 3 to 5 μmol/L of erlotinib for 24 to 72 hours did not decrease Glut-1 levels in the cell lysate of 3 × 106 cells and on the membrane of whole cell (Fig. 2A and B). Moreover, the immunohistochemical study of CAL33 (Fig. 4C and D) and CAL166 (Supplementary Fig. S2) tumors from xenografted mice showed comparable levels of Glut-1 before and after treatment. These results showed that erlotinib did not modify glucose transport and, consequently, 18FDG uptake in these cell lines. Moreover, the data also suggest that the glucose transport capability is not altered in the remnant malignant cells, and that the cytostatic effect of erlotinib is not mediated by an inhibition of the glycolytic activity in malignant cells.

**18FDG uptake by CAL33 cells**

We then studied the 18FDG uptake by CAL33 cells. The 18FDG uptake was determined by measuring the radioactivity in the cell lysates after subcellular fractionation (membrane and cytosol fractions) of CAL33 cells exposed to 18FDG for 30 minutes. The radioactivity values reported in Fig. 2C were established at the time of treatment and were corrected for the physical decay of 18F. We showed that 18FDG was detectable in the cytosolic fraction of CAL-33 cells exposed to 18FDG, suggesting that in CAL-33 cells, glucose transporters can translocate 18FDG from the extracellular domain to the cytosol.

![Fig. 1. Effects of erlotinib on the CAL33 proliferation pathway. Effects of erlotinib treatment (5 μmol/L) on P-EGFR (A; % of variation versus control quantification), ERK-1/2 (B), and p27kip1 (C and D; fold increase of variation versus control quantification) using Western blot (when indicated, EGF was added at 20 ng/mL during the 15 last min of the experiment). Data are representative of three independent experiments. FBS, fetal bovine serum.](https://www.aacrjournals.org/doi/fig/10.1158/1078-0432.CCR-09-2795)
Evaluation of $^{18}$FDG-PET/CT imaging to evaluate the early response of nude mouse xenografts to erlotinib

First, we checked the spatial resolution and sensitivity of our human PET-CT to validate its capabilities in imaging and detecting the $^{18}$FDG uptake activity of $1 \times 10^7$ cells 4 days after their xenografting. The pharmacokinetic study of the $^{18}$FDG mouse tumor uptake led us to determine the optimal window for in vivo imaging, which was between 60 and 80 minutes after the $^{18}$FDG injection (data not shown).

We then studied whether a rapid decrease in $^{18}$FDG uptake in erlotinib-treated mice could be detected by $^{18}$FDG-PET imaging. Both visual and semiquantitative analyses were carried out and are shown in Fig. 3.

We performed a series of protocols in which we imaged each time four mice bearing CAL33 or CAL166 tumors in both flanks (two control mice and two erlotinib-treated mice) before and after erlotinib treatment lasting 24 to 72 hours (72 hours only for CAL33). Our data showed significant and dramatic reductions in $^{18}$FDG uptake of 48% ($P < 0.0001$) and 36% ($P < 0.001$) for CAL33 and CAL166, respectively, after 24 hours of erlotinib treatment. This inhibition persisted at a significant level ($P < 0.05$) until 72 hours (64% inhibition). Here, we have shown that $^{18}$FDG-PET imaging enabled the early (24 hours) in vivo
Fig. 3. PET study of the effects of erlotinib on tumor $^{18}$FDG capture. A, nude mice bearing ($\rightarrow$) subcutaneous CAL33 or CAL166 tumors and visualized by PET using an i.v. injection of 9.85 ± 1.5 MBq of $^{18}$FDG 4 d after implantation (Before) and 24 to 72 h after oral treatment (After) with either saline (control group) or erlotinib (erlotinib group at 100 mg/kg). B and C, histogram representations for CAL33 (B) and CAL166 (C) of the variation of the TBR (tumor-to-background ratio of FDG uptake signal using ROIs of the same size drawn on cross-sectional images) before and after (24-72 h) saline or erlotinib treatment orally. Data are representative of four (CAL33) or two (CAL166) independent experiments, each done in triplicate.
evaluation of erlotinib treatment effects resulting in $^{18}$FDG uptake inhibition, which underlies the inhibition of cellular metabolism, which in turn is linked to tumor inhibition. As observed for EGFR activity inhibition, the $^{18}$FDG uptake inhibition in CAL166 is lower than that in CAL33 (36% versus 48%), which is related to the lower sensitivity to erlotinib of this cell line.

**Pathologic analysis of both preclinical and clinical studies**

The immunohistochemical analyses revealed a significant reduction of the phosphorylated form of ERK-1/2 when we compared the patients before and after erlotinib treatment ($P = 0.047$; Fig. 4). Phospho-EGFR showed a reproducible and important reduction in the mouse model.
that was also observed in patients; surprisingly, the reduction was statistically nonsignificant (Supplementary Fig. S2 for CAL166; Fig. 4). No significant change was observed in the levels of total EGFR (P = 0.61). More interestingly, no change was observed on the Glut-1 expression level (P = 0.2) even when we compared “molecular responder” patients to “molecular nonresponder” patients (P = 0.42 and P = 0.33, respectively; Fig. 4). These results are in total agreement with our previous in vitro data and support the rationale for using 18FDG-PET as a surrogate marker of the biological effects of erlotinib.

**Patient study**

This study included 18 patients (1 female and 17 male) with a mean age of 58.5 ± 10.4 years. Table 1 summarizes the patients’ characteristics and clinical outcomes after the treatment. Capillary blood glucose level at the time of 18FDG injection was, on average, 1.02 ± 0.21 g/mL (0.6–1.43 g/mL).

The parameters related to the 18FDG uptake within the entire tumors and their alterations after the exposure to erlotinib are summarized in Supplementary Table S1. On average, the treatment led to a statistically significant reduction by 17.9% and 28.8% of SUVbwmax and BV, respectively (Fig. 5A).

Among these 18 patients, immunohistochemical analyses of phospho-ERK1/2 showed that 11 patients had a molecular response (MR) and 7 were considered as non-MR. Alterations were more pronounced for BV than for SUVbwmax (Fig. 5C). Thus, considering the alteration in phospho-ERK1/2 protein as the gold standard, the accurate way to assess the metabolic response relied on the δBV rather than the ΔSUV variations, as δBV seemed significantly higher for patients having a MR (P = 0.0109; Fig. 5B).

Using receiver operating characteristic analysis of the performance of δBV values for the prediction of a MR, an area under the curve of 0.92 (95% confidence interval, 0.769–1.07; P = 0.003) was observed (Fig. 5D). A cutoff value of δBV = -16% gives the diagnosis of MR a sensitivity of 100% and a specificity of 86%.

**Discussion**

In non–small cell lung cancers and colorectal cancer, it is well established that EGFR or Kras mutations are predictive factors for the effects of EGFR inhibitors (EGFR-TKIs; ref. 14). In HNSCC, there are no currently established markers or surrogate markers of the responses to EGFR-targeted therapies (e.g., erlotinib; refs. 2, 15). EGFR mutations seem to be relatively rare in HNSCC (16), and indeed, we did not find any relevant mutations of the EGFR catalytic domain, neither in the clinical study we published (7) nor in the CAL33 cell line we used in this study. Kras mutations are relatively low or absent in HNSCC (17, 18). In this study, we looked at the in vitro effects of erlotinib on its molecular target (EGFR) and regulated proliferation pathways in the HNSCC cell line CAL33. As described in the literature, we showed that erlotinib inhibited EGFR phosphorylation. This inhibition is associated in vivo and in vitro with the inhibition of the proliferation signal transduction pathway, which results in p27kip1 upregulation and phospho-ERK-1/2 inhibition, leading to cell growth inhibition (19). These results allowed us to fix ERK-1/2 phosphorylation as a marker of the biological and molecular effects of erlotinib in our model, defining the cell MR to erlotinib treatment for the whole of the study.

A major problem for oncologists is the detection of any early specific biological effects of the targeted therapies in patients (2).

The conventional radiographic modalities are not adapted for the early evaluation of the therapeutic effectiveness of cytostatic drugs. Because these agents prevent tumor growth without necessarily inducing significant tumor regression, assessment based strictly on sequential measurement of tumor size may not accurately reflect the viable tumor cell fraction in a residual mass. Warburg’s findings underpin the principles of tumor imaging with 18FDG-PET (6). The increased metabolism of tumors for glucose and its analogues, such as 18FDG, is the basis for PET imaging in oncology. Glucose and its analogues are transported into the cell by membrane transporters of the Glut family. Thus, the glycolytic activity as an indicator of the effects of EGFR-targeted therapies seems relevant as a link between EGFR inhibition and the control of cell proliferation. For instance, the phosphatidyl-inositol-3-kinase/Akt/mammalian target of rapamycin cascade is one of the signaling pathways activated by tyrosine kinase receptors (EGFR in particular) that regulate antiproliferative and apoptotic functions and is also involved in the regulation of cell metabolism (3).

Experimental models of xenografts of gastrointestinal stromal tumors have shown that FDG-PET might enable alterations in glucose metabolism to be observed before the cytostatic effects of imatinib mesylate, an EGFR-TKI (20). Cullinan et al. (21) have shown, in another xenograft model of gastrointestinal stromal tumors, that imatinib treatment induced a decrease in FDG uptake together with an early (4 hours posttreatment) decrease in Glut-1 transcription and expression. This metabolic effect, which has not been observed in the resistant cell line, preceded the cell cycle block and apoptosis of the treated cells. Moreover, Su et al. (22) have observed that, in non–small cell lung cancer lines treated with the TKI gefitinib, there is an immediate “metabolic” response (after 4 hours of treatment) that is linked to a decrease in FDG uptake but is also associated with Glut-3 transporter expression together with the inhibition of EGFR phosphorylation and Akt phosphorylation.

In this study, we evaluated Glut-1 expression. We did not observe any significant decrease in Glut-1 expression with any of the experimental techniques used: Western blot, immunofluorescence assay, or immunohistochemical analysis of cell lines, mouse xenografts, and patients’ tumors. Moreover, the representation to the membrane...
of the Glut-1 transporter was also studied by immunofluorescence using a disconsolation procedure (data not shown). Until now, in HNSCC, there are no reports showing a decrease in Glut-1 by EGFR-TKI. Elsewhere, there are studies reporting that there are no significant correlations between $^{18}$FDG accumulation and Glut-1 expression in HNSCC (23, 24). These findings suggest that $^{18}$FDG-PET does not underestimate the residual disease after HNSCC.

**Fig. 5.** Effects of erlotinib on human tumor tissue. A, metabolic response with the mean tumor value ± 1 SD of SUV$_{bw}$ (square) and BV (circle) before (filled symbol) and after (open symbol) erlotinib treatment. *, two-tailed Wilcoxon signed-rank test of pooled data. BW, body weight. B, facial sagittal section of a $^{18}$FDG-PET-CT fusion of a patient with an oropharyngeal neoplasm before (I) and after (II) 21 d of erlotinib. Patient #13, molecular responder and metabolic responder ($\Delta$SUV = −17.8% and $\delta$BV = −27.3%). C, metabolic response (points, mean; bars, SEM) assessed using the SUV$_{max}$ ($\Delta$SUV$_{max}$) and the BV ($\delta$BV) between two groups of patients, those with a significant alteration of the phospho-ERK-1/2 protein (Mr, molecular responder) and those without significant alteration of the phospho-ERK-1/2 protein (nMr, non-molecular responder). D, receiver operating characteristic curve of the performance of $\delta$BV for the prediction of MR ($\delta$BV > 16.29%, sensitivity of 100%).
treatment with erlotinib because Glut expression seems to be conserved in remnant malignant cells.

Here, both the animal model and the patient tumor data showed that the alterations in $^{18}$FDG uptake are associated with tumor responses at the molecular level, with the downregulation of P-ERK-1/2, and with the clinical benefits observed in patients (7) without underestimating the residual disease because of the nonmodification of Glut-1 expression in malignant cells from patients and tumor xenografts. Taken together, our results show that FDG uptake can be considered as an early and reliable marker to assess the efficacy of an EGFR-TKI. However, evaluation according to the variation in SUV ($\Delta$SUV) or the variation in BV ($\Delta$BV) compared with MR led to conflicting conclusions. Using P-ERK1-2 inhibition as control, $\Delta$SUV$_{max}$ seemed to be less accurate than $\Delta$BV for the diagnosis of the MR to erlotinib with $^{18}$FDG-PET/CT. Despite its limitations, the SUV is the most frequently encountered parameter used for treatment monitoring with PET (10). Boucek et al. (25) demonstrated in phantom studies that the evaluation of the metabolic volume was more accurate than the SUV$_{max}$. Moreover, the study of Daisne et al. (26) showing that BV defined by FDG-PET provided a reliable evaluation of the real volume of head and neck cancer agrees with our conclusion about the accuracy of the use of BV instead of SUV.

It is clear that there are many methodologic approaches in performing PET image segmentations. We acknowledge that the method used in this study may be less accurate than the one used by Daisne et al. (26) or Boucek et al. (25), as the influence of the different noise-to-signal ratios is not taken into account for threshold determination (21, 22). Nevertheless, the method used in this study is available on commercialized clinical software, and according to Krak et al. (27), it seemed to be a good compromise between simplicity, user independence, reproducibility, and accuracy.

There are still some discrepancies between the molecular and metabolic responses. Smith-Jones et al. (28) showed that $^{18}$FDG-PET may be limited in detecting the cytostatic response to targeted therapies because of its lack of specificity. This could lead to the use of a more specific marker of cell proliferation, such as 3-deoxy-3-18F-fluorothymidine (29). Indeed, Atkinson et al. (30) reported that 3-deoxy-3-18F-fluorothymidine allowed anti-EGFR inhibitor therapy in squamous cell carcinoma to be monitored. However, 3-deoxy-3-18F-fluorothymidine is not commercially available. The present study provides strong evidence that FDG is a suitable marker of the effects of TKIs.

Conclusion

$^{18}$FDG-PET/CT enables the early evaluation of the efficacy of erlotinib treatment both in preclinical models of HNSCC and in patients. This is the first preclinical and clinical study to assess that $^{18}$FDG-PET/CT using the daily conventional clinical procedures is a reliable way to determine the early biological effects of erlotinib. In the head and neck cancer model used in this study, the inhibition of $^{18}$FDG uptake was in agreement with the MR (inhibition of ERK phosphorylation). Moreover, the differential MRs and the metabolic effects observed implicate EGFR pathway disruption (ERK1-2 inhibition) as the mechanism driving $^{18}$FDG-PET/CT changes. The expression of glucose transporters was not altered in malignant cells whether from patients or tumor xenografts. These data establish the use of the metabolic tumor volume in $^{18}$FDG-PET/CT for the early evaluation of the biological effects of EGFR-TKI in HNSCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank the Institut Claudius Regaud animal facility, John Woodley and Daniela Oswald for medical writing support, and Julia Nalis and Cyril Laulet for the TEP imaging acquisitions.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 10/21/2009; revised 06/23/2010; accepted 07/13/2010; published OnlineFirst 08/17/2010.

References


Preclinical and Clinical Evidence that Deoxy-2-[\textsuperscript{18}F]fluoro-D-glucose Positron Emission Tomography with Computed Tomography Is a Reliable Tool for the Detection of Early Molecular Responses to Erlotinib in Head and Neck Cancer

Sébastien Vergez, Jean-Pierre Delord, Fabienne Thomas, et al.

Clin Cancer Res  Published OnlineFirst July 26, 2010.

Updated version Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-09-2795

Supplementary Material Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2010/08/31/1078-0432.CCR-09-2795.DC1

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.