Small Molecule-based Chemotherapeutic Approach in p16-positive and -negative HNSCC *In Vitro*

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Abstract. *Background: Incidence of oropharyngeal head and neck squamous cell carcinoma (HNSCC) induced by the human papilloma virus (HPV) is rising. HNSCC is the sixth most common neoplasia worldwide. The survival rate remains poor, thus innovative therapy approaches are necessary. Everolimus, an inhibitor of the mammalian target of rapamycin, as well as the multi-tyrosine kinase inhibitors sorafenib (targeting vascular endothelial growth factor receptor, platelet-derived growth factor receptor and RAF) and sunitinib (targeting vascular endothelial growth factor receptor, platelet-derived growth factor receptor, stem cell factor receptor, RET proto-oncogene and colony-stimulating factor), have shown a remarkable antitumor effect against various tumor entities, with moderate side-effects. These drugs are administered orally, which should lead to higher patient compliance and less hospitalisation. Aim: This study sought to evaluate the expression of PDGFR α/β and hypoxia-inducible factor-1α (HIF-1α) and their alterations induced by everolimus, sorafenib and sunitinib in chemonaïve HPV-positive and HPV-negative HNSCC. To our knowledge, this is the first in vitro study to investigate such cases. Materials and Methods: We incubated HPV-positive CERV196 and HPV-negative HNSCC 11A and 14C cells for 2 to 8 days with increasing concentration of drugs. Expression of PDGFR α/β and HIF-1α was measured by enzyme-linked immunosorbent assay and compared to a chemonaïve controls. Results: Our study showed that PDGFR α/β and HIF-1α were expressed in all three cell lines. Incubation with everolimus, sorafenib or sunitinib led to a decrease in PDGFR α/β and HIF-1α expression, depending on the HPV status. A statistically significant alteration of PDGFR α/β was detected in CERV196 only. Thus, HPV-positive HNSCC exhibited a higher sensitivity to the drugs used compared to HPV-negative HNSCC 11A and 14C tumor cells. A significant reduction of HIF-1α was measured for HNSCC 11A and 14C only. An escalation of drug concentration had no significant effect. Conclusion: We showed that these novel agents led to a significant reduction of PDGFR and HIF-1α, depending on the HPV status. HPV positivity is associated with increased chemosensitivity and may be associated with better locoregional control and overall patient survival compared to HPV negativity. Further studies are necessary to investigate the efficacy and safety of these agents in the treatment of HPV-positive and -negative HNSCC in vivo.*

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Recent data from the International Agency for Research on Cancer (IARC) report 631,000 cases of head and neck squamous cell carcinoma (HNSCC) and approximately 352,000 associated deaths in 2008. HNSCC is the sixth most common cancer worldwide and varies widely in aggressiveness and response to treatment (1). Despite advances in surgery, radiation and chemotherapy, the five-year survival rate is still poor (2). The incidence of HNSCC has decreased in recent years, which may be due to a reduced prevalence of tobacco abuse (3). Smoking and alcohol abuse remain the most important risk factors and have synergistic effects (4, 5). In contrast, the incidence of oropharyngeal cancer, especially of the tonsils and tongue base, is rising in patients aged 20-44 years (6).

The oncogenic potency of human papilloma virus (HPV) is well-known in carcinomas of the uterine cervix. Since risk factors for HPV infection are promiscuity and unsafe sex, HPV infection is associated with anogenital carcinoma and oropharyngeal cancer (7). The rising incidence of HPV-associated oropharyngeal cancer illustrates the importance of its viral etiology (8, 9). HPV-positive HNSCC seems to be different from HNSCC associated with tobacco and alcohol consumption in molecular and clinical aspects (10-12). HPV

oncogenes suppress inflammatory signaling as one potential mechanism of immune escape (13). They also degrade tumor-suppressor proteins such as p53, which regulates the expression of angiogenic inhibitors and suppresses pro-angiogenic factors (14, 15). HPV positivity appears to be associated with higher sensitivity to radiation and chemotherapy (16, 17).

Various cell types make up the tumor stroma. They build the extracellular matrix (ECM) (18). ECM cells and the tumor secrete angiogenic factors that facilitate invasion and tumor progression (19, 20). Angiogenic peptides, such as platelet-derived growth factor (PDGF), regulate cell survival and induce cell proliferation. Its receptor, PDGFR is a tyrosine kinase receptor, activated by ligand receptor interaction. PDGF and PDGFR expression are increased in various malignant tumors, such as breast cancer, melanoma and HNSCC (21, 22). PDGF promotes the formation of a well-vascularized stroma and tumor proliferation in HNSCC (23).

An important factor for tumor neovascularisation is tissue hypoxia. The cellular response to hypoxia is mediated by the expression of hypoxia-inducible factor (HIF). It influences the expression of several angiogenic peptides and promotes the formation of a vascular system in tumors (24, 25). Aberrant tumor vessels have structural and functional abnormalities that may also disturb the distribution of oxygen and nutrients to the tumor cells. Hypoxic tumor cells are less vulnerable to radiation because reactive oxygen species stabilize radiation damage. This reduces therapy response and prognosis (26-28). A reduction of aberrant tumor vessels may paradoxically improve oxygenation and radiosensitivity of tumor cells (29, 30). Expression of HIF-1α is correlated with high levels of vascular endothelial growth factor (VEGF) (31). Hypoxic tumor cells acquire pro-survival pathways (32). Inhibitors of angiogenesis, such as sunitinib, transiently improve tumor oxygenation and reduce microvessel density. Radiation during a period of improved oxygenation results in a delay of tumor growth and increases the antitumor effect (29). A possible mechanism of HIF-1α suppression by sunitinib and sorafenib could be the inhibition of the rat sarcoma (RAS)/rapidly accelerated fibrosarcoma (RAF) cascade, in the case of sunitinib *via* stem cell factor receptor (c-KIT) inhibition (33).

New pharmacological approaches are necessary given the poor prognosis of HNSCC. The drugs tested in the present study have the advantage of oral application, moderate side-effects and a tumor-targeted mechanism of action.

Everolimus (Afinitor®) is an inhibitor of the mammalian target of rapamycin (mTOR) (34). Besides utilization as an immunosuppressant to prevent rejection of organ transplants (35), it is currently part of therapy regimes for metastatic renal cell, esophageal and gastric cancer, breast and hepatocellular carcinoma, as well as HNSCC (36-38). mTOR inhibitors overcome drug resistance in solid tumors against other VEGF/VEGFR-targeted therapies (36). mTOR is a phosphatidylinositol 3-kinase and regulates proliferation and cell survival by alteration of DNA transcription and protein synthesis (39). For instance, mTOR induces VEGF expression and therefore angiogenesis (40, 41). It acts as an integration center for various intracellular signaling pathways frequently dysregulated in cancer and stimulates expression of HIF-1α and VEGF (37, 39). This is consistent with the broad synergistic effects of other targeted agents as well as chemotherapy and radiation (42-44). An activated mTOR pathway is frequently found in locoregional invasion of lymph nodes in HNSCC. mTOR inhibition with sirolimus and everolimus diminishes lymphangiogenesis in the primary tumor and prevents dissemination to the cervical lymph nodes (45). Like sunitinib, mTOR inhibitors seem to normalize aberrant tumor vasculature and improve tumor oxygenation, which provides a temporal window for enhanced sensitivity to radiation (30).

Sunitinib (Sutent®) and sorafenib (Nexavar®) are multi-tyrosine kinase inhibitors targeting VEGFR, PDGFR, c-KIT, RET and colony-stimulating factor (CSF), and VEGFR, PDGFR and RAF kinases respectively. Sunitinib and sorafenib are established in the treatment of gastrointestinal stromal tumors (GIST), renal cell, hepatocellular carcinoma, pancreatic neuroendocrine tumors, breast, lung, thyroid, colorectal cancer and melanoma (46-50). In clinical trials, sunitinib as well as sorafenib have been evaluated for HNSCC therapy (51-54). Sorafenib showed synergistic effects with other targeted agents, chemotherapy and radiation. Combination therapy was explored with promising results in tumors such as gastric, breast, colorectal and ovarian cancer (55, 56).

Several studies on the application of everolimus, sunitinib and sorafenib in the treatment of HNSCC exist (43, 44, 51-53, 57-59); however, none of them has examined the difference between HPV-positive and -negative HNSCC. This study sought to evaluate the expression of PDGFR α/β and HIF-1α in HPV-positive (CERV196) and HPV-negative (HNSCC 11A and 14C) HNSCC cell lines and the impact of everolimus, sorafenib and sunitinib on the expression pattern of PDGFR α/β and HIF-1α. To our knowledge, this is the first *in vitro* study investigating the effect of these small molecule agents on HPV-positive and -negative HNSCC.

Materials and Methods

Cell lines. The cell lines HNSCC 11A and 14C originated from a human SCC of the oropharynx and larynx and were received from Dr T.E. Carey (University of Michigan, Ann Arbor, MI, USA). The CERV196 cells came from a SCC of the uterine cervix and are HPV16-positive (CLS, Eppelheim, Germany). Dulbecco's modified essential medium (DMEM) (Fisher Scientific and Co., Pittsburgh, PA, USA) combined with 10% fetal calf serum (FCS) and antibiotics (Life Technologies Inc., Gaithersburg, MD, USA) were used as medium for HNSCC 11A and 14C. CERV196 cells were

kept in Eagle's minimum essential medium with 2 mM L-glutamine, 10% FCS and Earle's balanced salt solution (BSS) with 1.5 g/l sodium bicarbonate, 0.1 mM amino acids and 1.0 mM sodium pyruvate. Cell cultures were carried out at 37˚C in a fully-humidified atmosphere with 5% CO_2. Everolimus, sorafenib and sunitinib were made up in dimethylsulfoxide (DMSO) at the time of use. The cells were exposed to different drug concentrations ranging from 1.0-25.0 μmol/ml for 2 to 8 days. These drug concentrations were chosen after the alamarBlue (AbD Serotec, Oxford, UK) cell proliferation assay was conducted, which measured the proliferation of HNSCC tumor cell lines quantitatively and indicated the relative cytotoxicity of the studied drugs.

Enzyme-linked immunosorbent assay (ELISA) for total HIF-1α and PDGFR α/β. After incubation with different drug concentrations, the cells were washed with phosphate-buffered saline (PBS) and then 350 μl of lysis buffer were added to each well. Afterwards, the lysed cells were vortexed at 2–8˚C for 30 min and microcentrifuged at 14,000 ×*g* for 5 min. The supernatant was pipetted into a clean tube.

HIF-1α levels were determined with a sandwich ELISA (R&D Systems, Wiesbaden, Germany). The system used a solid-phase monoclonal antibody and an enzyme-linked polyclonal antibody against HIF-1α. According to the manufacturer's directions, each ELISA was performed with 100 μl of supernatant. All analyses and calibrations were carried out twice. The calibrations on each microtiter plate included a recombinant human HIF-1α standard that was provided in the kit. The optical density was measured using a microplate reader at a wavelength of 450 nm. Wavelength correction was set to 540 nm, and concentrations are reported as relative units of optical density within a range of 0.0001 and 1.0.

The sandwich ELISA (R&D Systems, Wiesbaden, Germany) for human PDGFR used a solid-phase capture antibody specific for human PDGFR. Afterwards, unbound material was washed away, and a detection antibody specific for total PDGFR was added using a standard streptavidin-horseradish peroxidase (HRP) format. The capture antibody was diluted 1:180 (4 μg/ml), then 100 μl of the capture antibody were added to each well and plates were incubated overnight. The contents of each well were then aspirated, and the wells were washed three times with 400 μl of Tween buffer. Then 300 μl of block buffer were added to each well and plates were incubated for 1–2 h followed by another Tween buffer wash. The detection antibody was diluted to a concentration of 500 ng/ml, and 100 μl of the detection antibody were added to each well for 2 h. The cells were washed again and 100 μg of streptavidin-HRP were added to each well followed by 20 min of incubation at room temperature. Afterwards, 100 μl of substrate solution were added to each well for 20 min followed by 50 μl of stop solution. According to the manufacturer's instructions, each ELISA was carried out with 100 μl of supernatant. Microplate reader settings were the same as described above. The interassay coefficient of variation reported by the manufacturer was below 10%. The range of detection for PDGFRα was 312 to 20,000 pg/ml and for PDGFRβ 250 to 16,000 pg/ml. All analyses and calibrations were carried out twice.

Measuring total protein. Total protein was measured with the DC Protein Assay (BioRad, Hercules, CA, USA). Cells were incubated, lysed and centrifuged as previously described. Dilutions of protein standard were prepared according to the instructions of the manufacturer. Measurement was performed on 100 μl of protein standard or cell supernatant with a spectrophotometer set to 750 nm, and concentrations are reported as μg/ml.

Statistical analysis. Statistical analysis was carried out in cooperation with PD Dr C. Weiss, Institute of Biomathematics, Faculty of Medicine, Mannheim, Germany. A p-value ≤0.05 was considered statistically significant. The two-coefficient variance test (SAS Statistics, Cary, NC, USA), Dunnett's test and Scheffe's test were used.

Results

Total protein assay. In order to differentiate a decrease in protein expression of the target variable from a cytotoxic effect (apoptosis) of the drugs, expression levels were compared using the total protein content of the cell lysates. The quotient of the protein expression level and total protein content revealed a discrete fluctuation but no increase irrespective of the HPV status or the applied drugs. This indicates that any observed alteration of expression cannot be explained by apoptosis of the cells solely due to a cytotoxic effect of the applied drugs (data not shown).

An escalation of drug concentration had no statistically significant impact on expression levels of PDGFR α/β or HIF-1α when compared to the negative control; therefore, only the data for 25 μmol/ml are shown for simplification. The lowest tested concentration was 1.0 μmol/ml.

ELISA for PDGFR α/β expression in HNSCC 14C, 11A and CERV196 cells. CERV196 exhibited higher expression of PDGFRα compared to HPV-negative HNSCC. A statistically significant decrease of PDGFRα expression in CERV196 cells was detected for everolimus after 197 h of incubation (p=0.005) and for sunitinib after 48, 120 and 197 h (p=0.05, 0.011 and 0.03). Sorafenib did not significantly affect the expression of PDGFRα in CERV196 cells.

HNSCC 11A and 14C cell lines showed no statistically significant alteration of expression on drug treatment with the exception of everolimus in HNSCC 11A cells after 72 h of incubation (p=0.038). Although no significant alteration was detected for HPV-negative HNSCC, there was a clear trend towards decreased expression after incubation with the three drugs. Comparison of the effects of the drugs revealed no significant advantage for any of them. Data are shown in Table I.

PDGFRβ was expressed at lower levels in all cell lines compared to PDGFRα in the negative control. Again, expression levels in CERV196 cells were higher compared to HPV-negative HNSCC. A statistically significant reduction of PDGFRβ was detected in CERV196 cells for everolimus after an incubation time of 48 and 197 h (p=0.025 and 0.032), for sunitinib after an incubation time of 72 h (p=0.024) and for sorafenib after an incubation time of 48

Table I. *Enzyme-linked immunosorbent assay of platelet-derived growth factor receptor-α expression in head and neck squamous cell carcinoma 11A, 14C and CERV196 cell lines after incubation with everolimus, sunitinib and sorafenib compared to the negative control (statistical significance is shown in bold).*

Time of incubation (h)	Negative control	Everolimus (25 μmol/ml)		Sunitinib (25 μmol/ml)		Sorafenib (25 μmol/ml)	
	Mean value	Mean value	*p*-Value	Mean value	*p*-Value	Mean value	*p*-Value
HNSCC 11A							
48	1009.333	873	0.141	575.333	0.873	462.667	0.916
72	918.333	840	**0.038**	516.667	0.624	594	0.427
120	980.667	792.333	0.344	423.667	0.445	529.333	0.599
197	1018	770	0.371	355.333	0.925	300.333	0.868
HNSCC 14C							
48	1023.667	763	0.938	440	0.096	502.667	0.637
72	1032	762.667	0.82	373.333	0.258	374.333	0.546
120	1091.667	730	0.356	286.333	0.157	408	0.388
197	1016	608.333	0.778	176.333	0.337	306	0.516
CERV 196							
48	1446	1164.667	0.079	997.667	**0.05**	1148.667	0.067
72	1373	1129	0.077	795.333	0.085	1143.333	0.102
120	1358.667	1247.667	0.052	485	**0.011**	1026.667	0.095
197	1400	965.333	**0.005**	473	**0.03**	756	0.053

Table II. *Enzyme-linked immunosorbent assay of platelet-derived growth factor receptor-β expression in head and neck squamous cell carcinoma 11A, 14C and CERV196 cell lines after incubation with everolimus, sunitinib and sorafenib compared to the negative control (statistical significance is shown in bold).*

Time of incubation (h)	Negative control	Everolimus (25 μmol/ml)		Sunitinib (25 μmol/ml)		Sorafenib (25 μmol/ml)	
	Mean value	Mean value	*p*-Value	Mean value	*p*-Value	Mean value	*p*-Value
HNSCC 11A							
48	82.333	64.333	0.316	36.333	0.681	47	0.905
72	80.333	68.667	0.169	35.0	0.647	44	0.697
120	83.667	67.667	0.311	46.0	0.718	32	0.773
197	84.333	65.333	0.221	29.333	0.588	28.333	0.692
HNSCC 14C							
48	100	72.333	0.551	52.0	**0.027**	56.667	0.502
72	94	76	0.87	35.0	0.151	41.667	0.545
120	96.667	71	0.25	28.667	0.248	38.333	0.429
197	96.333	59.667	0.084	26.333	0.627	23	0.692
CERV 196							
48	152	99.667	**0.025**	62.0	0.206	89.333	**0.038**
72	142	113.667	0.057	57.333	**0.024**	79.667	0.053
120	153	94.333	0.059	45.0	0.121	71	0.156
197	143.667	85.333	**0.032**	33.0	0.146	63	**0.009**

and 197 h (p=0.038 and 0.009). In HNSCC 11A cells, no significant alteration of expression was determined. The same result was found in HNSCC 14C cells with the exception of sunitinib after an incubation time of 48 h (p=0.027). Expression levels fell in HPV-negative HNSCC cells after incubation with each of the drugs. A comparison of the drugs did not show a significant advantage for any of them. Data are shown in Table II.

ELISA for HIF-1α expression in HNSCC 14C, 11A and CERV196 cells. HIF-1α was expressed in all cell lines irrespective of their HPV status. In CERV196 cells, the expression of HIF-1α was higher than that of HPV-negative HNSCC cells. In HNSCC 11A cells, a statistically significant reduction of HIF-1α expression was found on treatment with sorafenib after an incubation time of 48 and 197 h (p=0.005 and 0.011). Everolimus and sunitinib had no significant

Table III. *Enzyme-linked immunosorbent assay of hypoxia-inducible factor-1α expression in head and neck squamous cell carcinoma 11A, 14C and CERV196 cell lines after incubation with everolimus, sunitinib and sorafenib compared to the negative control (statistical significance is shown in bold).*

Time of incubation (h)	Negative control	Everolimus (25 μmol/ml)		Sunitinib (25 μmol/ml)		Sorafenib (25 μmol/ml)	
	Mean value	Mean value	*p*-Value	Mean value	*p*-Value	Mean value	*p*-Value
HNSCC 11A							
48	0.3	0.197	0.917	0.167	0.366	0.155	**0.005**
72	0.317	0.168	0.945	0.134	0.159	0.074	0.153
120	0.302	0.124	0.998	0.063	0.110	0.074	0.193
197	0.312	0.133	0.47	0.029	0.087	0.053	**0.011**
HNSCC 14C							
48	0.302	0.145	**0.018**	0.105	**0.001**	0.184	0.115
72	0.295	0.139	**0.001**	0.134	**0.013**	0.132	**0.017**
120	0.296	0.117	**0.009**	0.335	0.821	0.115	0.076
197	0.262	0.088	**0.005**	0.065	**0.011**	0.093	**0.004**
CERV 196							
48	0.561	0.42	0.131	0.387	**0.012**	0.522	0.072
72	0.577	0.35	0.083	0.2	0.09	0.255	0.491
120	0.596	0.201	0.558	0.054	0.942	0.108	0.976
197	0.637	0.233	0.778	0.017	0.478	0.04	0.829

impact on HIF-1α expression in HNSCC 11A cells. In CERV196 cells, we only detected a significant decrease of expression levels on treatment with sunitinib after 48 h of incubation (p=0.012), but there was a clear trend towards decreased expression in an incubation time-dependent manner. Interestingly, we found a statistically significant decrease of HIF-1α expression levels in HNSCC 14C cells with all applied drugs. Everolimus reduced HIF-1α expression regardless of the time of incubation with p-values from 0.001-0.018. Sunitinib significantly reduced HIF-1α expression after 48, 72 and 197 h of incubation (p=0.001, 0.013 and 0.011). Sorafenib led to a significant reduction of HIF-1α expression after 72 and 197 h of incubation (p=0.017 and 0.004). Data are shown in Table III.

Discussion

The vascularization of a tumor is a pivotal factor for tumor progression, invasion and migration, leading to lymph node or distant metastasis. Intracellular hypoxia is a potent inducer of the expression of angiogenic factors mediated by HIF-1 and -2 (60, 61). Therefore, it is reasonable to target pro-angiogenic factors in anticancer therapy in order to prevent or decelerate the aforementioned process. Riedel *et al*. demonstrated a correlation between the microvessel density in a tumor and its recurrence or metastasis (62, 63). A set of angiogenic factors released either by the tumor or the stroma regulate vascularization and neo-angiogenesis (19, 64). Suppression of PDGFRα/β or HIF-1α during anticancer therapy could diminish tumor vascularization. PDGFRα/β suppression prevents neo-angiogenesis and consequently delays tumor growth. Tumor vessels have structural and functional abnormalities that disturb the distribution of oxygen and nutrients to the tumor cells. A reduction of aberrant tumor vessels and chaotic blood flow may paradoxically improve oxygenation and radiosensitivity of tumor cells during combined radio- and chemotherapy (29, 30). Given the poor prognosis of advanced-stage HNSCC, development of new therapy approaches is urgently required. This is the first study to scrutinize the effect of the mTOR inihibitor everolimus and two multi-tyrosine kinase inhibitors sunitinib and sorafenib on HNSCC cells according to the HPV status of the tumor cells.

The results showed that expression of the surrogate markers PDGFRα/β and HIF-1α were considerably higher in HPV-positive CERV196 cells than in HPV-negative HNSCC 11A and 14C cells. These findings are consistent with previously published results (65, 66). Apart from very few exceptions, everolimus, sunitinib and sorafenib had no significant impact on the expression of PDGFRα/β in HNSCC 11A or 14C cells. In CERV196 cells on the other hand, we found a significant decrease of PDGFRα/β expression to a variable extent for all applied drugs. CERV196 cells were characterized by higher susceptibility to small molecule-induced decrease of PDGFRα/β expression. In contrast to this study, previous trials reported that HPV-positive CERV196 cells exhibited lower chemosensitivity towards a targeted anti-angiogenic therapy with imatinib as well as standard chemotherapeutic drugs such as 5-fluorouracil and docetaxel when compared with

HPV-negative HNSCC (65, 66). Therefore, novel multi-tyrosine kinase inhibitors such as sunitinib and sorafenib, as well as the mTOR inhibitor everolimus, appear to be more effective in reducing PDGFRα/β expression *in vitro* compared to established tyrosine kinase inhibitors such as imatinib. Clinical trials confirmed that everolimus, sunitinib and sorafenib are effective against malignant diseases resistant to imatinib, such as acute myeloid leukemia, GIST and melanoma (67-69). Sunitinib and sorafenib directly inhibit the tyrosine kinase of PDGFRα/β (70, 71). Although everolimus is not an inhibitor of PDGFRα/β, incubation with everolimus did lead to a decrease of PDGFRα/β expression; the cellular mechanism for this remains unclear. As previously mentioned, mTOR works as an integration center for various intracellular signaling pathways, for instance, the angiogenic signaling pathways of HIF-1α (37, 39). It can be assumed that broad inhibition of several tyrosine kinases downstream of the receptor molecule is more effective than the sole inhibition of one receptor tyrosine kinase. Cetuximab, a monoclonal antibody against the epidermal growth factor receptor (EGFR), was one of the first agents used in targeted therapy for HNSCC. It has been reported that mutations of *EGFR* may cause resistance to cetuximab during therapy (72); therefore, it is reasonable to use agents that affect the signaling cascade downstream of the receptor. Multi-tyrosine kinase inhibitors have the advantage of affecting more than one target of the downstream signaling cascade. Mutations that abolish the effect of these drugs simultaneously are rather unlikely.

Interestingly, the results for HIF-1α contrast those for PDGFRα/β: In HNSCC 14C cells, a decrease of HIF-1α expression was found for all applied drugs. Yuan *et al*. showed that hypoxia induces mTOR-dependent HIF-1α expression and that the maintenance of HIF-1α levels under re-oxygenation can be averted by mTOR inhibitors such as everolimus (73). A decrease of HIF-1α expression induced by sorafenib and sunitinib can be explained by inhibition of the RAS/RAF cascade that induces HIF-1α (33). Sunitinib prevents activation of this cascade by inhibition of c-KIT, sorafenib by direct inhibition of RAF kinase. In the case of HNSCC 11A cells, HIF-1α expression significantly decreased after incubation with sorafenib. Since HIF-1α is an important signaling molecule for the induction of angiogenesis and the development of vasculature in tumors, these findings suggest a significant anti-angiogenic effect of everolimus, sunitinib and sorafenib in HNSCC 11A cells and especially 14C cells by reducing HIF-1α expression. We found no significant alteration of HIF-1α expression in CERV196 cells with one exception; however, there was a trend towards declining HIF-1α levels for everolimus, sunitinib and sorafenib in an incubation time-dependent manner. As previously mentioned, viral oncogenes of HPV are potent inductors of the expression of pro-angiogenic factors such as PDGFRα/β and VEGF but also HIF-1α (13). It is possible that virally transformed SCCs are less dependent on hypoxia as a stimulus for angiogenesis induced by HIF-1α because of a direct induction of pro-angiogenic factors by viral oncogenes. Actually, we found higher expression of HIF-1α in CERV196 cells compared to HPV-negative HNSCC cells. Since all cell cultures were kept under the same normoxic conditions, this could be an indicator for an induction of HIF-1α by viral oncogenes. Li and colleagues previously reported this mechanism of angiogenesis for non-small cell lung cancer (74).

Previously published Phase II studies by Choong *et al*., Fountzilas *et al*. and Machiels *et al*. investigated the use of sunitinib as a monotherapy in metastatic and recurrent HNSCC. Sunitinib had little or no antitumor activity in HNSCC *in vivo*. None of these studies advocated the use of sunitinib as a single-agent in HNSCC therapy (51-53). Similar data were published for the use of sorafenib in monotherapy (57, 58). However, none of these studies differentiated between HPV-positive and -negative HNSCC. A combination therapy with sunitinib, cetuximab and radiation, on the other hand, significantly reduced tumor proliferation according to a study published by Bozec and colleagues (43). Although sunitinib and sorafenib are well-tolerated, their anticancer activity in a monotherapy appears to be low and they should be part of a combination therapy. Everolimus was part of a combination therapy with cisplatin and docetaxel in a phase I study. The combination showed antitumor activity against several tumor types but further clinical trials are necessary (44, 59).

As far as we are aware of, this is the first study to investigate the effect of everolimus, sunitinib and sorafenib on HPV-negative and -positive SCC *in vitro*. It confirmed significant anti-angiogenic effects and differentiated the susceptibility of HPV-positive and HPV-negative SCC to targeted therapy. HPV-positive CERV196 cells exhibited increased susceptibility towards reduced PDGFRα/β expression induced by everolimus, sunitinib and sorafenib compared to HPV-negative HNSCC 11A and 14C cells. Thus, a greater anti-angiogenic effect of everolimus, sunitinib and sorafenib may be expected in HPV-positive SCC. Significant alteration of HIF-1α was observed in HNSCC 11A and 14C cells but not in CERV196 cells when exposed to the three drugs. A possible explanation for this could be the direct induction of angiogenic factors by viral oncogenes leading to a smaller contribution of angiogenic stimuli induced by hypoxia. However, CERV196 cells exhibited a trend towards reduced HIF-1α levels in an incubation time-dependent manner. Everolimus, sunitinib and sorafenib displayed anti-angiogenic effects dependent on the HPV status of HNSCC cells. Further studies are mandatory to evaluate the importance of everolimus, sunitinib and sorafenib in HNSCC therapy and their possible

implementation in established chemotherapeutic regimes. Our findings support distinguishing HPV-positive from -negative HNSCC in the assessment of these targeted agents in further clinical trials.

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