Co-treatment of wild-type EGFR head and neck cancer cell lines with afatinib and cisplatin

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Abstract. Treatment of head and neck squamous cell carcinoma (HNSCC) remains challenging. Non-surgical approaches typically comprise radiotherapy and antineoplastic chemotherapy, of which platinum-based agents are the most common. Similar to other malignancies, targeted therapies have an increasing role in the treatment of head and neck cancer. The overexpression of epidermal growth factor receptor (EGFR) is a useful target for specific therapeutic strategies. Resistance to EGFR-directed therapies, including cetuximab, is partly mediated by the activation of alternative receptors and pathways. Therefore, other members of the ErbB family, including human epidermal growth factor receptor (HER)2 and HER4, may have important therapeutic roles. The aim of the present study was to investigate the efficacy of afatinib, an EGFR/HER2/HER4 tyrosine kinase inhibitor, in combination with cisplatin in HNSCC cell lines. The cisplatin concentration used was set at cell line-specific half maximal inhibitory concentration values. Since the vast majority of head and neck cancers do not exhibit any EGFR tyrosine kinase domain mutations, five human EGFR wild-type HNSCC cell lines were used in the present study. For statistical analyses, non-parametric Mann-Whitney tests were conducted. The present study detected a concentration-dependent efficacy of afatinib. In three out of the five cell lines (PCI-9, PCI-52 and PCI-68), 0.625 μM afatinib in combination with cisplatin exerted significant antiproliferative effects. In the two other cell lines (PCI-1 and PCI-13), significant effects were observed following treatment with ≥1.25 μM afatinib. Notably, compared with the findings of previous studies, cell lines (PCI-9 and PCI-52) less vulnerable to erlotinib or gefitinib were more vulnerable to the afatinib/cisplatin combination, and vice versa. Differences in the treatment success of erlotinib/gefitinib (targeting only EGFR) and afatinib (targeting EGFR, HER2 and HER4) may be explained by mutations in the EGFR. Therefore, afatinib treatment may be considered an important therapeutic option for patients failing cetuximab treatment. In addition, the present study demonstrated significant enhancement of platinum-based therapies upon the addition of various afatinib concentrations. These results provide preclinical evidence to advocate further *in vivo* studies and clinical trials.

Introduction

The number of newly diagnosed cases of head and neck cancer worldwide is ~600,000 annually (1). In addition, head and neck cancer is the sixth most common type of solid cancer diagnosed worldwide (2). Since the majority of patients present with locally advanced or metastatic disease, intensive surgical therapy followed by adjuvant radiochemotherapy is often required. Usually, cisplatin is used as a powerful radiosensitizer, and therefore remains an integral part of head and neck cancer therapy (3). However, the cure rate of head and neck cancer remains at ~50% (4); therefore, there is an urgent requirement to improve treatment and survival rates. One possible approach involves the inhibition of epidermal growth factor receptor (EGFR)-related signaling. More than 90% of head and neck cancers exhibit EGFR overexpression (5,6). Notably, increased levels of EGFR are associated with a poorer prognosis (7), and less differentiated head and neck cancers exhibit higher levels of EGFR (8). Furthermore, EGFR expression is associated with resistance to radiotherapy, locoregional treatment failure, and an increased rate of distant metastases (9). These findings led to the development of agents directed against EGFR. In 2006, cetuximab, a chimeric immunoglobulin G_1 monoclonal antibody, was approved by the Food and Drug Administration for the treatment of recurrent/metastatic head and neck cancer (10). In addition to targeting EGFR by inhibiting the extracellular ligand binding domain, tyrosine kinase inhibitors (TKIs) suppress intracellular tyrosine kinase activity. Particularly in metastatic non-small cell lung cancer (NSCLC), agents such as gefitinib and erlotinib are widely used (11). However, in contrast to NSCLC,

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where tumors frequently harbor EGFR kinase domain mutations, head and neck cancer lesions predominantly possess wild-type EGFR (12,13). This finding may partly explain why erlotinib failed to improve the complete response rate or progression-free survival of locally advanced head and neck cancers treated with a cisplatin/radiotherapy-based regimen (14). At present, there are no markers predictive of response to anti-EGFR therapies, including monoclonal antibodies and TKIs. However, ongoing research may identify such markers, and more accurate patient selection will improve the benefits of TKI-based therapy in a subset of patients with head and neck cancer. Another aspect regarding the treatment failure of anti-EGFR approaches could be lateral signaling, as well as the activation of alternative receptors and downstream molecules, including other members of the ErbB family. Wheeler *et al* (15) demonstrated that human epidermal growth factor receptor (HER)2 and HER3 are strongly activated in cetuximab-resistant cells. Furthermore, EGFR upregulation led to increased dimerization with HER2 and HER3. Consequently, the inhibition of EGFR and HER2 resulted in decreased HER3 and phosphoinositide 3-kinase activity (15). At present, few *in vitro* studies concerning dual blockade of EGFR and HER2 have been conducted in head and neck cancer. Schütze *et al* (16) demonstrated a clear antiproliferative effect of afatinib, an EGFR/HER2/HER4 TKI. Notably, the radiosensitizing effect was only marginal; however, the study was conducted with only one cell line (FaDu) (16). To date, only one clinical trial regarding afatinib for the treatment of head and neck cancer has been published. Seiwert *et al* (17) compared afatinib treatment with cetuximab treatment in patients with recurrent or metastatic head and neck cancer who progressed after platinum-based therapy. The antitumor activity of afatinib was comparable to that of cetuximab; however, more patients in the afatinib group discontinued treatment due to adverse events.

In the present study, the rationale for combining afatinib with cisplatin was based on three considerations. Firstly, cisplatin serves as a radiosensitizer, and chemoradiotherapy remains the gold standard for the adjuvant treatment of head and neck cancer. Secondly, EGFR overexpression is known to contribute to radiotherapy resistance. Finally, EGFR overexpression leads to the activation of other ErbB family members, including HER2 and HER4.

To the best of our knowledge, the present study is the first to investigate the efficacy of afatinib in combination with cisplatin in wild-type EGFR head and neck cancer cell lines.

Materials and methods

Cell lines. The cell lines used in the present study (Table I) were provided by the Cancer Institute, University of Pittsburgh (Pittsburgh, PA, USA) (18). As described previously, the cells were cultured in a humidified atmosphere containing 5% CO_2/95% air at 37˚C and the medium was changed 2-3 times per week (19,20). The cells were cultured in Dulbecco's modified Eagle medium (Gibco; Fisher Scientific Deutschland, Schwerte, Germany) supplemented with 10% fetal calf serum (Life Technologies GmbH, Darmstadt, Germany), 1% penicillin/streptomycin (Life Technologies GmbH) and 1% glutamine (Biochrom KG, Berlin, Germany).

Mutational analysis of EGFR tyrosine kinase domain. For mutational analysis of the EGFR tyrosine kinase domain, DNA was isolated from the HNSCC cell lines using a Roche DNA Isolation kit (Roche Diagnostics Deutschland GmbH, Mannheim, Germany), according to manufacturer's protocol. Subsequently, isolated DNA samples were amplified by polymerase chain reaction (PCR) using the following allele-specific primers from Eurofins Genomics (Ebersberg, Germany): EGFR exon 18, forward (F) 5'-CCATGTCTG GCACTGCTTTCC-3', EGFR exon 18, reverse (R) 5'-AAG GACTCTGGGCTCCCCACC-3'; EGFR exon 19, F 5'-ACC CAGATCACTGGGCAGCATG-3', EGFR exon 19, R 5'-AGC AGCTGCCAGACATGAGAAAAG-3'; EGFR exon 20, F 5'-CAGCCCTGCGTAAACGTCCCTG-3', EGFR exon 20, R 5'-GGAGCGCAGACCGCATGTGAGG-3'; EGFR exon 21, F 5'-ACCCTGAATTCGGATGCAGAGC-3', and EGFR exon 21, R 5'-ATACAGCTAGTGGGAAGGCAG-3'. PCR was performed on a Primus 96 Plus cycler (Peqlab Biotechnologie GmbH, Erlangen, Germany) with an annealing temperature of 65˚C for 35 cycles. All further PCR reagents, including the Taq DNA polymerase PCR buffer and the dNTPs were purchased from Thermo Fisher Scientific, Inc. (Darmstadt, Germany). Subsequently, the amplified DNA was visualized in 2% agarose gels containing ethidium bromide, and purified using column affinity chromatography. The purified PCR products were sequenced using a 16-capillary electrophoresis instrument (3130XL GeneScan; Thermo Fisher Scientific, Inc., Darmstadt, Germany).

Treatment with afatinib and cisplatin. A total of 1×10^4 cells from each cell line were seeded per well. Cisplatin was purchased from TEVA GmbH (Radebeul, Germany) and stored according to the manufacturer's protocol. Afatinib was purchased from Selleckchem (distributed by Absource Diagnostics GmbH, München, Germany) and stored according to the manufacturer's protocol. The afatinib concentrations used in the study (0.3125, 0.625, 1.25, 2.5, 5.0, 10.0 μM) were derived from a $\log_2$ dilution. These concentrations are based on the findings of Mukohara *et al* (21); the clinically relevant maximum serum concentration of EGFR TKIs, such as gefitinib, was reported to be ~1 μM. Cisplatin concentrations were fixed in all experiments. In our previous analysis, the cisplatin half maximal inhibitory concentration (IC_{50}) values for all five cell lines used in the present study (Table II) was investigated (unpublished data). The IC_{50} values for cisplatin ranged between 1 and 14 μM. In huge panels of human cancer cells, concentrations similar to these have been reported (22). Following an overnight incubation in standard medium, medium containing a fixed concentration of cisplatin and the variable concentrations of afatinib was added to the cells, and the cultures were incubated for a further 72 h.

Crystal violet assay. Crystal violet (1 g) was diluted in 1 L double-distilled water containing 20% methanol. Subsequently, the drug-containing medium was removed from the cells, and 50 μl crystal violet was added to the wells. After 15 min, the 96-well plates were washed with distilled water. Using a microplate reader (Tecan Spectra Rainbow microplate reader; Tecan Deutschland GmbH, Crailsheim, Germany), the optical

Table I. Name, origin and TNM status of the five cell lines used in the present study.

Cell line	Origin	TNM
PCI-1	Laryngeal carcinoma of the glottis of a male patient	pT2N00M0G2
PCI-9	Primary carcinoma at the base of the tongue of a male patient	pT4N3M0G2
PCI-13	Oral squamous cell carcinoma of the retromolar triangle of a male patient	pT4pN1M0G3
PCI-52	Primary carcinoma of the aryepiglottic fold of a male patient	pT2N0M0G2
PCI-68	Primary tongue carcinoma of a male patient	pT4N0M0G1

TNM, Tumor Node Metastasis.

density (OD) was measured at a wavelength of 595 nm. All experiments were performed at least three times.

Statistical analysis. Statistical analysis of the data was performed using Microsoft Excel 2007 (Microsoft Corporation, Redmond, WA, USA) and Prism 6.04 (GraphPad Software, Inc., La Jolla, CA, USA). Two statistical aspects were investigated. Initially, the treatment efficacy was compared between cisplatin monotherapy and combination therapy with afatinib in each cell line. Secondly, significant differences in treatment efficacy between the five cell lines were determined. Due to the lack of a normal distribution and the number of measurements, a nonparametric Mann-Whitney test was performed. All of the experiments were repeated at least three times. $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

Mutational analysis of the EGFR tyrosine kinase domain. Wild-type exons 18, 19 and 21 were detected in all cell lines. The silent mutation Q787Q was identified in exon 20 in all cell lines. In addition, PCI-9 harbored the T785T mutation, which, similar to Q787Q, is a silent mutation with no functional relevance (Table III).

Treatment efficacy in PCI-1 cells. The fraction of viable cells following treatment with a fixed concentration of cisplatin (14 μM) and 0.3125 μM afatinib was 100.4% [standard deviation (SD)±2.6%]. When the cells were treated with cisplatin and 0.625 μM afatinib, the viable fraction was 103.7% (SD±4.9%). The fraction of viable cells following treatment with 1.25 μM afatinib and the fixed concentration of cisplatin was 81.1% (SD±6.1%), which was significantly reduced, as compared with the control cells ($P<0.0001$). Following treatment with cisplatin and 2.5 μM afatinib, the viable fraction was 69.3% (SD±8.8%), as compared with the control cells. The fraction of viable cells when treated with fixed cisplatin and 5 μM afatinib was 65.9% (SD±10.9%). Following treatment with cisplatin and the highest concentration of afatinib (10 μM), the viable fraction was only 57.0% (SD±12.3%) (Fig. 1A).

Treatment efficacy in PCI-9 cells. The fraction of viable cells following treatment with a fixed concentration of cisplatin (14 μM) and 0.3125 μM afatinib was 92.3% (SD±5.4%). Following treatment with cisplatin and 0.625 μM afatinib, the

Table II. Cisplatin IC$_{50}$ values in various cell lines.

Cell line	Cisplatin IC$_{50}$ (μM)
PCI-1	14
PCI-9	14
PCI-13	1
PCI-52	5
PCI-68	14

IC$_{50}$, half maximal inhibitory concentration.

Table III. Mutation status of the epidermal growth factor receptor tyrosine kinase domain.

Cell line	Exon 18	Exon 19	Exon 20	Exon 21
PCI-1	wt	wt	wt (Q787Q)	wt
PCI-9	wt	wt	wt (T785T, Q787Q)	wt
PCI-13	wt	wt	wt (Q787Q)	wt
PCI-52	wt	wt	wt (Q787Q)	wt
PCI-68	wt	wt	wt (Q787Q)	wt

wt, wild-type.

viable fraction was 92.4% (SD±3.8%), which was significantly reduced, as compared with the control ($P=0.003$). The fraction of viable cells treated with 1.25 μM afatinib and the fixed concentration of cisplatin was 78.2% (SD±3.2%). Following treatment with cisplatin and 2.5 μM afatinib, the viable fraction was 67.1% (SD±2.8%), as compared with the control cells. The fraction of viable cells following treatment with 5 μM afatinib and the fixed concentration of cisplatin was 51.0% (SD±2.9%). When the cells were treated with cisplatin and the highest concentration of afatinib (10 μM), the viable fraction was only 40.4% (SD±5.1%) (Fig. 1B).

Treatment efficacy in PCI-13 cells. The fraction of viable cells following treatment with a fixed concentration of cisplatin (1 μM) and 0.3125 μM afatinib was 98.7% (SD±6.1%). When the cells were treated with cisplatin and 0.625 μM afatinib,
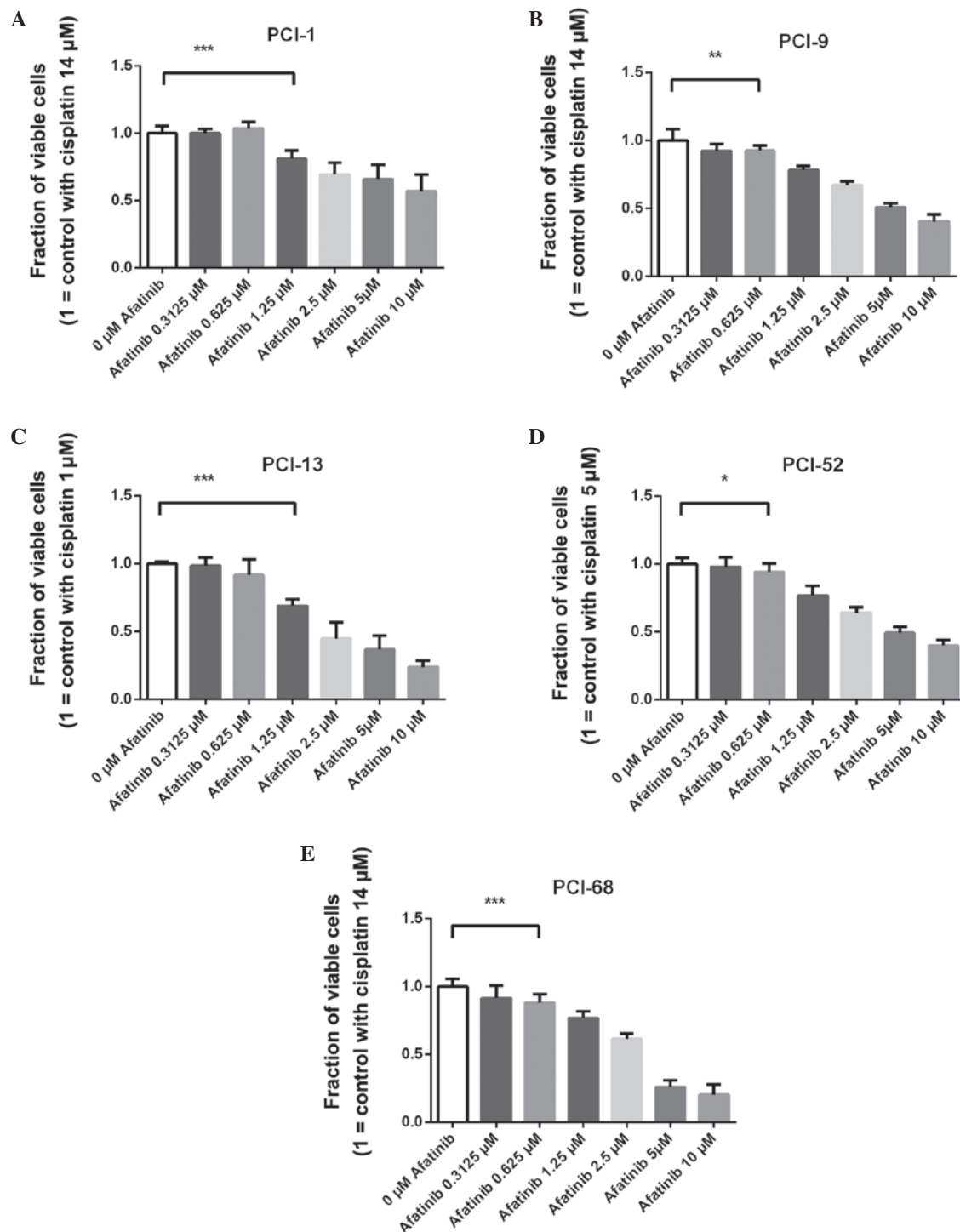
Figure 1. Treatment efficacy of afatinib in combination with the cell line-specific fixed concentrations of cisplatin, as measured by crystal violet assay. The white bar indicates the control cells treated with cell-specific cisplatin half maximal inhibitory concentration values (=1.0). The fraction of viable (A) PCI-1, (B) PCI-9, (C) PCI-13, (D) PCI-52 and (E) PCI-68 cells. In all cell lines, a concentration-dependent effect of combined afatinib and cisplatin treatment was detected. In the PCI-9, PCI-52 and PCI-68 cells, ≥0.625 μM afatinib resulted in a significantly lower viable fraction, as compared with the control. In the PCI-1 and PCI-13 cells, ≥1.25 μM afatinib resulted in a significantly lower viable fraction. Data are presented as the mean ± standard deviation. The horizontal bracket indicates the first concentration with a significantly different number of viable cells, as compared with the control. Concentrations above the first significantly different concentration were also significant. *P≤0.05, **P≤0.01, ***P≤0.001.

the viable fraction was 91.7% (SD±11.3%). Following treatment with 1.25 μM afatinib and the fixed concentration of cisplatin, the fraction of viable cells was 69.1% (SD±4.8%), which was significantly reduced, as compared with the control (P<0.0001). Following treatment with cisplatin and

2.5 μM afatinib, the viable fraction was 44.8% (SD±11.8%), as compared with the control cells. The fraction of viable cells following treatment with 5 μM afatinib and the fixed concentration of cisplatin was 36.8% (SD±10.4%). When the cells were treated with cisplatin and the highest concentration
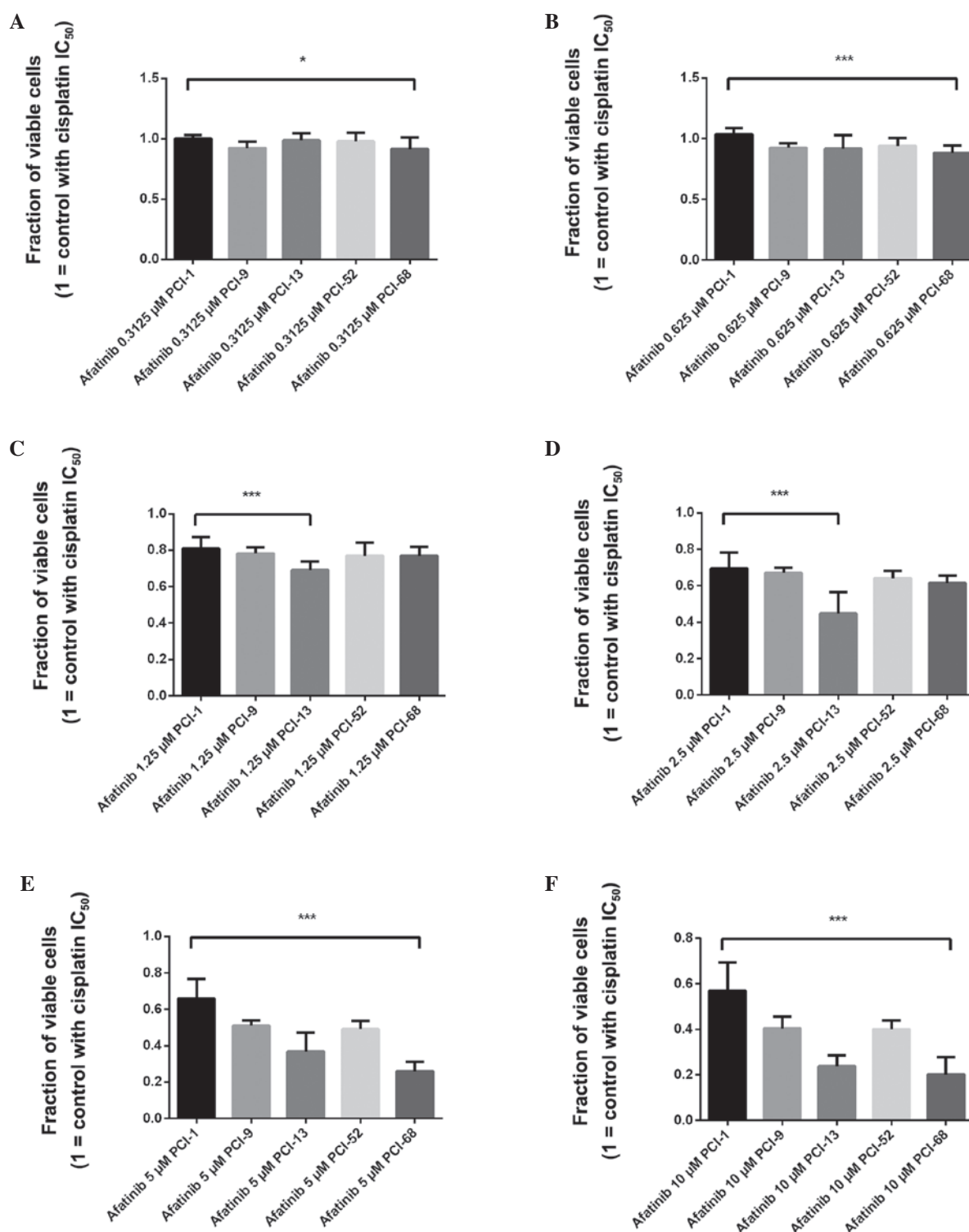
Figure 2. Comparison of various afatinib concentrations (in combination with fixed doses of cisplatin) across all cell lines. The efficacy of cisplatin combined with (A) 0.3125 μM (B) 0.625 μM, (C) 1.25 μM, (D) 2.5 μM, (E) 5 μM and (F) 10 μM afatinib. In response to all combination afatinib/cisplatin concentrations, PCI-1 cells exhibited the weakest effect. The highest efficacy differed in relation to the concentration. For 0.3125 μM, 0.625 μM, 5 μM and 10 μM afatinib, PCI-68 cells were the most vulnerable. For 1.25 μM and 2.5 μM afatinib PCI-13 cells exhibited the highest effect. Data are presented as the mean ± standard deviation. The horizontal bracket indicates the significant difference between the highest and the lowest treatment effects. *P≤0.05, ***P≤0.0001. IC$_{50}$, half maximal inhibitory concentration.

of afatinib (10 μM), the viable fraction was only 23.8% (SD±4.8%) (Fig. 1C).

Treatment efficacy in PCI-52 cells. The fraction of viable cells following treatment with a fixed concentration of cisplatin

(5 μM) and 0.3125 μM afatinib was 98.1% (SD±6.7%). When the cells were treated with cisplatin and 0.625 μM afatinib, the viable fraction was 94.2% (SD±6.5%), which was significantly reduced, as compared with the control cells (P=0.0213). The fraction of viable cells following treatment with 1.25 μM

afatinib and the fixed concentration of cisplatin was 77.0% (SD±7.1%). Following treatment with cisplatin and 2.5 μM afatinib, the fraction of viable cells was 64.1% (SD±4.0%), as compared with the control cells. The fraction of viable cells following treatment with 5 μM afatinib and the fixed concentration of cisplatin was 49.2% (SD±4.4%). Following treatment with cisplatin and the highest concentration of afatinib (10 μM), the fraction of viable cells was only 40.0% (SD±3.9%) (Fig. 1D).

Treatment efficacy in PCI-68 cells. The fraction of viable cells following treatment with the fixed concentration of cisplatin (14 μM) and 0.3125 μM afatinib was 91.6% (SD±9.5%). When the cells were treated with cisplatin and 0.625 μM afatinib, the viable fraction was 88.2% (SD±6.4%), which was significantly reduced, as compared with the control (P=0.0001). The fraction of viable cells following treatment with 1.25 μM afatinib and the fixed concentration of cisplatin was 77.0% (SD±4.7%). Following treatment with cisplatin and 2.5 μM afatinib, the fraction of viable cells was 61.4% (SD±4.3%), as compared with the control cells. The fraction of viable cells following treatment with 5 μM afatinib and the fixed concentration of cisplatin was 26.0% (SD±5.0%). Following treatment with cisplatin and the highest concentration of afatinib (10 μM), the viable fraction was only 20.2% (SD±7.6%) (Fig. 1E).

Statistical analysis of the highest and lowest efficacy of combination therapy in all cell lines. For all of the investigated concentrations, the highest and lowest efficacy of afatinib treatment in combination with cell line-specific cisplatin concentration differed significantly. In all experiments, afatinib exhibited the lowest efficacy in the PCI-1 cell line. The treatment efficacy ranges between the cell lines increased with the higher concentrations of afatinib. Comparing the effect of 0.3125 μM afatinib (in combination with the cell-specific fixed dose of cisplatin) the Mann-Whitney test revealed a significant difference (P=0.0115) between the PCI-68 (91.6%) and PCI-1 (100.4%) cell lines (Fig. 2A). In addition, a highly significant difference (P<0.0001) was noted between the PCI-68 (88.2%) and PCI-1 (103.7%) cell lines following treatment with 0.625 μM afatinib and cisplatin (Fig. 2B). Following treatment with cisplatin and 1.25 μM afatinib, a significant difference (P<0.0001) was detected between the cell line with the highest efficacy (PCI-13: 69.1%) and the cell line with the lowest efficacy (PCI-1: 81.1%) (Fig. 2C). Comparing the effects of 2.5 μM afatinib (in combination with the cell-specific fixed dose of cisplatin), the Mann-Whitney test revealed a significant difference (P<0.0001) between the PCI-13 (44.8%) and PCI-1 (69.3%) cells (Fig. 2D). Following treatment with 5 μM afatinib and cisplatin, a significant difference (P<0.0001) was detected between the cell line with the highest efficacy (PCI-68: 26.0%) and the cell line with the lowest efficacy (PCI-1: 65.9%) (Fig. 2E). Furthermore, a significant difference (P<0.0001) was detected between PCI-1 (57.1%) and PCI-68 (20.2%) cells (Fig. 2F) in response to cisplatin and the highest concentration of afatinib (10 μM).

Discussion

Squamous cell carcinoma of the oral cavity is the most common type of head and neck cancer (23). Similar to other malignancies, overall survival is associated with the extent of local tumor spread, regional lymph node metastases and distant metastases. Based on the literature, the cumulative five-year survival rate for head and neck cancer has been ~50% for more than 30 years (24). Unfortunately, ~60% of patients present with locally advanced or metastatic disease (25). In these patients, multimodal treatment, which typically comprises surgery, radiotherapy and chemotherapy, is commonly applied. In terms of targeted therapies, the EGFR has an important role in head and neck cancer (5); however, single agent therapy against EGFR with cetuximab in patients with recurrent or metastatic head and neck cancer exhibits limited response rates of ~13% (26). At present, this treatment failure is attributed to alternative receptor activation, mainly by other members of the ErbB family, including HER2 (15,27). In addition to monoclonal antibodies, including cetuximab and trastuzumab, which inhibit ErbB family receptors, the TKI are powerful agents that inhibit signaling. Unfortunately, erlotinib, a reversible first-generation EGFR TKI, failed to improve treatment results in patients with locally advanced head and neck cancer (14). In this context, afatinib, an irreversible EGFR/HER2/HER4 second-generation TKI, may improve outcome in head and neck cancer therapy.

Given the extensive research being conducted on afatinib monotherapy in human cancer cell lines (28), the present study aimed to explore the effects of combination therapy with a widely used agent. By investigating five wild-type EGFR head and neck cancer cell lines, the present study demonstrated that afatinib enhances platinum-based chemotherapy. In all cell lines used in the present study, the growth inhibiting effects of afatinib were concentration-dependent.

Notably, differences were noted between the cell lines. In all cell lines, significant treatment effects could be observed using concentrations achieved for other EGFR TKIs in a clinical setting (21). By comparing the results of the present study to those of previous studies by our group, we observed the lowest efficacy of afatinib in the PCI-1 cell line, which exhibits the best response to EGFR antibodies, including cetuximab and panitumumab (19). In our previous study, the impact of EGFR knockdown on cetuximab and panitumumab efficacy was investigated. Notably, knockdown of EGFR expression enhanced anti-EGFR treatment efficacy (20), and this effect was strongest in PCI-1 cells. In addition, PCI-1 cells also exhibited the best response to erlotinib and gefitinib in the same panel of cell lines used in the present study (data not shown).

According to the growth assay, afatinib efficacy in the PCI-52 cell line did not differ, as compared with the other cell lines. In previous studies, this cell line exhibited the lowest response to cetuximab, panitumumab, erlotinib and gefitinib (19,29). This finding indicated that predictions regarding the efficacy of anti-EGFR treatment in head and neck cancer remain challenging. However, in cancer exhibiting cetuximab, erlotinib and gefitinib failure, afatinib may serve as an additional treatment option. This hypothesis was addressed by Seiwert *et al* (17), which indicated that disease control may be achieved by switching from cetuximab to afatinib treatment, and vice versa, in cases of progressive disease.

In conclusion, the present study demonstrated that afatinib in combination with platinum agents may exhibit considerable

potential to enhance response rates in head and neck cancer, especially in patients that have previously experienced cetuximab failure. Further preclinical and clinical investigations are required to identify predictive markers for anti-EGFR/HER2 and HER4 treatment, and to identify a subset of patients who will benefit from targeted therapy.

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