Iressa (ZD 1839) Inhibits Phosphorylation of Three Different Downstream Signal Transducers in Head and Neck Cancer (SCCHN)

MARKUS HAMBEK1, MEHRAN BAGHI1, HELENA BAUMANN1, KLAUS STREBHarD2, OLIVER ADUNKA1, WOLFGANG GSTÖTTNER1 and RAINALD KNECHT1

1Department of Otorhinolaryngology and 2Department of Gynecology and Obstetrics, School of Medicine, J. W. Goethe University, 60590 Frankfurt, Germany

Abstract. Proliferation of squamous cell carcinoma of the head and neck (SCCHN) depends on epidermal growth factor receptor (EGFR) expression. The EGFR activates different signal pathways leading to gene transcription in the nucleus due to cell cycle progression and proliferation of tumor cells. AKT, STAT3 and MAPK play central roles in these pathways. However, they are not only regulated by the EGFR. We therefore investigated whether a specific inhibitor of the EGFR tyrosine kinase (ZD 1839 or Iressa) is able to inhibit phosphorylation of these three signals at the same time. Western blot analysis of pretreated SCCHN cells revealed that ZD 1839 greatly reduces the amount of phosphorylated AKT, STAT3 as well as MAPK. Surprisingly, this effect was not dose-dependent between the concentration range of 5.15 to 41.2 ÌM/ml. We conclude that Iressa has a high potency to inhibit nuclear gene transcription responsible for cell cycle progression. Furthermore, dose reduction of Iressa in the case of toxicity may not severely influence the response to treatment.

Squamous cell carcinoma of the head and neck (SCCHN) is the sixth most common malignant tumor worldwide. Whereas significant progress has been made in defining the molecular mechanisms of SCCHN progression, the overall survival of patients suffering from these tumors has not changed significantly in recent years. As SCCHN typically overexpresses the epidermal growth factor receptor (EGFR), downstream signal transduction from the activated EGFR tyrosine kinase is thought to play a major role in proliferation of SCCHN cells (Figure 1). The EGFR family consists of four members: EGFR, ErbB2, ErbB3 and ErbB4 (1). The members of the EGFR family, particularly EGFR and ErbB2, are implicated in various forms of human cancers and serve as both prognostic markers and therapeutic targets. EGFR contains an extracellular ligand-binding domain, a single transmembrane region and an intracellular domain harboring intrinsic tyrosine kinase activity (2). Activation of the receptor tyrosine kinase requires ligand-induced dimerization that allows reciprocal transphosphorylation of residues within the catalytic domain, leading to enzymatic activation and autophosphorylation of cytoplasmic tyrosine residues. It is known that this tyrosine kinase phosphorylation leads to activation of signal transducers via the Ras/ERK/MAPK pathway. Recent studies have revealed, however, that EGFR activation may influence different signal transducers, for example the STAT proteins (signal transducers and transcription activators). These were identified in the last decade as transcription factors that were critical in mediating virtually all cytokine-driven signaling (3-5). In addition to their central roles in normal cell signaling, recent studies have demonstrated that diverse oncoproteins can activate specific STATs (particularly STAT3 and STAT5) and that constitutively activated STAT signaling directly contributes to oncogenesis (6).

Furthermore, involvement of STAT3 in chemoresistance of tumors has been described recently. The inactive cytoplasmic STATs are activated by phosphorylation, then translocate to the nucleus and activate target gene transcription (7-9). Constitutive activation of STAT3 has been detected in a wide variety of other cancers, including breast, prostate, renal cell, melanoma, ovarian, lung, leukemia, lymphoma and multiple myeloma (10). Upon ligand-induced kinase activation and protein tyrosine phosphorylation, STAT3 is recruited via its SH2 domain to tyrosine-phosphorylated motifs within receptor complexes and is itself phosphorylated on tyrosine 705 within its COOH terminus. Our understanding of the molecular
details of its recruitment and activation by the EGFR are incomplete. Recently, it has been shown that STAT3 directly binds to the cytoplasmic domain of the EGFR (11).

Characterization of signal pathways which regulate apoptosis have identified phosphoinositide 3-kinase (PI-3K) as a transducer of survival signals. The serine/threonine kinase, AKT, is a major target of PI-3K. AKT is present in the cytosol of unstimulated cells in a low activity conformation. Activation of PI-3K generates 3'-phosphorylated phosphoinositides which induce translocation of AKT to the plasma membrane (12-15). A number of AKT substrates, including BAD, caspase 9, apoptosis signal-regulating kinase 1 (ASK1), and the forkhead transcription factors FKHR, FKHRL1 and AFX play a role in cell survival (16-23). It is known that AKT, as well as STAT3 and MAPK are not only regulated by the EGFR. For example, STAT3 activation also depends on IL-2 signals. We therefore investigated whether a specific inhibitor of the EGFR tyrosine kinase (ZD 1839) is able to inhibit phosphorylation of these three signals at the same time.

Materials and Methods

To determine the signal transduction proteins STAT3, MAPK and AKT, we used tumor material from SCCN cell cultures (Detroit 562, UMSCC24, SCC 1624 and UMSS 10B). In vitro SCCN cells were treated with four different concentrations of ZD 1839 (Iressa, see below). To elucidate the effects of Iressa on signal transduction the following formulae were used:

\[ \text{Activation index (x)} = \frac{P^* \text{ phosphorylated protein (treatment group)}}{P^* \text{ whole protein (treatment group)}} \]

\[ \text{Activation index (y)} = \frac{P^* \text{ phosphorylated protein (treatment group)}}{P^* \text{ whole protein (treatment group)}} \]

The degree of dephosphorylation (z) has been defined as x=yz.

**ZD 1839 (Iressa).** AstraZeneca (Macclesfield, UK) kindly provided gefitinib (ZD 1839, Iressa).

**Iressa-treated cell cultures.** Three different SCCHN cell lines were used in this investigation. Detroit 562 and SCC 1624 were obtained from ATCC (American Type Culture Collection), UM-SCC 24 and UM-SCC 10B were kindly provided by Thomas Carey, University of Michigan, Ann Arbor, MI, USA. Cell lines were incubated with ZD 1839 for 24 and 48 hours using four different doses (5.15; 10.3; 20.6; 41.2 μM/mL DMEM). Thereafter, protein from tumor cells were isolated directly for Western blot analysis.

**Western blot analysis.** Whole cell extracts (50 μg / lane) were electrophoresed through 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel and were transferred onto a Hybond-C-super...
Figure 2. Inhibition of STAT3 phosphorylation in Detroit 562 cells through the tyrosine kinase blockade. The Western blot of phosphorylated STAT3 protein is displayed at two different time-points (24 and 48 h) and four different doses of ZD 1839. Compared to the untreated control, phosphorylated STAT3 is significantly down-regulated. ZD 1839 effects on pSTAT3 expression are dose- and time-dependent, as calculated by the formula in the text. Dephosphorylation of STAT3 is significant at each dose level compared to the control (p<0.05).

Figure 3. Inhibition of AKT phosphorylation in Detroit 562 cells through tyrosine kinase blockade. The figure displays the Western blot of phosphorylated AKT protein at two different time-points (24 and 48 h) and four different doses of ZD 1839. Compared to the untreated control, phosphorylated AKT is significantly down-regulated. ZD 1839 effects on pAKT expression are comparable at 24 h and 48 h. Dephosphorylation of AKT is significant compared to the control (p<0.02) at each dose level.

Figure 4. Inhibition of MAPK phosphorylation in Detroit 562 cells through the tyrosine kinase blockade. The Western blot of phosphorylated MAPK protein is displayed at two different time-points (24 and 48 h) and four different doses of ZD 1839. Compared to the untreated control, phosphorylated MAPK is significantly down-regulated. ZD 1839 effects on pMAPK expression are stronger at time-point 24 h than at 48 h. Dephosphorylation of MAPK is significant compared to the control (p<0.04) at each dose level.
Table I. The degree of dephosphorylation ($z$) as calculated by the formula $x y z^2$: $x$: activation index of the control group; $y$: activation index of the treatment group. This gives a complete overview of signal transduction blockade on different cell lines at different time-points after treatment. For example, the strongest dephosphorylation of MAPK occurred 24 h after treatment of Detroit 562 cells. In UMSCC 10 B cells phosphorylated AKT was not detected. Effects of ZD 1839 on signal transduction blockade, as described in the Results section, were significant ($p<0.05$).

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>MAPK ($z$)</th>
<th>STAT3 ($z$)</th>
<th>AKT ($z$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Detroit 562 (x)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Detroit 562 24 h (y)</td>
<td>1/8</td>
<td>1/2.1</td>
<td>1/2.5</td>
</tr>
<tr>
<td>Detroit 562 48 h (y)</td>
<td>1/2.5</td>
<td>1/0.65</td>
<td>1/2.27</td>
</tr>
<tr>
<td>Control UMSCC 24 (x)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>UMSCC 24 24 h (y)</td>
<td>1/1.25</td>
<td>1/1.25</td>
<td>1/3</td>
</tr>
<tr>
<td>UMSCC 24 48 h (y)</td>
<td>1/1.25</td>
<td>1/1.25</td>
<td>1/3</td>
</tr>
<tr>
<td>Control UMSCC 10B (x)</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>UMSCC 10B 24 h (y)</td>
<td>1/2.5</td>
<td>1/4</td>
<td>1</td>
</tr>
<tr>
<td>UMSCC 10B 48 h (y)</td>
<td>1/5.2</td>
<td>1/2.35</td>
<td>1</td>
</tr>
<tr>
<td>Control SCC 1624 (x)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>SCC 1624 24 h (y)</td>
<td>1/5.2</td>
<td>1/1.05</td>
<td>1/0.4</td>
</tr>
<tr>
<td>SCC 1624 48 h (y)</td>
<td>1/5</td>
<td>1/1.1</td>
<td>1/1.05</td>
</tr>
</tbody>
</table>

Discussion

In the present study, the effects of tyrosine kinase inhibition on downstream signaling of AKT, STAT3 and MAP kinase in head and neck carcinomas are described. Increasing evidence supports the critical role of STAT3 in tumor transformation and tumor progression. Extensive surveys of primary tumors and cell lines derived from tumors indicate that inappropriate activation of specific STATs occurs with surprisingly high frequency in a wide variety of human cancers (24). It has been shown that STAT3 binds to the cytoplasmic domain of the EGFR (11). As well as STAT3, MAP kinase is known to react as a consequence of EGFR activation. MAP kinase is known to be activated via a signal transduction cascade of different receptor tyrosine kinases as well as integrins and ion channels. STAT3 has been shown to be activated via IL-6 receptor signaling, but also has been recently detected in the activated EGFR complex (11, 24), especially in the region between amino acid residues 1061 and 1123. The serine/threonine kinase, AKT, is a major target of PI-3K. AKT is present in the cytosol of unstimulated cells in a low activity conformation. Activation of PI-3K generates 3'-phosphorylated phosphoinositides which induce translocation of AKT to the plasma membrane (12-15). A number of AKT substrates, including BAD, caspase 9, apoptosis signal-regulating kinase 1 (ASK1), and the forkhead transcription factors FKHR, FKHRL1, and AFX play a role in cell survival (16-23). ZD 1839 highly reduces the amount of phosphorylated AKT, STAT3 as well as MAPK. Surprisingly, this effect was not found to be dose-dependent between the concentration range of 5.15 to 41.2 µM/ml (IC$_{50}$: 20.6 µM/ml). It is known from proliferation studies (Hambek et al., unpublished data) that there are differences in efficiency between those concentrations, when treatment was interrupted after the first incubation, but not within repeated applications of ZD 1839. This suggests that there may be a wider dose range of effective Iressa treatment, when continuously applied. We conclude that ZD 1839 has a high potency to inhibit gene transcription in the nucleus responsible for cell cycle progression. Furthermore, dose reduction of Iressa in case of toxicity may not severely influence response to treatment.
References


Received December 23, 2004
Accepted April 6, 2005