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Apoptosis-related Proteins Are Altered by Selective Tyrosine Kinase Inhibitors and Everolimus in HPV-dependent SCC

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Abstract. *Background: Head and neck squamous cell cancer (HNSCC) affects the oral cavity and the pharynx. The aim of the study was to investigate the effects of selective tyrosine kinase inhibitors (TKIs) erlotinib, gefitinib, nilotinib and dasatinib and the mammalian target of rapamycin (mTOR) inhibitor everolimus on the expression of apoptosis-related proteins caspase-3, FAS cluster of differentiation (CD)-95 and FAS ligand in human papilloma virus (HPV)-dependent squamous cancer. Materials and Methods: Two HPV-negative cell lines (UMSCC-11A/-14C) and one HPV-positive cell line (CERV196) were incubated with TKIs or everolimus and protein concentrations of target proteins were analyzed with enzyme-linked immunosorbent assay (ELISA). Results: Caspase-3 was affected by the tested TKIs in HPV-positive SCC, whereas FAS CD95 and FAS ligand were influenced in HPV-negative SCC. Discussion: This is the first study to analyze the influence of TKIs and everolimus on key proteins of apoptosis. Our results provide novel information contributing to a better understanding of the cell biology of HPV-dependent HNSCC and might contribute to the discovery of novel pharmaceutical treatment strategies for HNSCC.*

Head and neck squamous cell cancer (HNSCC) is a malignancy affecting the entire upper aerodigestive tract and mostly occurs in the oral cavity and the pharynx. The main risk factors for HNSCC are tobacco and alcohol consumption (1). Despite a worldwide reduction of tobacco and alcohol consumption, the incidence of HNSCC is increasing (2, 3). HNSCC is the sixth most common type of cancer with approximately 800,000 new cases per year and a mortality rate of 430,000 cases per year (4-6). The mortality rate is 40-50%, which leads to a poor overall prognosis of HNSCC (6). The increasing incidence of oropharyngeal SCC in Western countries is associated with an oral infection with high-risk subtypes of the human papilloma virus (HPV) family (7, 8). This novel factor has influenced the tumor–node–metastasis (TNM) staging manual for oropharyngeal SCC, which was updated in 2017 to include p16INK4A immunostaining as a surrogate for the HPV status of oropharyngeal SCC (9). Currently, the prevalence of HPV in HNSCC is approximately 20% and is still increasing (10). Most HPV infections are transmitted through oral sexual intercourse and remain subclinical. High-risk subtype HPV 16 and 18 are detected in 90% of HPV-associated HNSCC and lead to an overexpression of the viral oncogenes *E6* and *E7* in the host genome which induces genomic instability and cell proliferation (11, 12). Patients with HPV-related HNSCC are typically younger (30–50 years) and are often diagnosed with advanced stages of disease because of early lymphogenic neck metastases at a small primary tumor size (13-15). However, HPV-related HNSCC has a more favorable prognosis than HPV-negative HNSCC but recent studies did not show promising results regarding the de-escalation of treatment (16-18).

Most therapy regimens of HNSCC treatment include a combination of surgery, radiation and chemotherapy. New therapy approaches include targeted therapies such as antibodies against epidermal growth factor receptor (EGFR) and checkpoint inhibitors. Modulation of key signaling

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pathways promote and accelerate cancer development (19). The deregulation of several tyrosine kinases affects tumor cell signaling cascades and promotes tumor progression. Selective tyrosine kinase inhibitors (TKI) compete for the catalytic ATP-binding site and potently inhibit the biochemical function of the targeted tyrosine kinases (20). Several TKIs have been investigated in multiple tumor entities (21). Nilotinib and dasatinib inhibit breakpoint cluster region (BCR)–ABL and are effective in chronic myelogenous leukemia treatment (22, 23). Other TKIs, such as gefitinib and erlotinib, have been approved for the treatment of non-small-cell lung cancer and selectively inhibit EGFR (24-26). Everolimus is a potent inhibitor of mammalian target of rapamycin (mTOR) and is used in the treatment of several malignant tumor types (27, 28). It has also been shown that everolimus has significant potential in a possible treatment of HPV-positive HNSCC (29). However, the exact mechanism of action of these targeted therapies is not yet fully understood because different tumors often react differently to targeted therapies. A critical question includes the influence of TKIs and other targeted therapy approaches on apoptosis and apoptosis-related mechanisms. Significant key proteins of apoptosis include the FAS cluster of differentiation (CD)-95 [also known as FAS receptor or apoptosis antigen 1 (APO1)], FAS ligand and caspase-3. FAS CD95 is located on the cell surface and carries a cytoplasmic death domain (DD) like other receptors of the death receptor family. The DD is essential for the initiation of apoptosis (30-32). The FAS CD95 DD binds to the FAS ligand and assembles the death-inducing signaling complex, which consists of FAS CD95, the adaptor molecule FAS-associated with a death domain (FADD), procaspase 8, procaspase 10 and the caspase 8/10 regulator cellular FLIP (c-FLIP) (32, 33). Activated caspase 8 initiates the apoptotic cascade by splitting various intracellular proteins with the subsequent activation of caspase-3 (34, 35). As a result, the expression of FAS CD95 is down-regulated whereas that of FAS ligand is up-regulated. This mechanism is also described in HNSCC (33).

The aim of this study was to investigate the effects of small-molecule TKIs erlotinib, gefitinib, nilotinib and dasatinib and the mTOR-inhibitor everolimus on the expression of apoptosis-related proteins *in vitro* in HPV-positive and -negative squamous cancer cell lines.

Materials and Methods

Experimental design. 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, MI, USA) and one human HPV16-positive cell line (CERV196; Cell Lines Service GmbH, Eppelheim, Germany) were used for the *in vitro* experiments. The HPV-negative cell lines originated from a primary laryngeal SCC of the epiglottis (UMSCC 11A) and a skin metastasis of an oral cavity SCC of the floor of the mouth after surgery and radiochemotherapy (UMSCC 14C). The HPV16-positive cell line originated from a cervical SCC. Cell cultures of UMSCC 11A and UMSCC 14C were prepared with Eagle's minimum essential medium (Gibco, Life Technologies, Carlsbad, CA, USA), containing 2 mM of L-glutamine and 10% fetal calf serum and were supplemented with antibiotics/antimycotics, according to the instruction manual (Gibco, Life Technologies). The cell cultures of CERV196 were prepared with Eagle's minimum essential medium (Gibco, Life Technologies), 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% of fetal bovine serum (Gibco, Life Technologies). All cell cultures were incubated under standardized conditions at 37˚C, with 5% CO_2 and 95% humidity. New cell passages were created by adding a phosphate-buffered saline solution supplemented with 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (Sigma Aldrich; Merck KGaA, Darmstadt, Germany) at 37˚C for 5 min.

The drugs erlotinib, gefitinib, nilotinib, dasatinib and everolimus were donated by Professor R.D. Hofheinz (Oncological Department, University Hospital Mannheim, Medical Faculty Mannheim, University of Heidelberg, Germany). The drugs were stored at room temperature and dissolved in dimethylsulfoxide. A total of 20 μmol/l of each drug was added to cell cultures (confluency was 70%), and the cells were incubated at 37˚C for 24, 48, 72 and 96 hours. The same cultivation durations were applied to untreated cells as a negative control. The cell proliferation assay (alamarBlue©; AbD Serotec, Raleigh, NC, USA) was performed using 96-well microtiter plates. The experiments were repeated at least three times (n=3).

Enzyme-linked immunosorbent assay (ELISA) for FAS CD95, FAS ligand and caspase-3. Protein concentrations were measured using a Sandwich ELISA according to the manufacturer's instructions. DuoSet ELISAs (R&D Systems, Inc., Minneapolis, MN, USA) were used for FAS CD95, FAS ligand and caspase-3. Optical density was determined with an 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 78.88 pg/ml for FAS CD95, 1.04 pg/ml for FAS ligand and 604.67 pg/ml for caspase-3. The interassay coefficient of variation given by the manufacturer was below 10%.

Statistical analysis. Mean values were used for statistical analysis. They are presented±standard deviation. The two coefficient variance test and Dunnett's test were applied using SAS statistics software (Version 9.3; SAS Institute, Inc., Cary, NC, USA). A value of $p \leq 0.05$ was considered to be statistically significant. Statistical analysis was performed with the expertise of Dr. S. Hetjens (Department of Medical Statistics, Biomathematics and Information Processing, Medical Faculty Mannheim, University of Heidelberg, Germany)

Results

FAS CD95. FAS CD95 was detected in all three tested cell lines, with the expression levels increasing with culture time in untreated cells, except for CERV196, where protein expression was not detectable after 24 and 48 h and was 11.39±3.5 pg/ml after 72 h and 1.75±2.03 pg/ml after 96 h. Overall, the concentration of FAS CD95 was highest in HPV-negative UMSCC 11A cells. After 24 h, a reduction of

Table I. *FAS cluster of differentiation (CD)-95 expression (pg/ml) in UMSCC 11A, UMSCC 14C and CERV196 after incubation with nilotinib, dasatinib, erlotinib, gefitinib and everolimus compared to the negative control.*

Cell line		Negative control	Nilotinib (20 μmol/l)		Dasatinib (20 μmol/l)		Erlotinib (20 μmol/l)		Gefitinib (20 μmol/l)		Everolimus (20 μmol/l)	
	Incubation time (h)	Mean	Mean	*p*-Value	Mean	*p*-Value	Mean	*p*-Value	Mean	*p*-Value	Mean	*p*-Value
UMSCC-11A	24	6.79	8.27	0.982	13.72	0.143	9.32	0.867	0.67	0.220	0.08	0.162
	48	10.58	8.64	0.978	22.02	**0.038**	9.59	0.999	15.22	0.619	8.59	0.975
	72	57.94	25.20	**0.001**	21.61	**0.001**	17.78	**0.001**	15.95	**0.001**	9.91	**0.001**
	96	78.88	23.68	**0.001**	16.81	**0.001**	24.28	**0.001**	16.97	**0.001**	2.00	**0.001**
UMSCC-14C	24	4.17	0.69	0.237	0.63	0.225	4.68	0.998	1.39	0.417	3.76	0.999
	48	8.65	7.97	0.977	12.46	0.051	4.88	0.052	4.20	**0.022**	1.88	**0.001**
	72	11.62	9.59	0.751	0.00	**0.003**	0.00	**0.003**	7.05	0.132	5.95	0.051
	96	11.99	9.62	0.958	0.18	**0.041**	0.00	**0.038**	0.72	0.052	12.82	>0.99
CERV-196	24	0.00	0.00	>0.99	6.12	0.114	7.94	**0.033**	16.40	**0.001**	5.97	0.126
	48	0.00	0.40	>0.99	1.35	>0.99	23.69	**0.001**	1.97	0.988	4.33	0.791
	72	11.37	7.47	0.346	12.93	0.934	8.33	0.561	2.79	**0.011**	1.41	**0.004**
	96	1.75	13.02	**0.001**	0.00	0.843	1.68	>0.99	11.39	**0.002**	12.10	**0.001**

Data are mean values. Significance is marked in bold ($p<0.05$).

expression was seen after incubation with gefitinib and everolimus, although it was not statistically significant. After 48 h, only the addition of dasatinib led to a significant increase ($p=0.038$) of FAS CD95. The addition of nilotinib, dasatinib, erlotinib, gefitinib or everolimus led to a significant (all $p<0.001$) decrease of FAS CD95 after 72 and 96 h in the UMSCC 11A cell line.

Regarding the UMSCC 14C cell line, the data were not as uniform. No significant effect was seen when nilotinib was added, although nilotinib led to a reduction of FAS CD95 expression at all measured time points. The addition of dasatinib also led to a significant reduction of protein levels after 72 and 96 h ($p<0.05$). The addition of erlotinib led to fluctuating protein expression, with a significant reduction of protein level only after 72 h ($p<0.05$). Gefitinib led to a decrease of FAS CD95 expression after all measured time points. However, the effect was only significant after 48 h ($p<0.05$). A similar pattern was observed after incubation with everolimus, with a significant decrease of protein expression after 48 h ($p=0.001$).

In the HPV16-positive cell line CERV196, it is difficult to make conclusions regarding the alteration of FAS CD95 expression because no measurable protein expression was detected in the negative control after 24 and 48 h. Interestingly, FAS CD95 expression was detected after incubation with all tested drugs in CERV196 cells after 24 and 48 h. The addition of all tested drugs led to a decrease of protein expression in CERV196 after 72 h, with significant effects after incubation with gefitinib ($p<0.05$) and everolimus ($p<0.01$). Interestingly, FAS CD95 expression decreased again after 96 h in comparison to the results after 72 h in the negative control. With the exception of dasatinib and erlotinib, all tested drugs led to a significant increase of protein expression after 96 h in CERV196 cells. Data are presented in Table I.

FAS ligand. Expression of FAS ligand was detected in all three tested cell lines. Expression levels increased over the course of 96 h in UMSCC 11A and UMSCC 14C cells, with one exception after 72 h, when a lower concentration was measured in UMSCC 14C cells. In the culture of HPV-positive cells, FAS was detected for the first time after 72 h in the negative control and then increased at 96 h. Significant changes in protein levels of FAS ligand in UMSCC 11A cell culture were seen following the addition of dasatinib, erlotinib, gefitinib and everolimus. No significant changes were seen in FAS ligand expression levels after adding nilotinib. The addition of dasatinib, erlotinib, gefitinib and everolimus led to significantly decreased of FAS ligand expression after 96 h ($p<0.01$). Interestingly, its protein expression was significantly increased after 48 h when dasatinib or everolimus were added. At the other measurement points, there was no significant change in FAS ligand protein level. Regarding UMSCC 14C cells, only the addition of erlotinib led to a significant increase after 24 h. At later time points, no significant changes were measured in the erlotinib-treated cultures. Interestingly, nilotinib also led to a constant increase of FAS ligand expression in UMSCC 14C cells, although this effect was not statistically significant.

In the HPV16-positive cell line CERV196, conclusions regarding changes in FAS ligand expression are hard to draw

Table II. *FAS ligand expression (pg/ml) in UMSCC 11A, UMSCC 14C and CERV196 after incubation with nilotinib, dasatinib, erlotinib, gefitinib and everolimus compared to the negative control.*

Cell line		Negative control	Nilotinib (20 μmol/l)		Dasatinib (20 μmol/l)		Erlotinib (20 μmol/l)		Gefitinib (20 μmol/l)		Everolimus (20 μmol/l)	
	Incubation time (h)	Mean	Mean	*p*-Value	Mean	*p*-Value	Mean	*p*-Value	Mean	*p*-Value	Mean	*p*-Value
UMSCC-11A	24	0.01	0.35	0.785	0.45	0.595	0.42	0.664	0.58	0.376	0.00	>0.99
	48	0.02	0.29	0.074	0.72	**0.001**	0.02	>0.99	0.28	0.093	0.39	**0.013**
	72	0.45	0.38	0.999	0.50	0.999	0.09	0.627	0.04	0.518	0.97	0.297
	96	1.04	0.97	0.989	0.05	**0.001**	0.41	**0.006**	0.46	**0.010**	0.12	**0.001**
UMSCC-14C	24	0.10	0.10	>0.99	0.00	0.856	0.67	**0.002**	0.00	0.856	0.03	0.948
	48	0.10	0.29	0.065	0.26	0.117	0.11	0.999	0.02	0.612	0.00	0.428
	72	0.02	0.25	0.099	0.02	>0.99	0.00	0.999	0.13	0.674	0.17	0.361
	96	0.26	0.27	>0.99	0.00	0.318	0.00	0.318	0.02	0.385	0.49	0.409
CERV-196	24	0.00	0.00	>0.99	0.21	0.048	0.31	**0.004**	0.86	**0.001**	0.17	0.135
	48	0.00	0.00	>0.99	0.08	0.988	1.90	**0.001**	0.61	0.028	0.04	>0.99
	72	0.24	0.42	0.315	0.89	**0.001**	0.27	0.999	0.04	0.198	0.00	0.101
	96	0.55	1.04	0.543	0.00	0.454	0.00	0.454	0.66	0.998	0.52	>0.99

Data are mean values. Significance is marked in bold ($p<0.05$).

because no measurable protein expression was detected in the negative control until 72 h had passed. FAS ligand protein levels fluctuated in treated cells. After 72 h, only dasatinib led to a significant increase of protein expression in CERV196 cells ($p<0.001$). No other significant changes were seen after 72 and 96 h. The addition of everolimus to the CERV196 cell line cultures resulted in no observable changes. The results are presented in Table II.

Caspase-3. Caspase-3 expression was detected in all three tested cell lines. The protein levels of caspase-3 were the highest in comparison to the other tested proteins. In the untreated UMSCC 11A cell line, expression increased from 24 to 48 h, decreasing slightly after 72 h and then increasing again to a maximum level after 96 h. In the UMSCC 11A cell line, we observed that the addition of all the tested drugs except nilotinib led to reduced protein concentrations after 72 h, with significant effects after incubation with erlotinib and gefitinib ($p<0.05$). All the tested drugs led to a decrease of caspase-3 expression after 96 h in UMSCC 11A, with significant effects after incubation with nilotinib and everolimus ($p<0.05$). Nilotinib, dasatinib and erlotinib treatment of UMSCC 11A cells led to significantly increased protein expression levels after 24 h ($p<0.05$). The addition of dasatinib and nilotinib also significantly increased caspase-3 expression after 48 h in UMSCC 11A cells. A similar effect was observed after incubation with gefitinib and everolimus, although this effect was not significant. Erlotinib treatment of UMSCC 11A cells resulted in a statistically significant decrease after 72h ($p<0.05$). With the exception of dasatinib incubation after 96 h, no significant changes in caspase-3 expression could be seen in the UMSCC 14C cells. The expression of caspase-3 in the UMSCC 14C cells was irregular. Therefore, no valid conclusion regarding the effect of the tested drugs could be made.

In the untreated HPV-positive cell line CERV196, protein concentrations increased in the first 72 h but then decreased again after 92 h to a similar level to that after 48 h. All tested drugs led to an increase of caspase-3 expression level after 24h in CERV196 cells. The effect was significant after incubation with dasatinib, erlotinib, gefitinib and everolimus ($p<0.05$). A statistically significant increase of caspase-3 expression was observed after incubation with erlotinib after 48 h ($p<0.01$). In addition, a statistically significant increase of protein expression could be seen after incubation with Dasatinib after 72 h ($p<0.01$). Protein expression was also significantly increased in HPV-positive CERV196 cells after incubation with nilotinib, gefitinib and everolimus after 96 h ($p<0.05$). The results are presented in Table III.

Discussion

In order to improve the prognosis and treatment of HNSCC, intensive efforts have been made to establish individualized therapeutic approaches. Cetuximab targets EGFR and was the first available monoclonal antibody for a targeted therapy approach in HNSCC (36). EGFR is a transmembrane member of the ERBB receptor tyrosine kinase family. Activation of EGFR through binding of one of its ligands, EGF, transforming growth factor-alpha or amphiregulin, leads to receptor kinase activation and results in the activation of the RAS/rapidly accelerated fibrosarcoma (RAF) mitogen-

Table III. *Caspase-3 (pg/ml) expression in UMSCC 11A, UMSCC 14C and CERV196 after incubation with nilotinib, dasatinib, erlotinib, gefitinib and everolimus compared to the negative control.*

Cell line		Negative control	Nilotinib (20 μmol/l)		Dasatinib (20 μmol/l)		Erlotinib (20 μmol/l)		Gefitinib (20 μmol/l)		Everolimus (20 μmol/l)	
	Incubation time (h)	Mean	Mean	*p*-Value	Mean	*p*-Value	Mean	*p*-Value	Mean	*p*-Value	Mean	*p*-Value
UMSCC-11A	24	113.67	509.00	**0.043**	604.67	**0.012**	578.67	**0.016**	288.00	0.570	62.33	0.993
	48	161.00	436.33	**0.002**	554.67	**0.001**	80.67	0.503	262.67	0.304	274.67	0.221
	72	378.67	410.00	0.986	277.33	0.431	162.00	**0.025**	138.67	**0.013**	238.67	0.215
	96	402.00	83.00	0.009	263.00	0.358	384.33	>0.99	385.67	>0.99	154.33	**0.042**
UMSCC-14C	24	200.67	98.00	0.651	135.67	0.905	349.33	0.338	63.67	0.407	208.33	>0.99
	48	279.00	327.67	0.941	418.33	0.266	346.00	0.828	259.33	0.999	267.00	>0.99
	72	247.00	407.33	0.233	20.67	**0.060**	184.33	0.897	393.67	0.300	327.33	0.780
	96	458.67	440.67	>0.99	114.67	**0.043**	252.00	0.306	289.33	0.474	511.67	0.986
CERV-196	24	66.67	90.00	0.996	309.00	**0.016**	315.00	**0.014**	438.67	**0.001**	222.33	0.183
	48	128.67	100.00	0.994	206.33	0.760	498.33	**0.002**	110.00	0.353	269.33	0.282
	72	347.00	388.33	0.486	486.00	**0.002**	339.67	0.999	131.33	**0.001**	110.00	**0.001**
	96	120.67	340.33	**0.010**	47.00	0.605	152.67	0.970	383.33	**0.002**	414.33	**0.001**

Data are mean values. Significance is marked in bold ($p<0.05$).

activated protein kinase, phosphoinositide-3-kinase/protein kinase B and Janus kinase/signal transducer and activator of transcription-3 pathways. These pathways promote cell proliferation, stimulate angiogenesis, activate invasion and metastasis and inhibit apoptosis (37). EGFR is overexpressed in up to 90% of HNSCCs. The overexpression of EGFR in HNSCC is correlated with a poor prognosis and resistance to radiation (38, 39). Other drugs targeting EGFR, either by the humanized antibody panitumumab or by small-molecule TKI, have failed to improve clinical outcomes in the treatment of HNSCC. Moreover, erlotinib and gefitinib did not improve clinical outcomes in a curative therapy protocol compared to standard radiochemotherapy (40-47). Other TKIs, such as nilotinib, have been extensively studied *in vitro* but are not yet included in *in vivo* approaches (48, 49). A recent meta-analysis of gefitinib in advanced HNSCC showed that gefitinib did not prolong overall survival or progression-free survival but gefitinib alone is equivalent to methotrexate or methotrexate plus fluorouracil and tends to improve the quality of life of patients with recurrent disease (50). The inhibition of mTOR does not exhibit relevant clinical activity in HNSCC. Overall response rates remain modest and the optimal clinical setting has yet to been established (51-53).

The available clinical studies have not found significant effects of TKI and mTOR on HNSCC, but in other tumors entities, such as chronic myelogenous leukemia and non-small-cell lung cancer, TKIs are approved for primary treatment and have demonstrated promising results (22-26). Accordingly, we designed the present study to obtain new insights to better understand these contradicting effects. These novel insights might lead to the better selection of patients who are suitable for treatment with TKI or everolimus or to the discovery of new drug combinations. To the best of our knowledge, apoptosis-related proteins have not yet been investigated under the influence of TKI and everolimus in HNSCC *in vitro*.

FAS CD95 and FAS ligand. The expression of FAS CD95 is down-regulated and FAS ligand is up-regulated in HNSCC, which indicates reduced apoptosis in HNSCC (33). While the physiological relevance of this activity in cancer cells is not fully understood, the altered expression of FAS ligand and FAS CD95 by cancer cells might be due to a mechanism of resistance to CD95-mediated apoptosis (54). This suppression of protein expression might play a role in the failure of TKI therapy. In contrast to treated cells, FAS CD95 expression was not observed in untreated HPV-positive tumor cells within the first 48 h. The highest expression was measured after 48 h in erlotinib-treated cells. Overall, the added TKIs and everolimus seemed to inhibit FAS CD95 expression in HPV-positive cells, with the exception of treatment with dasatinib after 72 h. This observation is in contrast to the expression of HPV-negative cells in which FAS CD95 was inhibited after 72 and 96 h following initial induction within the first 48 h. Our results show increased expression of FAS CD95 in UMSCC 11A and reduced expression in UMSCC 14C for TKIs that inhibit EGF (erlotinib, gefitinib), and reduced expression in both HPV negative cultures for TKI that inhibit BCR–ABL (nilotinib, dasatinib) respectively. Despite these inconsistent findings, nearly all TKIs increased the expression of FAS CD95 in the HPV-positive cell line and reduced it in HPV-negative cells.

Overall, chemotherapy-naive tumor cells showed an increase of FAS CD95, but the expression levels were divergent in HPV-positive and HPV-negative cells. TKIs and everolimus seem to intervene in the mechanism of apoptosis and related FAS CD95 expression. It has already been shown that HNSCC cells can escape FAS-mediated cell death and do not express FAS ligand but rather FAS CD95 (55). Our data suggest that the tested TKIs and everolimus are capable of inhibiting this apoptosis-escape mechanism. FAS ligand is expressed on HNSCC and promotes T-lymphocyte apoptosis (56). A systematic review and meta-analysis by de Ruiter *et al*. confirmed the favorable, prognostic role of $CD3^+$ and $CD8^+$ T-cell infiltration in patients with HNSCC and found an association between $FOXP3^+$ tumor-infiltrating T-lymphocytes and improved overall survival (57). The tested TKIs and everolimus significantly inhibited FAS ligand expression over time in HPV-positive cells. In particular, the EGF-inhibiting TKIs erlotinib and gefitinib led to increased FAS ligand expression in the HPV-positive cell line. TKIs and everolimus also inhibited the apoptosis of the above-mentioned T-lymphocytes. Hence, it can be assumed that apoptosis not only of tumor cells but also of tumor-infiltrating immune cells are affected by TKIs. FAS ligand and FAS ligand gene therapy in mice and *in vitro* have been shown to be efficient in HNSCC treatment (58). There seems to be intracellular and extracellular storage of FAS ligand, and blocking the activity of extracellular FAS ligand was found to have no effect on cell viability (59). Therefore, it may be necessary to target the intracellular proteins or the mRNAs of FAS and FAS ligand (60). Our data indicate that the tested TKIs and everolimus influence apoptosis via the FAS CD95 and FAS ligand pathway, with an emphasis on HPV-negative tumor cells. Overall, the current data on FAS CD95 and FAS ligand in HNSCC remains somewhat contradictory, but the growing evidence now suggests that cancer cells do not lose their ability to express FAS or FAS ligand when they are active (60).

Caspase-3. Overall, the drugs appeared to have varying impacts on the tested cancer cells, thus emphasizing the different roles of HPV-dependent tumor cells.

Apoptosis might be dysregulated in SCC cells, which prevents a uniform and predictable expression pattern of apoptosis-related proteins, such as caspase-3. In patients with oral tongue squamous cell carcinoma, the combination of either cleaved caspase-3 or higher caspase-3 expression or both were associated with poor disease-free survival rates (61). Dysregulated apoptosis has been shown to induce a variety of diseases, including cancer (62, 63). An impaired apoptotic mechanism allows cancer cells to survive and promotes tumor angiogenesis, with subsequent tumor formation and metastasis (64). Tumor cells also evade apoptotic mechanisms to acquire resistance against treatments (65, 66). Furthermore, it has been shown that caspase-3 was a biomarker for tumorigenesis in HNSCC of the tongue (61). This observation may explain the varying results of our cancer cell cultures regarding caspase-3, FAS ligand and FAS CD95.

Targeting the EGF receptor leads to a change in apoptosis in oral SCC. In a study by Iwase *et al*., EGFR inactivation by cetuximab and the TKI tyrphostin AG1478 induced FAS-mediated apoptosis in the HSC-2 cell line through the down-regulation of c-FLIP. The inhibition of caspase-3 or -8 but not -9 reduced the effect of EGFR inhibitors on FAS-mediated apoptosis of HSC-2 cells. These results support the hypothesis that pharmacological inhibition of the EGFR signaling pathway is a potent strategy for treating HNSCC (67). It has also been shown that the induction of apoptosis of oral SCCs can be accomplished through a combination therapy of ultrasound with cetuximab-coated albumin microbubbles (68).

Limitations of this study are particularly evident, such as initial protein expression of FAS and FAS ligand not being detected in the HPV-positive cell cultures. Future research projects with clinically applied drug combinations and other TKIs might be carried out to provide a better understanding of the limited effectiveness of TKIs and everolimus in HNSCC treatment as well as their influence on apoptosis.

In conclusion this is the first study to analyze the influence of TKIs and everolimus on caspase-3, FAS ligand and FAS CD95 as key proteins of apoptosis. We found that the tested drugs influenced the expression of the proteins in various ways. Caspase-3 seems to be the main apoptosis marker in HPV-positive cells, while FAS CD95 and FAS ligand have a primary influence in 11A HPV negative cultures. These results contribute to a better understanding of the cell biology of HPV-positive and HPV-negative HNSCC and provide valuable information for possible novel treatment strategies in HNSCC with the addition of or combination with TKIs and mTOR inhibitors.

Conflicts of Interest

The Authors declare that they have no conflicts of interest.

Author's Contributions

Richard Birk, Benedikt Kramer and Christoph Aderhold conceived and planned the experimental design. Benedikt Kramer, Lena Huber and Christoph Aderhold carried out the experiments. Richard Birk and Benedikt Kramer led in writing the article. Richard Birk, Lena Huber, Christoph Aderhold, C. Emika Mueller, Stephan Hoch, Claudia Scherl, Nicole Rotter, Angela Schell, Anne Lammert and Benedikt Kramer contributed to the interpretation of the results, provided critical feedback and improved the article. All Authors carefully read and commented on the article.

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