Targeted Therapies in HPV-positive and -negative HNSCC – Alteration of EGFR and VEGFR-2 Expression *In Vitro*

BENEDIKT KRAMER[1], CLEMENS HOCK[1], RICHARD BIRK[1], ALEXANDER SAUTER[1], BORIS A. STUCK[2], KARL HÖRMANN[1], JOHANNES DAVID SCHULTZ[1] and CHRISTOPH ADERHOLD[1]

[1]*Department of Otorhinolaryngology Head and Neck Surgery, University Hospital Mannheim, Medical Faculty Mannheim, University Heidelberg, Mannheim, Germany*
[2]*Department of Otorhinolaryngology Head and Neck Surgery, University Hospital Essen, University Duisburg-Essen, Essen, Germany*

Abstract. *Background: Angiogenesis plays a crucial role in the formation and progression of tumor growth in head and neck squamous cell carcinoma (HNSCC). The tyrosine kinase receptors epidermal growth factor receptor (EGFR) and vascular endothelial growth factor receptor (VEGFR) are essential for mediation of pro-angiogenic signals. Nilotinib, dasatinib, erlotinib and gefitinib are tyrosine kinase inhibitors and approved as targeted therapies for several tumor entities other than HNSCC. In this study, we sought to evaluate the alteration of EGFR and VEGFR-2 expression by these tyrosine kinase inhibitors with respect to the human papillomavirus (HPV)-status in squamous cell carcinoma (SCC) tumor cells. Materials and Methods: Expression patterns of EGFR and VEGFR-2 were determined by enzyme linked immunosorbent assay (ELISA) in HNSCC 11A, HNSCC 14C and p-16-positive CERV196 tumor cell lines. These cells were incubated with nilotinib, dasatinib, erlotinib and gefitinib (5-20μmol/l) and compared to a chemonaive control. The incubation time was 24, 48, 72 and 96 h. Results: All tested substances led to a statistically significant reduction ($p<0.05$) of EGFR protein expression levels in HPV-negative cells compared to the negative control. Surprisingly, a statistically significant increase in VEGFR-2 expression was observed after exposure to all tested substances especially after exposure to erlotinib treatment. Conclusion: Nilotinib, dasatinib, erlotinib and gefitinib cause significant changes in protein expression of EGFR and VEGFR-2 in vitro. Besides the anti-angiogenic impact of the substances, as shown for the decrease of EGFR expression, we also observed an increase of VEGFR-2 expression. These contradictive effects could be interpreted as a compensatory up-regulation by the tumor cell.*

Abbreviations: ATP: Adenosine triphosphate; BCR-ABL: fusion protein - breakpoint cluster region protein and Abelson murine leukemia viral oncogene; BSS: balanced salt solution; CERV: cervical squamous cell carcinoma tumor cell line; cKIT: Mast/stem cell growth factor receptor; CSF: colony stimulating factor; DMEM: dulbecco's modified essential medium; DMSO: dimethylsulfoxide; EGF: epidermal growth factor; EGFR: epidermal growth factor receptor; ELISA: enzyme-linked immunosorbent assay; FDA: food and drug administration; FCS: fetal calf serum; HNSCC: head and neck squamous cell carcinoma; HPV: human papillomavirus; HRP: horseradish peroxidase; IL-6: interleukin-6; MAPK: mitogen-activated protein kinase; NSCLC: non-small cell lung cancer; PBS: phosphate-buffered saline; PDGFR: platelet-derived growth factor receptor; PI3K: phosphatidylinositol 3-kinase; PI3K–AKT: intracellular signaling pathway regulating cell cycle; PTEN: phosphatase and tensin homolog; Src: sarcoma tyrosine kinase; TGF-α: transforming growth factor α; TRK: tyrosine kinase receptor; VEGF: vascular endothelial growth factor; VEGFR: vascular epidermal growth factor receptor.

Correspondence to: Dr. med. Benedikt Kramer, Department of Otorhinolaryngology Head and Neck Surgery, University Hospital Mannheim, Theodor-Kutzer-Ufer 1-3, 68167 Mannheim, Germany. Tel: +49 6213831600, Fax: +49 6213833827, e-mail: benedikt.kramer@umm.de

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Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide with a global incidence of more than 680,000 cases and a 5-year prevalence of over 1,680,000 cases (1). The most common risk factors for the development of HNSCC are tobacco and alcohol abuse (2, 3). Even though our understanding of tumor growth has increased and despite multimodal interdisciplinary therapeutic approaches against HNSCC, the 5-year survival rate has improved only marginally during the past 40 years (4, 5). Therapy of HNSCC includes surgery, radiation, chemotherapy and immunotherapy. To date, therapeutic options in advanced-stage tumor disease or cases with distant metastasis are limited (6).

Human papillomavirus (HPV) is known to be involved in the formation of several types of cancer, including carcinoma

of the uterine cervix and oropharynx. HPV-related HNSCC is reported with a prevalence of more than 20% (7, 8). Despite a global decrease of tobacco use, the incidence of oropharyngeal cancer is rising (9), which indicates the importance of HPV infection and its oncogenic potency. Among a large group of more than 100 subtypes, the high-risk HPV-types 16 and 18, mostly transferred through unprotected sex, seem to be of exceeding relevance in tumor formation. In this context, the direct stimulation of vascular endothelial growth factor (*VEGF*) gene plays a crucial role that can be modulated through HPV oncoprotein E6 independently of tumor-suppressor protein p53. This protein is a crucial regulator in the expression of angiogenic inhibitors (10). Molecular, as well as clinical, characteristics differ between HPV-positive and HPV-negative oropharyngeal cancers (11). HPV positivity is said to be associated with a better response to radiation and chemotherapy (12).

The molecular basis of tumor cell formation includes several processes, including neovascularization and inhibition of apoptosis (13). Endothelial growth factor receptor (EGFR) is a receptor tyrosine kinase overexpressed in several tumor entities, including breast cancer and squamous cell carcinoma. In HNSCC, overexpression of EGFR has been demonstrated in 40-80% (14). EGFR activation through natural ligands is accomplished by ligand-induced conformational change in EGFR; such ligands are epidermal growth factor (EGF), amphiregulin and transforming growth factor alpha (TGF-α) (15). Receptor activation leads to signal transduction *via* several molecular pathways, including Ras/Raf/mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase-protein kinase B (PI3K–AKT) (16). EGFR overexpression is generally associated with a poor prognosis (17); in HNSCC, in particular, the level of EGFR expression is affiliated with the rate of survival (18). EGFR can be inhibited with monoclonal antibodies in terms of targeted therapy. Currently, the EGFR inhibitor cetuximab is the only monoclonal antibody approved for HNSCC by the Food and Drug Administration (FDA). It is approved for patients with advanced-stage tumor disease in combination with radiation therapy or in patients with distant metastasis or recurrent disease. Mutated variants of EGFR can cause constitutive activation of the receptor and can be a possible mechanism of resistance against cetuximab (19). Yet, the response of HPV-associated HNSCC to EGFR inhibition remains unclear and is discussed controversially (20, 21).

VEGF and its receptors VEGFR-1, -2 and -3 are crucial for the proliferation and differentiation of endothelial cells (22). The growth of blood vessels (angiogenesis), as well as the formation of a circulatory system (vasculogenesis), is essential for tumor progression, invasion and formation of metastases (23, 24). VEGFRs are cell surface receptors and occur in various kinds of malignant tumors (25, 26). The "angiogenic switch" describes the imbalance of angiogenic promotion and inhibition. VEGF is said to be associated with this process, which can result in the progression of solid tumors (27, 28). In the context of HPV-related cervical neoplasia, there also seems to be an association with HPV oncoprotein E5 as a regulator of VEGF expression *in vitro* (29).

The dysregulation of tyrosine kinases is often involved in the formation of tumors. Small molecule tyrosine kinase inhibitors have been developed to selectively target tyrosine kinases that are crucial for tumor progression by inactivating the enzyme through competitive inhibition of the adenosine triphosphate (ATP) binding site (30). Small molecule-targeted therapy has been established in various cancerous diseases.

Nilotinib belongs to the group of second-generation tyrosine kinase inhibitors and acts through inhibition of the breakpoint cluster region protein and Abelson murine leukemia viral oncogene (BCR-ABL), platelet-derived growth factor receptor (PDGFR) and the mast/stem cell growth factor receptor (cKIT) (31). Nilotinib was designed as an alternative for non-responders to first generation BCR-ABL inhibitor imatinib in the treatment of chronic myeloid leukemia. The *BCR-ABL* oncogene is formed by a reciprocal translocation between chromosomes 9 and 22 called Philadelphia chromosome (32). The first orally bioavailable alternative to imatinib was dasatinib with a shorter half-life; this drug mediates its inhibitory effects *via* BCR-ABL through cKIT and PDGFR and *via* Sarcoma tyrosine kinase (Src)-inhibition (33). Tumor proliferation and invasion, as well as angiogenesis are closely associated with Src expression (34). It has also been established that EGFR-degradation is a possible mechanism for dasatinib-induced apoptosis (35).

EGFR inhibition can be accomplished by small-molecule inhibitor gefitinib (36). Gefitinib was approved for the therapy of non-small cell lung cancer (NSCLC) for the first time. The relevant mechanism of action seems to be a competitive inhibition of ATP binding to EGFR and consequent inhibition of autophosphorylation (37). This effect leads to a decrease in the expression of proangiogenic proteins, such as VEGF (38). Erlotinib is a selective and reversible inhibitor of EGFR by reducing EGFR autophosphorylation in tumor cells and by blocking cell-cycle progression at the G1 phase (39). Erlotinib is presently applied for advanced or metastatic NSCLC and metastatic pancreatic cancer (40, 41).

Based on the molecular mechanisms of nilotinib, dasatinib, erlotinib and gefitinib and the well-examined effects in several tumor entities, a similar effect can be expected in HNSCC but has not yet been demonstrated *in vitro*. Therefore, the aim of the study is the evaluation of the expression of EGFR and VEGFR-2 in HPV-positive and -

negative squamous cell carcinoma cells *in vitro* and the determination of modifications of their expression patterns under these targeted therapy agents.

Materials and Methods

Cell lines. In our laboratory we are using two different HPV-negative cell lines originating from oropharyngeal and laryngeal SCC (HNSCC 11A and HNSCC 14C) gratefully obtained from Dr. T.E. Carey (University of Michigan, Ann Arbor, MI, USA). CERV196 cell line was provided from poorly differentiated squamous cell carcinoma cells of the uterine cervix and is positive for HPV-16 (CLS, Eppelheim, Germany). CERV196 cells were cultured in Eagle's minimum essential medium with 2 mM L-glutamine and Earle's balanced salt solution (BSS) adjusted to contain 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate and 10% fetal bovine serum. Cell cultures were incubated at 37˚C in a fully humidified atmosphere with 5% CO_2 using Dulbecco's modified essential medium (DMEM) (Fisher Scientific and Co., Pittsburgh, PA, USA) supplemented with 10% fetal calf serum (FCS) and antibiotics (Life Technologies Inc., Gaithersburg, MD, USA).

Nilotinib, dasatinib, gefitinib and erlotinib were gratefully provided by Prof. Dr. Hofheinz, Oncological Department, University Medical Centre Mannheim, Medical Faculty Mannheim, University of Heidelberg. The substances were stored at room temperature and dissolved in dimethyl sulfoxide at the time of use. The cell lines were incubated with different concentrations of all substances (5, 10 and 20 μmol/l) for 24, 48, 72 and 96 h. For the negative control, the cell lines were incubated 24, 48, 72 and 96 h with no substance added. The alamarBlue® (AbD Serotec, Oxford, UK) cell proliferation assay was used for quantitative measures of proliferating HNSCC tumor cells to establish the relative cytotoxicity of the tyrosine kinase inhibitors. The protrusions of the incubated cells were collected and stored at –20˚C for further analysis.

Enzyme-linked immunosorbent assay (ELISA) for total VEGFR-2 and EGFR. The cells were incubated and rinsed with phosphate-buffered saline (PBS). After lysing the cells, a process of spinning the cells with a vortex and microcentrifugation for 5 min, the supernatant was collected for further use. Determination of protein concentrations was performed with the ELISA technique. We used DuoSet IC Human Total VEGFR-2 (DYC1780) and DuoSet IC Human Total EGFR (DYC1854) (R&D Systems, Wiesbaden, Germany). The sandwich ELISA system used a solid-phase capture antibody specific for either VEGFR-2 or EGFR and a specific detection antibody with a standard streptavidin-horseradish peroxidase (HRP) format. The capture antibody was prepared by dilution to the working concentration (0.2 μg/ml for VEGFR-2; 0.05 μg/ml for EGFR). The capture antibody was then added to each well and incubation started. After collecting the volume of each well, the wells were washed three times with 400 μl of Tween buffer. The next step was to add 300 μl of block buffer to each well and another incubation for 1-2 h before washing with Tween buffer continued as previously described. Then, the detection antibody was diluted to its working concentration (0.2 μg/ml for VEGFR-2; 0.05 μg/ml for EGFR). One hundred μl of the detection antibody was added and plates were incubated for another 2 h at room temperature. Once again, the washing procedure was accomplished before 100 μl of streptavidin-HRP (diluted according to manufacturer's instructions) were added to each well followed by 20 min of incubation at room temperature. The wells were washed again. To start the reaction, 100 μl of substrate solution was added to each well for 20 min followed by 50 μl of stop solution. Subsequently, ELISA was performed with 100 μl of supernatant according to the manufacturer's directions. To validate the data obtained, the procedure was performed three times. The calibrations on each microtiter plate included recombinant human VEGFR-1, VEGFR-2 and EGFR standards that were provided in the manufacturers' kits. A microplate reader at wavelength of 450 nm (MRX - Elisa Reader; Dynatech, El Paso, TX, USA) was used for measuring the optical density. Wavelength correction was set to 540 nm and concentrations were reported as pg/ml. The range of detection was 62.5-4,000 pg/ml for VEGFR-2 and 312-20,000 pg/ml for EGFR. The interassay coefficient of variation reported by the manufacturer was below 10%.

Statistical analysis. To perform statistical analysis, means were generated and used for further analysis. The means of each experiment were compared to the means of the negative control to evaluate statistical significance. For all analyses, $p \leq 0.05$ was considered to be statistically significant. We used two-coefficient variance test (SAS Statistics, Cary, NC, USA) and Dunnett's test. Statistical analysis was performed in cooperation with Prof. Dr. C. Weiss, Institute of Biomathematics, Faculty of Medicine, Mannheim, Germany.

Results

EGFR expression levels in HNSCC 11A, 14C and CERV196. EGFR expression was seen in every cell line tested. Expression levels were nearly constant in all three cell lines. Statements related to statistically significant differences are referred to comparisons between experiments with the small molecule inhibitors to the negative control. We observed a statistically significant decrease of EGFR expression in HNSCC 11A and HPV-positive CERV196 cells induced by nilotinib, dasatinib, gefintib and erlotinib. Dasatinib showed the strongest effect in HNSCC 11A ($p<0.002$) with one exception after 24 h of incubation. In addition, it significantly decreased EGFR expression after 24 h ($p=0.004$) in CERV196. Statistically significant suppression of EGFR by nilotinib could be detected after 72 h for HNSCC 11A ($p=0.031$) and after 48 h for CERV196 ($p=0.006$). Erlotinib significantly suppressed EGFR expression in HNSCC 11A after 48 and 72 h ($p=0.048$ and 0.002) and in CERV196 after 48 and 96 h ($p<0.001$). A statistically significant effect for EGFR suppression induced by gefitinib could be seen after 72 and 96 h in HNSCC 11A ($p=0.007$ and 0.004) and after 24 and 96 h in CERV196 ($p=0.004$ and 0.006). For HNSCC 14C, there was a tendency towards a dasatinib-induced decrease of EGFR expression not reaching, however, statistical significance. Interestingly, gefitinib significantly increased EGFR expression after 48 h ($p<0.001$). For simplification, only the data for 20 μmol/l are shown in Table I and Figure 1.

Table I. *ELISA of EGFR expression in HNSCC 11A, 14C and CERV196 after incubation with nilotinib, dasatinib, erlotinib or gefitinib compared to the negative control.*

Incubation time (h)	Negative control	Nilotinib (20 μmol/l)		Dasatinib (20 μmol/l)		Erlotinib (20 μmol/l)		Gefitinib (20 μmol/l)	
	Mean	Mean	*p*-Value	Mean	*p*-Value	Mean	*p*-Value	Mean	*p*-Value
HNSCC 11A									
24	5423.7	5547.0	0.996	5173.7	0.505	5400.7	1.000	5190.0	0.800
48	6114.3	5153.7	0.256	4074.0	**0.002**	4610.3	0.048	5248.7	0.062
72	5972.0	5077.7	**0.031**	3291.3	**<0.001**	4406.3	**0.002**	4738.0	**0.007**
96	5597.0	4782.0	0.598	2710.0	**<0.001**	4349.0	0.188	3664.3	**0.004**
HNSCC 14C									
24	3782.3	2515.7	0.083	3338.7	0.831	4291.7	0.587	4528.3	0.993
48	4174.3	4518.3	0.991	4295.3	0.974	3655.3	0.889	5476.0	**<0.001**
72	5539.3	5636.7	0.929	4960.7	0.277	5097.3	0.317	5107.7	0.323
96	5220.3	5428.7	0.913	4920.3	0.932	5245.3	0.999	3694.7	0.742
CERV196									
24	4772.0	3507.0	0.343	3268.0	**0.004**	4619.3	0.984	3421.0	**0.004**
48	4589.3	3659.7	**0.006**	4471.7	1.000	2908.7	**<0.001**	4590.7	0.855
72	4708.3	4296.3	0.288	4867.7	0.400	4874.0	0.414	4946.7	0.951
96	5106.7	4423.3	0.118	4785.7	0.799	3448.7	**<0.001**	4028.7	**0.006**

Statistically significant differences (p<0.05) in bold.

Table II. *ELISA of VEGFR-2 expression in HNSCC 11A, 14C and CERV196 after incubation with nilotinib, dasatinib, erlotinib or gefitinib compared to the negative control (statistically significant differences (p<0.05) in bold).*

Incubation time (h)	Negative control	Nilotinib (20 μmol/l)		Dasatinib (20 μmol/l)		Erlotinib (20 μmol/l)		Gefitinib (20 μmol/l)	
	Mean	Mean	*p*-Value	Mean	*p*-Value	Mean	*p*-Value	Mean	*p*-Value
HNSCC 11A									
24	27.1	37.7	**0.036**	38.3	0.099	43.5	**0.022**	31.6	0.469
48	21.4	18.4	1.000	33.2	0.640	37.3	0.170	27.2	0.995
72	14.5	33.8	**0.002**	37.1	**<0.001**	39.6	**<0.001**	32.7	**<0.001**
96	22.9	12.7	0.893	21.6	1.000	38.0	0.037	40.5	**0.031**
HNSCC 14C									
24	18.7	36.0	**0.008**	44.7	**<0.001**	43.0	**0.001**	26.6	**0.029**
48	19.1	18.6	0.982	31.3	0.722	50.1	0.118	44.8	0.578
72	26.3	31.9	0.376	33.5	**0.030**	39.9	**0.013**	33.2	0.435
96	16.4	18.9	0.550	23.6	0.263	31.1	0.054	19.8	0.665
CERV196									
24	34.9	49.5	0.065	50.5	**0.018**	44.9	**0.029**	31.1	0.911
48	32.2	28.9	1.000	41.5	0.804	57.8	0.146	45.4	0.786
72	27.9	35.4	0.584	41.3	**0.043**	44.7	0.062	38.9	0.449
96	20.6	19.5	0.915	28.6	0.340	35.1	**0.014**	28.2	0.108

Statistically significant differences (p<0.05) in bold.

VEGFR-2 expression levels in HNSCC 11A, 14C and CERV196. VEGFR-2 expression was demonstrated in every cell line tested. Statements related to statistically significant differences are referred to comparisons between experiments with the small-molecule inhibitors to the negative control. Notably, VEGFR-2 expression levels were increased by all drugs tested in all cell lines employed. Nilotinib increased VEGFR-2 expression significantly after 24 and 72 h in HNSCC 11A (p=0.036 and 0.002) and after 24 h in HNSCC 14 C (p=0.008). For dasatinib, a statistically significant increase of VEGFR-2 expression could be detected after 72 h in HNSCC 11A (p<0.001), after 24 and 72 h in HNSCC 14C (p<0.001 and 0.003) and after 24 and 72 h in CERV196 (p=0.018 and 0.043). Tyrosine kinase inhibitors

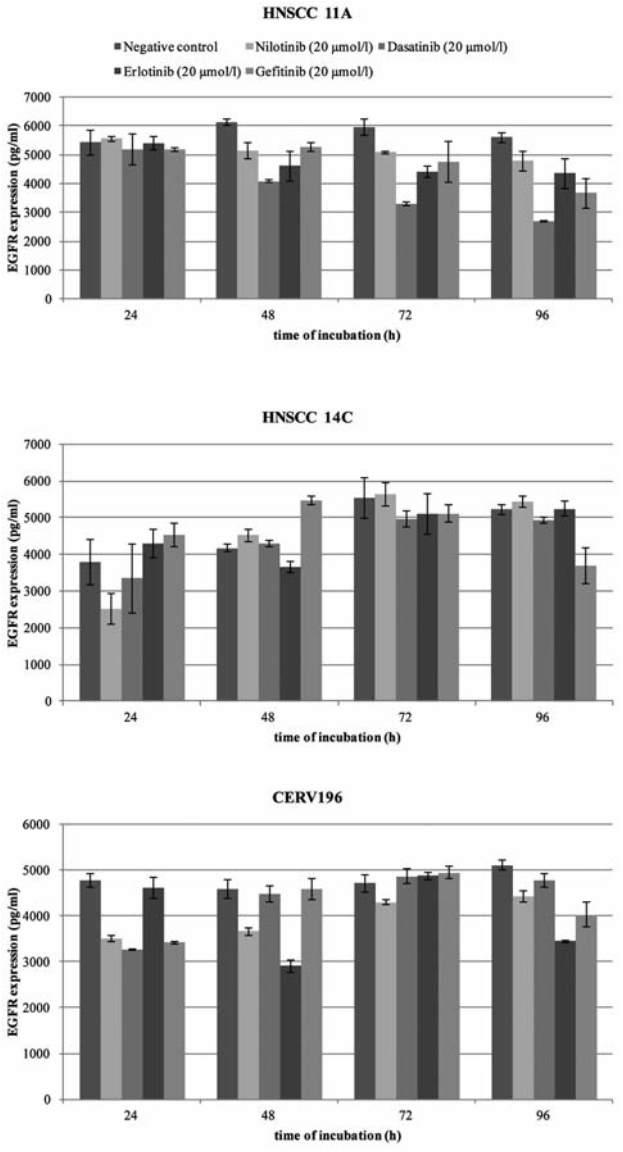

Figure 1. *EGFR expression in HNSCC 11A, 14C and CERV196 after incubation with nilotinib, dasatinib, erlotinib or gefitinib compared to the negative control. Data are mean values. Standard deviation is indicated.*

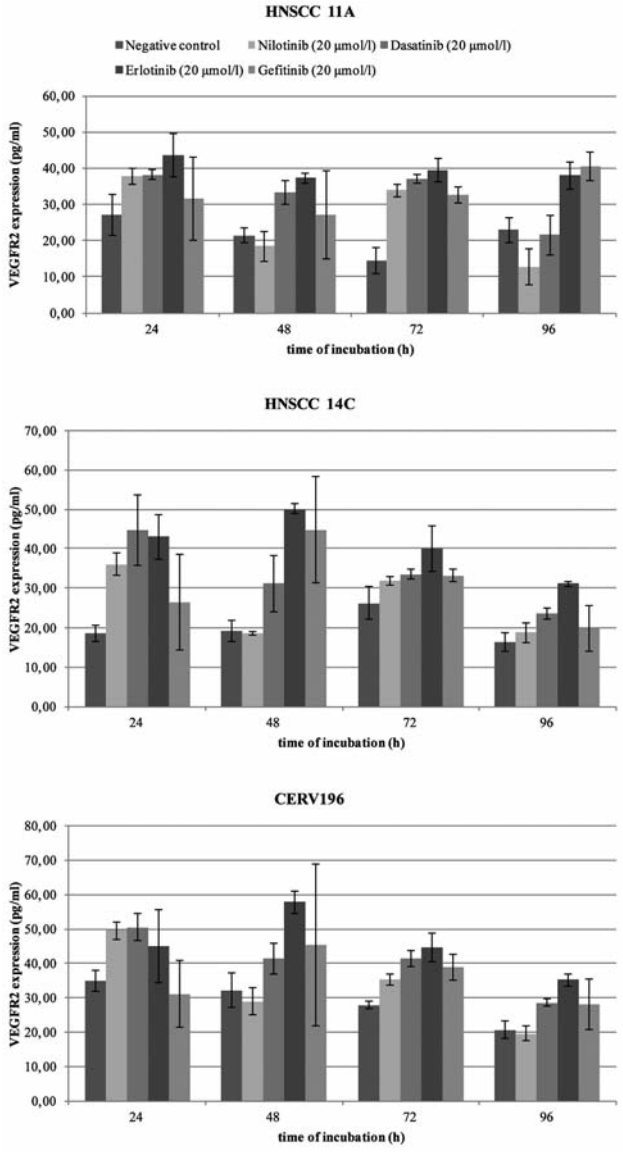

Figure 2. *VEGFR-2 expression in HNSCC 11A, 14C and CERV196 after incubation with nilotinib, dasatinib, erlotinib or gefitinib compared to the negative control. Data are mean values. Standard deviation is indicated.*

erlotinib and gefitinib significantly increased levels of VEGFR-2 as well. Erlotinib significantly increased VEGFR-2 protein levels after 24, 72 and 96 hours in HNSCC 11A (p=0.022; <0.001 and 0.037). Significantly higher levels of VEGFR-2 could also be seen after 24 and 72 h in HNSCC 14C (p=0.001 and 0.013) and after 24 and 96 h in CERV196 (p=0.029 and 0.014). Gefitinib also significantly increased VEGFR-2 expression after 72 and 96 h in HNSCC 11A (p=0.031 and <0.001) and after 24 h in HNSCC 14C (p=0.029). For simplification, only the data for 20 µmol/l are shown in Table II and Figure 2.

Discussion

This study was undertaken to investigate the alteration of expression patterns of EGFR and VEGFR-2 under the influence of small-molecule inhibitors nilotinib, dasatinib, gefitinib and erlotinib in HPV-positive and -negative SCC cell lines. EGFR- and VEGFR-2-induced angiogenesis is a crucial step for local tumor progression and the formation of lymphonodal, as well as distant metastases, and is, therefore, a major target in the pharmacological treatment of cancer cells.

EGFR expression. We demonstrated that all substances tested significantly reduce the expression of EGFR in HPV-positive and –negative squamous tumor cell lines in a time-dependent manner. Nilotinib and dasatinib are no direct inhibitors of EGFR but inhibitors of BCR-ABL, Src and PDGFR, which are able to form heterodimers with other tyrosine kinase receptors like EGFR, whereas erlotinib and gefinitib act through direct inhibition of EGFR (42).

As previously described, EGFR overexpression can be found in more than 40% of HNSCC (14, 19). Lin *et al*. showed that degradation of EGFR is a possible new mechanism for dasatinib-induced apoptosis in HNSCC cells (35). Our results support this hypothesis since we observed a strong reduction of EGFR expression in HPV-positive and -negative tumor cells by dasatinib. The strongest effect could be demonstrated for dasatinib in HNSCC 11A with a down-regulation of EGFR expression up to 52% after 96 h. This effect was also observed for nilotinib, although less pronounced. A recent study showed that dasatinib was effective at inhibiting cell proliferation by efficient inhibition of Src in NSCLC EGFR-expressing cells (43). These findings support the hypothesis that dasatinib is able to induce a significant alteration of EGFR expression in HNSCC, although it is not a direct inhibitor of EGFR.

The down-regulation of EGFR expression was also demonstrated for EGFR inhibitors erlotinib and gefitinib. Both compounds produced a statistically significant reduction of EGFR in HPV-positive and -negative tumor cells. Several Phase II/III trials have been performed using EGFR the tyrosine kinase inhibitors gefitinib and erlotinib with only moderate response in advanced staged HNSCC patients (44-46). A study of Abhold and coworkers revealed that the role of EGFR in HNSCC is more complex as it could be involved in the control of yet unknown key properties of cancer stem cells that are crucial for the development of cancer inception not directly targeted by gefitinib-induced EGFR inhibition (47). A possible explanation for the weak impact of these EGFR inhibitors could also be different mechanisms of drug resistance, which are not yet detected but are induced by HNSCC tumor cells when they come into contact with selective EGFR tyrosine kinase inhibitors. In this context, a recent study has shown a novel approach for the identification and possible compensation mechanisms of erlotinib-induced drug resistance by interleukin-6 in HNSCC (48). Related to the HPV status of a cancer cell, our results support the findings of Woodworth *et al*. who suggested a proapoptotic effect of erlotinib in p16 positive cells indicating a possible prevention of further tumor progression of HPV-infected cells (49).

Taken these results together, further studies to investigate novel possible mechanisms and understand the influence of EGFR expression and, also, the alteration of EGFR-dependent intracellular signal transduction by small molecules in HNSCC are needed. In addition, such findings could be useful steps to develop mechanisms to prevent drug resistance in HNSCC by a preselection for genetic susceptibility and, therefore, a better selection of patients who could profit from targeted therapies using different combinations of drugs, including erlotinib and gefitinib.

VEGFR expression. The development of a hypoxic microenvironment induced by the rapid growth of the tumor cells is known to be a strong promoter of angiogenesis. VEGF is critical for the mediation of a proangiogenetic signal as VEGFR-2 is involved in endothelial cell proliferation, invasion and microvascular permeability. VEGFR-2 is expressed on the surface of endothelial cells and could be found in all investigated tumor cell lines. In this study, the expression of VEGFR-2 was detected in all tested cell lines independently of the HPV status. In our data, VEGFR-2 expression levels were considerably lower than VEGFR-1 expression levels (data not shown) (50, 51). This may be due to the fact that VEGFR-1 is not only expressed on endothelial cells like VEGFR-2 but also on various other cell types like monocytes and macrophages (52). Remarkably, VEGFR-2 expression levels were significantly increased by all tested compounds in HPV-positive and -negative tumor cells. The strongest effect was seen for erlotinib and dasatinib with a statistically significant increase of protein levels in every tested cell line. However, none of the applied substances acts as a direct inhibitor of VEGFR-2. Therefore, a possible mechanism for the increase of VEGFR-2 expression levels in the presence of the tested substances could be a compensatory up-regulation of pro-angiogenic factors like VEGFR-2. As a result, the secretion of these proangiongenic factors by active cancer cells could lead to an increased support for the formation of tumor vessels. This mechanism has been discussed in several studies (53, 54). Another possible mechanism for a VEGFR-2 up-regulation could be a drug-induced counter-regulation of the tumor cell itself, which results in a therapeutic resistance of the applied substances. To our knowledge, there are no published data investigating the influence of nilotinib, dasatinib, erlotinib and gefitinib on VEGFR-2 expression in HNSCC. Referring to p16-positive cells, it is already known that viral oncogenes can induce the expression of angiogenic factors, such as VEGF (50, 55). In this context, a VEGFR-2 increase could also be discussed by a drug-induced activation or stimulation of viral oncogenes, such as *E6* and *E7*. As a result, increased autocrine mechanisms of the HPV-transfected cell to excite the production of proangiogenic factors could work as evasive mechanisms to protect the cell from drug-induced stress and consequent dysregulation. A similar mechanism was postulated to be responsible for drug resistance in virally transformed oropharyngeal cancer cells against cetuximab by an increase of CD44/CD133 positive HPV-dependent cancer stem cells (56). However, this hypothesis needs to be proved in further studies.

To date, this is one of the first studies investigating the influence of nilotinib, dasatinib, erlotinib and gefitinib on the expression patterns of EGFR and VEGFR-2 in HPV-positive and -negative SCC cells *in vitro*. In conclusion, the results reveal new insights in the understanding of the interaction between EGFR and VEGFR-2 expression with small molecule tyrosine kinase inhibitors in HNSCC *in vitro*. The results also reveal possible new approaches for further studies to investigate potential new strategies, in addition to existing chemotherapeutic regimens for HPV-positive and -negative HNSCC. Yet, further *in vitro* and *in vivo* studies with established therapeutic options need to be performed to carve out the suitability of the tested drugs in HNSCC.

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