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FGF Expression in HPV16-positive and -negative SCC After Treatment With Small-molecule Tyrosine Kinase Inhibitors and Everolimus

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Abstract. *Background: Targeted therapies in the treatment of head and neck squamous cell carcinoma (HNSCC) are subject to extensive research. Different mutations of genes belonging to the fibroblast growth factor (FGF) family have been detected in HNSCC. In this study, we examined the expression of FGF1 and FGF2 after treatment with small-molecule tyrosine kinase inhibitors (TKIs) and an inhibitor of mechanistic target of rapamycin (mTOR) in vitro using human papillomavirus (HPV)-positive and -negative SCC lines. Materials and Methods: Cells of two human HPV-negative cell lines (UMSCC-11A/-14C) and one HPV-positive cell line (CERV196) were incubated with 20 μmol/l of erlotinib, gefitinib, nilotinib, dasatinib, or everolimus for 24-96 h. Cell proliferation was assessed by proliferation assay and the protein concentrations of FGF1 and FGF2 by sandwich enzyme-linked immunosorbent assay. For statistical analysis, the results were compared with those for untreated HPV-negative SCC cells. Results: FGF1 and FGF2 were detected in all three tested cell lines. The tested TKIs significantly ($p<0.05$ reduced) FGF1 expression in the UMSCC-11A cell line within the first 24 h. At later time points, the tested TKIs and everolimus significantly ($p<0.05$) increased FGF1 and FGF2 expression in HPV-negative and -positive cancer cell lines. The effect was stronger in the HPV-positive cell line. Conclusion: Alterations in FGF signalling are considered to be relevant drivers of tumourigenesis in some HNSCCs. Our results show that the expression of FGF1 and -2 can be influenced effectively by small-molecule TKIs and everolimus. Based on our data, future research should include combinations of specific FGF inhibitors, mTOR inhibitors and other TKIs in the treatment of HNSCC and research on FGF-mediated drug escape mechanisms.*

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Head and neck squamous cell carcinomas (HNSCC) are a heterogeneous group of malignancies that account for over 90% of all head and neck cancer. The development of new treatment options besides radiotherapy, chemotherapy, surgery and immunotherapy is the subject of extensive research. The increase in targeted therapies, such as the checkpoint inhibitors pembrolizumab and nivolumab or the epidermal growth factor receptor (EGFR) antibody cetuximab, has widened the treatment range, improved the overall survival rate for recurrent/metastatic cancer and lead to less toxic side-effects (1-3). Recently, research efforts have focused on HNSCC caused by human papillomavirus (HPV). By integrating viral DNA into the host genome, the viral oncogenes *E6* and *E7* are overexpressed and stimulate cell proliferation with genomic instability and the formation of tumours (4). HPV-related HNSCC has a better prognosis than HPV-negative HNSCC. However, recent study has shown that concerning overall survival, de-escalating therapy strategies were not superior to classic medical therapy options (5).

Here, we examined the effect of small-molecule tyrosine kinase inhibitors (TKIs) and the inhibitor of mechanistic target of rapamycin (mTOR) everolimus on the fibroblast growth factor (FGF) signalling pathway in HPV-positive and -negative squamous cancer cells (SCC). The FGF family consists of a variety of growth factors and receptors. Since the discovery of FGF1 and FGF2, more than 20 peptides have been added to the FGF family (6). FGF signalling is complicated and plays a

relevant role in many other connected signalling pathways (7). The FGF family is involved not only in embryonic development but also in tissue regeneration, homeostasis and many other metabolic functions in adult organisms (8, 9). When FGF signalling is disrupted, it can be the cause for metabolic disorders, congenital defects and cancer (6, 7). FGF ligands are polypeptides classified as canonical isoforms that bind to FGF receptors (FGFRs) and as non-canonical or intracellular isoforms that interact with voltage-gated channels (10). The ligands interact with cofactors, such as heparan/heparin sulphate proteoglycans or Klotho cofactors, which are responsible for the specificity of FGF signalling and binding affinity to FGF (11-13).

In this study, we examined the expression of FGF1 and FGF2, which are part of the FGF1 subfamily. FGF1 and FGF2 are directly translocated through the cell membrane because of the lack of signal peptides, and FGF1 is the only subtype that can interact with all four FGFRs (14, 15). The deregulation of FGF signalling is involved in the development of many types of solid carcinomas, including HNSCC (16-20). Different mechanisms, such as receptor dimerization, formation of fusion proteins, autophosphorylation of FGFR or permanently active kinases, lead to aberrant FGF signalling in cancer (17, 21-23). The amplification of *FGFR1* is strongly associated with the development of cancer but the up-regulation of most FGF isoforms is also involved in tumour development and growth (24, 25).

Some cancer treatment strategies designed to interfere with FGF signalling, including TKIs, are currently under experimental investigation. Multiple non-specific TKIs are already used in clinical applications (23). In this study, we examined the effects of selective TKIs erlotinib, gefitinib, nilotinib and dasatinib on the expression of FGF1 and FGF2 in HPV-positive and -negative SCC. Nilotinib and dasatinib are inhibitors of breakpoint cluster region (BCR)–Abelson murine leukaemia viral oncogene homolog 1 (ABL) that affect platelet-derived growth factor receptor and c-KIT, and are used in the treatment of chronic myeloid leukaemia (26). Erlotinib and gefitinib are mainly EGFR inhibitors approved for the treatment of non-small lung cancer. All the TKIs tested here exert effects on multiple tyrosine kinases, but only erlotinib and dasatinib directly affect FGFRs (27). These TKIs have been studied in the treatment of recurrent or metastatic HNSCC. To date, gefitinib and dasatinib have not been shown to have beneficial effects on overall or progression-free survival compared to standard chemotherapy (28, 29). Treatment with erlotinib showed the most promising effects in clinical trials (30, 31). Enhanced phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) is another key signalling pathway that is often deregulated in cancer, and the pharmaceutical inhibition of mTOR has been investigated in trials for different cancer types, including HNSCC (32). In the majority of HNSCCs, the mTOR pathway was found to be activated, especially in HPV-positive HNSCCs (33, 34). However, early trials on recurrent or metastatic HNSCC were discouraging: A positive effect on the tumour response rate with the combination of erlotinib and everolimus was not detected in a phase II trial (35, 36), and everolimus monotherapy was not effective (35, 36). Other combinations, such as docetaxel and an mTOR inhibitor, have achieved promising results in preclinical studies, and an mTOR inhibitor neoadjuvant monotherapy also obtained notable response rates (37, 38).

As far as we are aware, this study is the first to investigate the effects of erlotinib, gefitinib, nilotinib, dasatinib and everolimus on the expression of FGF1 and FGF2 *in vitro* using HPV-positive and -negative SCC lines.

Materials and Methods

Cell lines, drugs and study design. We examined two human HPV16-negative cell lines [University of Michigan squamous cell carcinoma (UMSCC), provided by T.E. Carey, Ph.D., University of Michigan, Ann Arbor, USA] and one human HPV16-positive cell line (CERV196; Cell Lines Service GmbH, Eppelheim, Germany). The HPV16-negative cell lines originally came from a primary SCC of the epiglottis (UMSCC-11A) and a skin metastasis of a floor of mouth SCC after surgery and radiochemotherapy (UMSCC-14C). The HPV16-positive cell line was derived from a cervix SCC. Eagle's minimum essential medium (Gibco, Life Technologies, Carlsbad, CA, USA) was used in the culture of UMSCC-11A and UMSCC-14C cells. The cultures contained 2 mM L-glutamine, 10% fetal calf serum and antibiotics/antimycotics according to the instruction manual (Gibco, Life Technologies). The same culture medium was used for CERV196 tumour cells but it was supplemented with 2 mM L-glutamine, 1.0 g/l sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 g/l sodium pyruvate and 10% foetal bovine serum (Gibco, Life Technologies). The cells were incubated under standardised conditions at 37˚C, with 5% CO_2 and 95% humidity. New passages of the cells were created through the addition of a phosphate-buffered saline (PBS) solution containing a combination of 0.05% trypsin and 0.02% ethylenediaminetetra-acetic acid (EDTA) (Sigma Aldrich; Merck KGaA, Darmstadt, Germany) at 37˚C for 5 min. Nilotinib, dasatinib, gefitinib, erlotinib and everolimus were kindly provided by Professor Dr. Hofheinz, Oncological Department, University Hospital Mannheim, Medical Faculty Mannheim, University of Heidelberg, Germany. The drugs were stored at room temperature and dissolved in dimethylsulfoxide at the time of the experiments. Tumour cells were incubated with 20 μmol/l of each drug at 37˚C for 24, 48, 72 and 96 h. Untreated cells served as a negative control.

Proliferation assay and enzyme-linked immunosorbent assay (ELISA) for FGF1 and FGF2. Cell confluency was 70%. Despite the cytotoxic properties of the protein kinase inhibitors, the experiments required drug concentrations in the range of 1-50 μmol/l to maintain a vital basis for the cells. Thus, we used the alamarBlue™ cell viability tests (AbD Serotec, Raleigh, NC, USA) to identify the optimal drug concentrations for experiments. No

significant difference was found in the results between the different drug concentrations, and thus we chose a concentration of 20 μmol/l. The alamarBlue™ cell proliferation assay was used according to the manufacturer's protocol. Measurement of the protein concentrations was achieved using sandwich ELISA according to the manufacturer's instructions. DuoSet® ELISA (R&D Systems, Inc., Minneapolis, MN, USA) was used for FGF1 (DY 232) and FGF2 (DYC 233). Optical density was measured with a MRX Microplate Reader (DYNEX Technologies, Chantilly, VA, USA) at a wavelength of 450 nm with a wavelength correction of 540 nm. The detection range was 125-8,000 pg/ml for FGF1 and 47-3,000 pg/ml for FGF2. The interassay coefficient of variation given by the manufacturer was below 10%. All experiments were repeated at least three times (n=3).

Statistical analysis. Data are presented as the mean values±standard deviation. The two coefficient variance test (SAS Statistics software, version 9.3; SAS Institute, Inc., Cary, NC, USA) and Dunnett's test were used. A p-value of 0.05 or less was considered statistically significant. Statistical analysis was performed with the assistance of Professor Dr. C. Weiss of the Institute of Biomathematics, Medical Faculty Mannheim, University of Heidelberg, Germany.

Results

FGF1 protein expression. FGF1 was detected in all three tested cell lines. The lowest expression level was found in the HPV-positive cell line CERV196.

Compared with those of the negative control, the expression levels of FGF1 fluctuated after incubation with the tested TKIs and everolimus in UMSCC-11A cells. The FGF1 levels mostly decreased in the first 72 h and then increased again after 96 h. This pattern was also observed in the negative control and after incubation with nilotinib, dasatinib and everolimus. In the UMSCC-11A cell line, treatment with nilotinib led to a significant decrease in expression after 24 h, and then the level aligned with those of untreated cells. No significant difference compared to the negative control was found after treatment with nilotinib in the UMSCC-14C cell line. Similarly, the other BCR–ABL inhibitor dasatinib significantly reduced the FGF1 level initially after 24 h in the UMSCC-11A cell line. Afterwards, the level increased, even reaching a significant difference from the negative control after 72 h. In the UMSCC-14C cell line, a significant increase in FGF1 expression was seen after 48 h treatment with dasatinib.

Erlotinib and gefitinib also induced a significant decrease in the expression of FGF1 in the UMSCC-11A cell line over the first 24 h. The level increased, reaching a significant difference from the control after 72 h. In the UMSCC-14C cell line, erlotinib first increased the expression level before reducing it, again with a significant difference after 72 h. Gefitinib led to a significant increase in expression after 24 h.

Everolimus did not induce significant changes in the expression of FGF1 in the HPV-negative cell lines.

None of the tested drugs led to a significant decrease in expression of FGF1 in the HPV-positive cell line CERV196. Nilotinib significantly increased the expression level after 24 and 48 h. At later time points, the expression levels aligned with those of the negative control. Treatment with dasatinib led to consistently higher expression levels than those of the negative control, reaching significance at 48 h and 72 h.

Treatment with erlotinib and gefitinib also led to higher expression of FGF1, reaching significance after 24, 48 and 96 h for erlotinib and after 48 and 72 h for gefitinib ($p \leq 0.05$). Treatment with everolimus induced consistently higher expression of FGF1 in HPV-positive cancer cells, leading to a significant difference from the negative control after 48-96 h.

FGF2 protein expression. FGF2 was detected in all three tested cell lines. The lowest expression level was found in the HPV-positive cell line CERV196.

Overall, FGF2 expression was lower than that of FGF1 in untreated cells.

In the UMSCC-11A cell line, nilotinib induced a significant increase in the expression of FGF1 after 24 h. Afterwards, the expression fluctuated with an increase reaching significance after 96 h. In the UMSCC-14C cell line, increasing expression levels were observed with a significant difference from the control after 72 and 96 h after treatment with nilotinib. Dasatinib significantly increased expression at all time points in the UMSCC-11A cell line but did not have a significant effect on the UMSCC-14C cell line. The expression patterns were similar after incubation of UMSC-11A cells with dasatinib, erlotinib and gefitinib and after the incubation of UMSCC-14C cells with erlotinib and gefitinib. A significant increase in expression at all time points was also observed when UMSCC-11A cells were treated with erlotinib. Expression levels of FGF2 were significantly higher than those of the control in UMSCC-14C cells. Gefitinib induced a significant increase in expression after 48-96 h in the UMSCC-11A cell line and after 24 and 72 h in the UMSCC-14C cell line.

Everolimus did not have a significant effect on FGF2 expression compared with the other tested drugs in HPV-negative SCC cells. Everolimus significantly increased FGF2 expression after the first 24 h in the UMSCC-11A cell line but it did not affect the expression in the UMSCC-14C cell line.

In the HPV-positive cell line, the effect of the tested drugs was not as strong as in the HPV-negative cells. A significant increase in expression was measured after 24 h after treatment with nilotinib and dasatinib, whilst erlotinib and gefitinib led to a significant increase in the FGF2 expression only after 96 h. Incubation with everolimus did not change the expression levels significantly, although the expression levels increased over time.

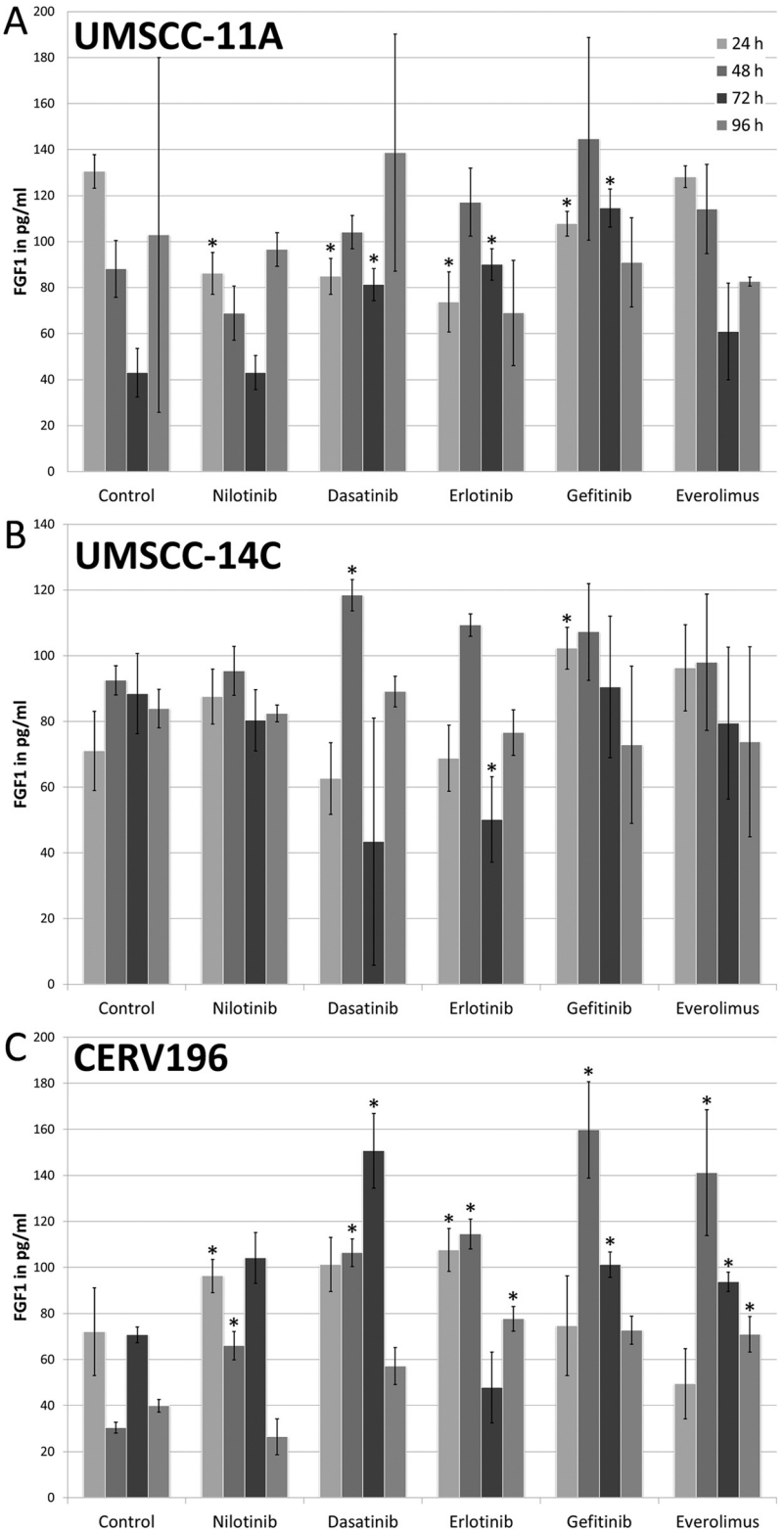

Figure 1. *Fibroblast growth factor 1 (FGF1) expression in human papillomavirus 16 (HPV16)-negative UMSCC-11A (A) and UMSCC-14C (B) and HPV16-positive CERV196 (c) cell lines after incubation for 24-96 h with small-molecule tyrosine kinase inhibitors nilotinib, dasatinib, gefitinib, erlotinib, or with everolimus, an inhibitor of mechanistic target of rapamycin, compared with that in untreated control cells. Data are the mean values. The standard deviation is indicated. *Significantly different at p≤0.05.*

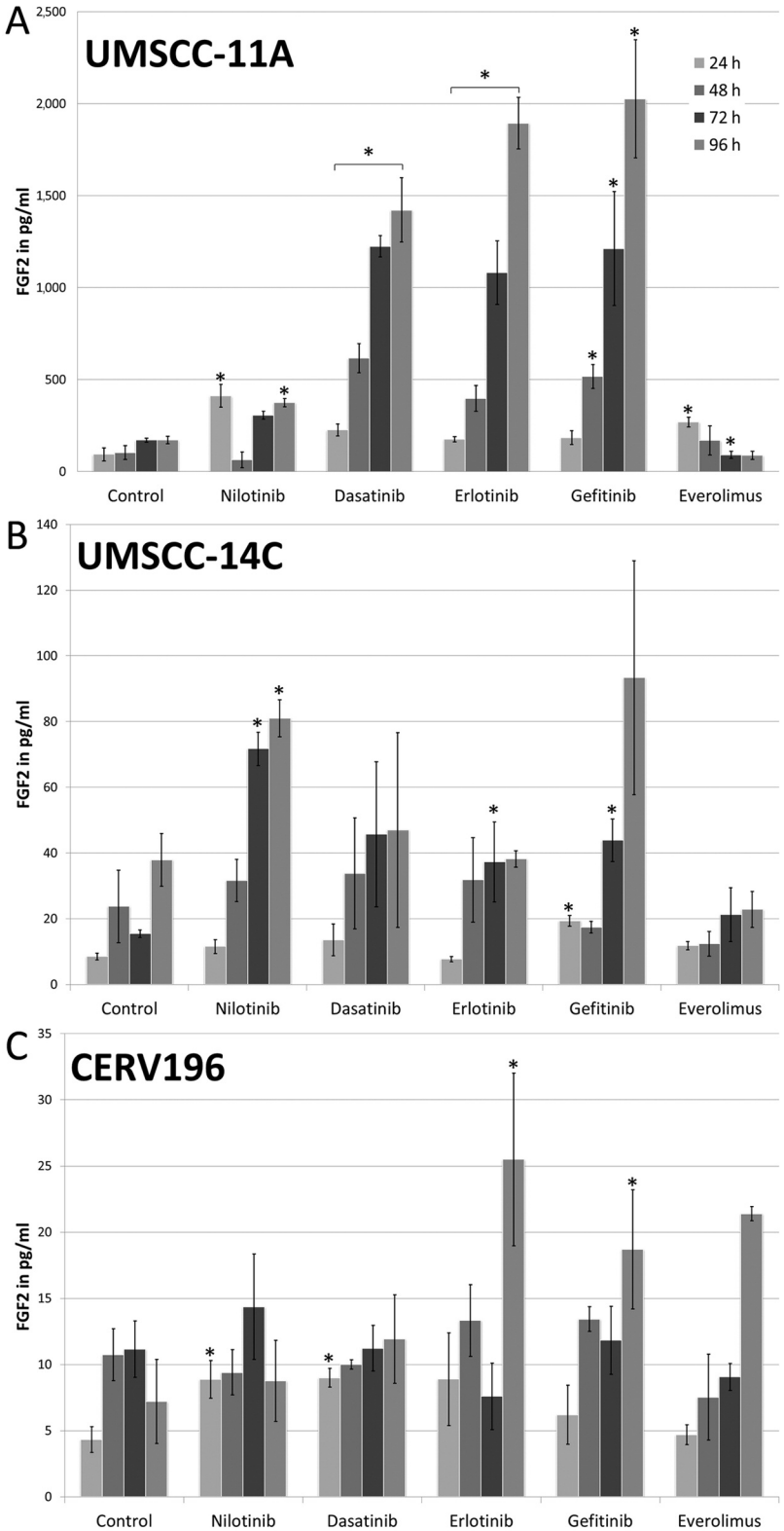

Figure 2. *Fibroblast growth factor 2 (FGF2) expression in human papillomavirus 16 (HPV16)-negative UMSCC-11A (A) and UMSCC-14C (B) and HPV16-positive CERV196 (c) cell lines after incubation for 24-96 h with small-molecule tyrosine kinase inhibitors nilotinib, dasatinib, gefitinib, erlotinib, or with everolimus, an inhibitor of mechanistic target of rapamycin, compared with that in untreated control cells. Data are the mean values. The standard deviation is indicated. *Significantly different at p≤0.05.*

Discussion

In this study, we investigated the effect of TKIs, namely nilotinib, dasatinib, gefitinib and erlotinib, and the mTOR inhibitor everolimus on the expression patterns of FGF1 and FGF2 in HPV-positive and -negative SCC cell lines. FGF-mediated signalling is crucial for local tumour progression and cancer development in HNSCC, and it serves as a possible pharmaceutical target in the medical treatment of HNSCC.

Overall, the tested TKIs and everolimus tended to increase FGF1 and FGF2 expression in the studied HPV-negative and -positive cancer cell lines. Previous studies of our group showed that the expression levels of other key signalling proteins involved in cancerogenesis, such as platelet-derived growth factor, vascular endothelial growth factor (VEGF) receptor and β-catenin, were significantly reduced by TKIs (39-41). The tested TKIs successfully reduced FGF1 expression within the first 24 h in the UMSCC-11A cell line. Apart from that, no decrease in expression could be discerned. Specifically, FGF1 was strongly affected by the tested TKIs with the most significant results in the HPV-positive cell line. Everolimus had little effect on the HPV-negative cells but increased the FGF1 expression in the HPV-positive cell line.

Mutations, rearrangements and amplifications of various genes or altered expressions of proteins in the FGF signalling pathway have been found in HNSCC. FGFR1/FGF3 mutation and FGF2/FGFR2/FGFR3 overexpression were detected in HSNCC (20, 42-46). However, the expression of FGF-related proteins varies greatly depending on the cell line and patient cohort. The co-expression of FGF2 and FGFR2 plays an important role in autocrine FGF signalling (45). Alterations in FGF signalling are considered to be relevant drivers of tumourigenesis in some HNSCCs, but the exact mechanisms remain the subject of research (42).

In our study, we found differences in the alteration of FGF expression levels after treatment with TKI or everolimus between the HPV-positive and -negative cell lines.

Although HPV-positive HNSCC has better prognosis, and increasing evidence shows that HPV-positive HNSCC can be considered a separate malignancy, current treatment regimens do not differ from those for HPV-negative HNSCC. The viral oncogenes *E6* and *E7* induce carcinogenic processes through viral host integration and the deregulation of tumour-suppressor genes, such as retinoblastoma (*RB*) and *p53* (47, 48). The discovery of other aberrant pathways was recently made possible by genome sequencing (49). HPV-positive HNSCC was found to harbour different mutations from smoking-associated tumours. These mutations mainly affect oncogene *PI3K*, an upstream ligand of the mTOR pathway (49). mTOR was also shown to be up-regulated in HPV-positive HNSCC cell lines compared with the HPV-negative cell lines (50). Therefore, investigating mTOR inhibitors, such as everolimus, as a possible supplement in the treatment of HPV-positive HNSCC is a valid approach to implementing new targeted therapies. A previous study of our group showed that everolimus successfully reduced mTOR-expression in HNSCC (50). In this study, everolimus led to a significant increase in FGF1 expression in the HPV-positive cell line, suggesting a connection between FGFR/FGF signalling and the mTOR pathway. This connection was also shown in other studies. Patients with breast cancer with *FGFR* or *FGF* amplification and alteration in the mTOR pathway who were treated with mTOR inhibitors had a longer period of stable disease and better response rates to treatment than those with only one deregulated pathway, thus suggesting that tumour cell growth is dependent on FGFR/FGF signalling when the mTOR pathway is disabled (51). In gastric cancer cells, specific FGFR inhibitor also inhibited mTOR signalling and impaired tumour cell growth more effectively in combination with an mTOR inhibitor than an mTOR inhibitor alone (52). Early clinical studies using an mTOR inhibitor alone or in combination with TKIs have not yet shown promising results in the treatment of HNSCC (35, 36). However, the mTOR pathway is considered one reason for drug resistance mechanisms and may reveal its full potential when an mTOR inhibitor is combined with other drugs. The results of a study examining the effect of neoadjuvant chemotherapy with docetaxel, fluorouracil and cisplatin demonstrated that the overexpression of phosphorylated mTOR is correlated with a poor response to this induction therapy. When mTOR inhibition was added to the regimen, the efficacy of docetaxel was significantly enhanced (37). A combination of taxane, carboplatin and an mTOR inhibitor was also tested in a phase II clinical trial in recurrent/metastatic HNSCC and led to a good response rate (53). In both studies, the differences between HPV-positive and -negative tumours were not examined. Similar to the effects of everolimus on the tested cell lines, the most consistent increase in FGF1 expression after treatment with TKIs was seen in the HPV-positive cell line. Erlotinib and dasatinib also significantly increased FGF2 expression in the HPV-negative cell line UMSCC-11A. These findings are not surprising, as erlotinib and dasatinib have been shown to exert effects on FGF signalling (27). Moreover, there are differences in FGF signalling between HPV-positive and -negative tumours. The HPV-associated oncoprotein E5 is involved in the down-regulation of the expression of FGFR2b, which leads to the overexpression of another FGFR2 variant, thereby altering the specificity of the FGFR ligands (54). This mechanism may explain the low expression levels of FGF2 in untreated HPV-positive cells in our study. The expression of FGF in HPV-positive cells was up-regulated by the addition of the tested TKIs, consistent with the results of other studies: Treatment of non-small-cell lung cancer cells with gefitinib

led to an increase in FGFR2 and FGFR3 expression and thus bypassed EGFR signalling to stimulate tumour growth and transformation (55). This activation can explain why use of initially effective drugs may rapidly lead to drug resistance. This resistance mechanism can be mediated through an autocrine loop of FGF ligand and receptor overexpression in the same cell (56).

Similar results were found when lung cancer cells were treated with the mitogen-activated protein kinase inhibitor trametinib: An increase in FGFR1 and ligand expression was found in the tumour cells, resulting in drug resistance. The addition of an FGFR-specific inhibitor enabled the further inhibition of cell proliferation (57). The same FGF-mediated resistance mechanism was shown for pancreatic cancer in a mouse model. Tumour growth and angiogenesis were initially impaired in response to VEGF inhibition. However, the induction of other growth factors, such as FGF, led to an induction of proliferation in later stages. The combination of VEGF inhibition with FGF inhibition through a specific ligand trap after 10 days managed to significantly reduce tumour growth compared with VEGF inhibition alone (58).

The effect of a combination therapy of EGFR- and FGFR-specific TKIs has already been examined in HNSCC cell lines. In cell lines dependent on the co-activation of both pathways, the combination of drugs was more effective in inhibiting growth than a single agent (45). The TKIs tested for FGF inhibition either compete for the ATP-binding site or impair downstream signalling through means such as preventing receptor autophosphorylation (59). Non-ATP-binding site inhibitors (*e.g.* L6123) are considered to be more specific, whereas the ATP-binding site inhibitors (*e.g.* dovitinib and nintedanib) affect multiple tyrosine kinase receptors (7, 60, 61). Currently, some non-selective FGFR/FGF inhibitors (*e.g.* lenvatinib and dovitinib) have been approved for cancer therapy or are finishing phase III clinical trials, but specific FGFR/FGF inhibitors, monoclonal antibodies and FGF ligand traps are still under consideration in early phase I/II clinical trials (62, 63). Proteins of the FGF pathway as biomarkers in HNSCC are also currently being investigated (46, 64). FGF2 and FGFR2 are reportedly up-regulated when pre-malignant oral lesions transform into oral cancer (64).

Based on our data, future research should include combinations of specific FGF inhibitors and mTOR inhibitors and other TKIs in the treatment of HNSCC. Each tumour seems to respond in a different way to specific treatment or treatment combination because of different patterns of mutations and amplifications of different proteins in HNSCC. This strengthens the need for biomarkers prior to treatment in order to select drugs with regard to the response to an individualised medical treatment.

In conclusion, our results show that proteins of the FGF pathway are affected by treatment with small-molecule tyrosine kinase and mTOR inhibitors in HPV-negative and -positive SCC. Therefore, they provide crucial information for future research on drug escape mechanisms and the development of targeted therapies for HNSCC.

Conflicts of Interest

All Authors declare that they have no conflicts of interest.

Authors' Contributions

Lena Huber, Benedikt Kramer and Christoph Aderhold conceived and planned the experimental design. Benedikt Kramer, Manuel Knuettel and Christoph Aderhold carried out the experiments. Lena Huber, Anne Lammert, Frederic Jungbauer, Christoph Aderhold, Claudia Scherl, Richard Birk and Benedikt Kramer contributed to the interpretation of the results. Lena Huber and Benedikt Kramer took the lead in writing the article. All Authors provided critical feedback, discussed the results and commented on the article.

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