Abstract. The anti-apoptotic protein BCL-xL and the cell cycle inhibitor p21CIP1/WAF1 were previously implicated in head and neck cancer. Several reports point to a role of the epidermal growth factor receptor (EGFR, ErbB-1, HER1) in regulating their expression. In the present study, we investigated the influence of EGFR on these tumor-associated factors. HNSCC cell lines were incubated with EGF or with the EGFR-specific kinase inhibitor AG1478. Western blot analysis and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) were deployed to measure BCL-xL and p21CIP1/WAF1 protein and mRNA levels. A dose-dependent rise of BCL-xL as well as p21CIP1/WAF1 protein was noted after incubation with EGF, whereas inhibition with AG1478 reduced basal expression levels. No influence on BCL-2 was seen. Interestingly, qRT-PCR revealed that p21CIP1/WAF1 but not BCL-xL transcript levels were induced after EGF treatment. Taken together, it can be stated that p21CIP1/WAF1 and BCL-xL but not BCL-2 levels are tightly regulated by EGFR in HNSCC cell lines. BCL-xL induction appears to be due to protein stabilization rather than transcriptional activation, which is the likely cause of p21CIP1/WAF1 induction. The noted variability in EGF response of HNSCC cells could reflect frequently observed variations in clinical response rates after implementation of anti-EGFR therapies.

For more than thirty years, there has been no significant prolongation in the five-year survival rate of patients with head and neck squamous cell carcinoma (HNSCC), representing the most frequent malignancy of the upper aerodigestive tract (1). Main reasons include tumor cell resistance to standard chemotherapeutics such as cisplatin (2). Overcoming therapy resistance is therefore a major task in cancer research. Progression of HNSCC and negative prognosis is tightly associated with expression of the epidermal growth factor receptor (EGFR, ErbB-1, HER1) (3). EGFR is a receptor tyrosine kinase and a powerful proto-oncogene that is found to be overexpressed in up to 100% of HNSCC tumors. This overexpression is due to amplification of the EGFR gene and causes EGFR to act as an oncogene (4). Resistance to chemotherapy is associated with this EGFR overactivation but also with overexpression of antiapoptotic molecules such as BCL-2 and BCL-xL (5, 6). Similarly, a correlation of EGFR with expression levels of the cell cycle inhibitor p21CIP1/WAF1 was also noted (7-9). Although these studies pointed to an association of EGFR with these tumor-relevant factors, only few investigated EGFR-activation and expression levels in HNSCC tumors in detail (10-12). Here it is interesting to mention that previous studies found survival of normal keratinocytes in culture to depend on EGFR-mediated up-regulation of BCL-xL, similarly this could also be true for keratinocyte-derived HNSCC tumor cells (13-15). The aim of this study, therefore, was to further investigate the influence of EGFR on the expression levels of BCL-2, BCL-xL and p21CIP1/WAF1 in HNSCC cells.

Materials and Methods

Cell lines and cell culture. The squamous cell carcinoma cell lines. UM-SCC-3, -14A, -27, and UT-SCC-24A and -26A were kindly provided by T. E. Carey (University of Michigan, MI, USA) and R. Grénman (University of Turku, Finland) respectively. The UMB-SCC-745, -864 and -969 (University of Marburg) cell lines were
Table I. HNSCC cell lines used in the study.

<table>
<thead>
<tr>
<th>HNSCC cell line</th>
<th>Origin of primary</th>
<th>Origin of specimen</th>
<th>TNM</th>
<th>Grading</th>
<th>Age (years)</th>
<th>Gender</th>
</tr>
</thead>
<tbody>
<tr>
<td>UM-SCC-3</td>
<td>Nasal columnella</td>
<td>Lymph node</td>
<td>T1N0M0</td>
<td>G1-G2</td>
<td>73</td>
<td>Female</td>
</tr>
<tr>
<td>UM-SCC-4</td>
<td>Tonsil</td>
<td>Primary tumor</td>
<td>T3N2M0</td>
<td>G3</td>
<td>47</td>
<td>Female</td>
</tr>
<tr>
<td>UM-SCC-14A</td>
<td>Floor of the mouth</td>
<td>Primary tumor</td>
<td>T1N0M0</td>
<td>G2-G3</td>
<td>58</td>
<td>Female</td>
</tr>
<tr>
<td>UM-SCC-27</td>
<td>Anterior tongue</td>
<td>Lymph node</td>
<td>T1N0M0</td>
<td>Unknown</td>
<td>62</td>
<td>Male</td>
</tr>
<tr>
<td>UMB-SCC-745</td>
<td>Oropharynx</td>
<td>Primary tumor</td>
<td>T4N2M0</td>
<td>G2</td>
<td>48</td>
<td>Male</td>
</tr>
<tr>
<td>UMB-SCC-864</td>
<td>Tongue</td>
<td>Primary tumor</td>
<td>T2N2M0</td>
<td>G2</td>
<td>59</td>
<td>Male</td>
</tr>
<tr>
<td>UMB-SCC-969</td>
<td>Tonsil</td>
<td>Primary tumor</td>
<td>T4N2M1</td>
<td>G2</td>
<td>67</td>
<td>Male</td>
</tr>
<tr>
<td>UT-SCC-24A</td>
<td>Anterior tongue</td>
<td>Primary tumor</td>
<td>T2N0M0</td>
<td>G2</td>
<td>41</td>
<td>Male</td>
</tr>
<tr>
<td>UT-SCC-26A</td>
<td>Hypopharynx</td>
<td>Lymph node</td>
<td>T1N2M0</td>
<td>G2</td>
<td>60</td>
<td>Male</td>
</tr>
</tbody>
</table>

derived from tumors of the oropharynx, tongue and pharynx (Table I) (16-18). Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) in the presence of penicillin and streptomycin.

Results

BCL-xL and p21CIP1/WAF1 expression depends on stimulation with EGF. BCL-xL, BCL-2 and p21CIP1/WAF1 expression was evaluated in five randomly selected HNSCC cell lines before and after incubation with 100 ng/ml EGF. All tested cell lines (UMB-SCC-745, -864, -969, UT-SCC-24A and -26A) demonstrated EGF-dependent induction of BCL-xL and p21CIP1/WAF1. There was no obvious influence of EGF on the expression level of BCL-2 (Figure 1).

HNSCC cell lines show variable sensitivity to EGF-mediated up-regulation of BCL-xL and p21CIP1/WAF1. To test the sensitivity of different HNSCC cell lines to EGF-dependent up-regulation of BCL-xL and p21CIP1/WAF1, we chose six HNSCC cell lines (UM-SCC-3, -14A, -27, UMB-SCC-745, -864, UT-SCC-26A) and incubated these with rising EGF concentrations (Figure 2). The HNSCC cell lines UM-SCC-14A, -27, UMB-SCC-745, -864 and UT-SCC-26A exhibited BCL-xL up-regulation starting at differing EGF levels (0.1-10 ng/ml) and reaching a maximum at about 100 ng/ml. Only a minor effect was visible in UM-SCC-3, which already exhibited high basal BCL-xL protein levels. Also, a clear EGF-dependent effect on p21CIP1/WAF1 was visible in all tested cell lines (Figure 2). The rise in p21CIP1/WAF1 expression was particularly well visible in the cell lines UMB-SCC-864, UM-SCC-3, -27 and UT-SCC-26A (Figure 2c-f). Similarly as observed for BCL-xL, the effect of EGF on p21CIP1/WAF1 expression appeared highly variable between the tested cell lines (Figure 2a-f).

Inhibition of EGFR autophosphorylation by AG1478 influences BCL-xL and p21CIP1/WAF1 expression levels. Previously, we demonstrated that incubation of HNSCC cells with 10 μg/ml of the specific EGFR kinase inhibitor AG1478 (tyrphostin)
completely inhibited EGFR phosphorylation (18). After treatment of the HNSCC cell line UMB-SCC-864 with rising concentrations of AG1478, the basal BCL-xL and p21CIP1/WAF1 levels dropped visibly at high (1000 and 10000 ng/ml) AG1478 concentrations (Figure 3a). Treatment of the six HNSCC cell lines UM-SCC-27, UMB-SCC-745, -864, -969, UT-SCC-24A and -26A with AG1478 (Figure 3b) visibly diminished basal expression levels of BCL-xL (Figure 3b; UMB-SCC-745, -864, -969, UT-SCC-24A) and p21CIP1/WAF1 (Figure 3b; UMB-SCC-969, UT-SCC-24A, -26A).

**EGF treatment of HNSCC cell lines induces p21CIP1/WAF1 but not BCL-xL transcript levels.** Quantitative RT-PCR was applied to further evaluate if the observed EGFR-dependent effect on BCL-xL and p21CIP1/WAF1 is due to a change in the mRNA transcript level. None of the tested HNSCC cell lines exhibited a significant change in the level of BCL-xL mRNA transcripts after treatment with EGF. However, p21CIP1/WAF1 transcripts rose in a dose-dependent manner after stimulation with the EGFR ligand, resembling the induction of protein expression as seen in the Western blot analysis (Figure 4a and b). It is interesting to note that UM-SCC-3, which had high basal BCL-xL protein levels also exhibited high basal transcript level when compared to UMB-SCC-864.

**Discussion**

The epidermal growth factor receptor (EGFR, ErbB-1, HER1) is overexpressed in a wide variety of epithelial tumors such as breast, ovary, kidney, colorectal and non-small cell lung cancer (NSCLC) and frequently also in HNSCC (3). All HNSCC cell lines tested in this study were previously shown to overexpress EGFR (16, 18).

**BCL-xL in HNSCC tumors.** Using immunohistochemistry, Pena and co-workers observed overexpression of BCL-xL in 52% and BCL-2 in 17% of HNSCC specimens and found that BCL-xL, in contrast to BCL-2, correlated with a lower prognosis of the disease (5). Similarly Bauer et al. selected for cisplatin sensitive and resistant HNSCC cell lines and found that resistant cell lines overexpressed BCL-xL and wt p53 (19). Furthermore they demonstrated that BH3 mimetic (–)-gossypol, a polyphenolic aldehyde derived from the cotton plant, inhibited BCL-xL, particularly in cisplatin-resistant HNSCC cells by binding to its BH3 domain and thereby sensitizing the tumor cells to apoptosis (20). With this treatment, 70-80% of the resistant cells entered programmed cell death. This again underlines the role of BCL-xL for HNSCC cell survival.
Figure 2. EGF treatment demonstrates a dose-dependent rise of BCL-xL and p21CIP1/WAF1 in HNSCC cell lines. The six HNSCC cell lines UMB-SCC-745, -864, UM-SCC-3, -14A, -27 and UT-SCC-26A were treated with rising concentrations of EGF (0.1, 1, 10, 100 and 1000 ng/ml) and BCL-xL and p21CIP1/WAF1 expression levels were monitored by Western blot analysis. In virtually all cell lines, rising EGF levels resulted in a concomitant up-regulation of BCL-xL and p21CIP1/WAF1 (a-f). Notice that the effect on BCL-xL was less obvious in UM-SCC-3 (e), which was the only tested cell line that expressed high basal BCL-xL levels. The reduction of BCL-xL levels at the highest tested EGF concentration (1000 ng/ml), particularly visible in UM-SCC-14A (b) and UMB-SCC-745 (a) cells, is likely due to EGF-mediated EGFR down-regulation as previously reported (18).
BCL-xL and EGFR. It was observed that EGFR and its ligands are required for survival of normal keratinocytes in culture. Jost et al. reported that BCL-xL but not BCL-2 regulation in epidermal keratinocytes is dependent on EGFR and required for survival of these epithelial cells in culture (21). They further demonstrated that EGFR-dependent BCL-xL expression was able to prevent ultraviolet light-induced apoptosis in keratinocytes (22). Similarly as described by Jost and colleagues for keratinocytes, a clear dependence of EGFR and BCL-xL protein levels was also observed in virtually all tested HNSCC cell lines. This observed EGF-mediated BCL-xL up-regulation was not found to be associated with a rise in BCL-xL transcript levels. This suggests that a mechanism other than EGFR-mediated transcripational activation, such as a prolongation of the protein’s half-life, is involved in EGFR-dependent BCL-xL regulation.

Regulation of p21CIP1/WAF1 by EGF. Up-regulation of p21CIP1/WAF1 frequently results in G1 cell cycle arrest (23). Previous studies observed a paradox up-regulation of p21CIP1/WAF1 in A431 cells after EGF stimulation with high EGF levels and contributed this effect to phosphorylation of the receptor (9). In this study, we found EGF-mediated up-

Figure 3. Inhibition of HNSCC cells with the EGFR-specific kinase inhibitor AG1478 reduces basal BCL-xL and p21CIP1/WAF1 expression levels. a: A reduction of basal BCL-xL and p21CIP1/WAF1 levels, the latter particularly visible, is noted after incubation of the HNSCC cell line UMB-SCC-864 with rising concentrations of AG1478. b: After treatment of UMB-SCC-745, -864, -969, UT-SCC-24A and -26A with inhibiting concentrations (≥10 μg/ml) of AG1478, a clear reduction of basal BCL-xL levels is visible in the UMB-SCC-745, -864, -969 and UT-SCC-24A cell lines. A marked decrease of p21CIP1/WAF1 appeared most prominently in UMB-SCC-969, UT-SCC-24A and -26A cells.

Figure 4. EGFR-dependent p21CIP1/WAF1 but not BCL-xL protein up-regulation correlates with a change in transcript levels. qRT-PCR was implemented to evaluate if the observed up-regulation of BCL-xL and p21CIP1/WAF1 proteins after treatment with EGF is due to a rise in mRNA transcript levels. The two HNSCC cell lines UM-SCC-3 (a) and UMB-SCC-864 (b) were treated with rising EGF concentrations. No significant change in BCL-xL transcript levels was observed in any of the tested cell lines. However, transcript levels for p21CIP1/WAF1 correlated well with the respective protein levels of the cell cycle inhibitor. Actin was used as an internal reference. Samples were tested as a triplicate (shown is the mean).
regulation of p21\textsuperscript{CIP1/WAF1} in virtually all tested HNSCC cell lines. The p21\textsuperscript{CIP1/WAF1} response of HNSCC cell lines to EGF stimulation varied between the tested cell lines, pointing to a variable response also in the original primary tumor.

**Potential impact of EGFR-dependent p21\textsuperscript{CIP1/WAF1} regulation on the cell cycle.** One could expect that up-regulation of p21\textsuperscript{CIP1/WAF1} would always inevitably result in cell cycle inhibition. However, the situation seems to be more complex. We recently demonstrated that in some cases, stimulation of HNSCC cell lines with EGF promoted cell proliferation and that in this case tumor cells even become more sensitive to cisplatin treatment (24). In the aforementioned study, the highly cisplatin-resistant cell line UT-SCC-26A, as well as two other HNSCC cell lines (UMB-SCC-745 and -864), were treated with rising levels of EGF in the same manner as described in the present study. In the present study, we have seen that p21\textsuperscript{CIP1/WAF1} is induced by EGF in all three cell lines. However, we also observed previously that only UMB-SCC-745 and -864 exhibited a concomitant reduction in proliferation that correlates with the behaviour of p21\textsuperscript{CIP1/WAF1} expression after EGF treatment (24). In sharp contrast, UT-SCC-26A reacted with a rise of cells in S and G2/M phases and cells became susceptible to cisplatin treatment. This cell line also exhibited a prominent up-regulation of the growth-promoting EGFR-dependent cyclin D1. It therefore seems that, depending on the tested HNSCC cell-type, EGFR activation can either inhibit or activate tumor cell proliferation. These observations are in agreement with other reports which found that the cell cycle inhibitors p21\textsuperscript{CIP1/WAF1} and p27\textsuperscript{KIP1} exhibit a reciprocal response to EGFR activation or inhibition (7). Depending on the consequences EGFR activation or inhibition has on both inhibitors, this could help explain why some cells are inhibited but other cells react with enhanced proliferation.

It was demonstrated that in HNSCC cells, expression of the anti-apoptotic protein BCL-x\textsubscript{L} and the cell cycle inhibitor p21\textsuperscript{CIP1/WAF1} is tightly regulated by EGFR. Since the EGFR-dependent effect on these proteins appeared highly variable, these findings may reflect clinical observations where HNSCC tumors exhibit highly variable response rates to chemoradiotherapy treatment. Therefore, the results further emphasize EGFR as a major target molecule in the treatment of head and neck cancer.

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**Conflicts of Interest Statement**

None declared.

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