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Expression Patterns of CD44 and AREG Under Treatment With Selective Tyrosine Kinase Inhibitors in HPV$^+$ and HPV$^-$ Squamous Cell Carcinoma

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Abstract. *Background: We investigated the expression patterns of cluster of differentiation (CD) 44 and amphiregulin (AREG), two signaling molecules essential for cell proliferation and differentiation, under the influence of selective tyrosine kinase inhibitors (TKIs) in human papillomavirus (HPV)$^+$ and HPV$^-$ squamous carcinoma cell lines. Materials and Methods: The protein expression of CD44 and AREG was determined by sandwich enzyme-linked immunosorbent assay in HPV$^-$ cell lines UMSCC-11A and UMSCC-14C, and HPV$^+$ CERV-196 cells after TKI treatment. Results: The expression of AREG and CD44 was dependent on the cell line's HPV status. AREG expression increased after incubation with nilotinib in HPV$^+$ tumor cells. The expression of CD44 was significantly influenced by all drugs; its expression under selective epidermal growth factor receptor inhibition was mostly reduced, whereas nilotinib led to an exceptional increase of CD44 expression. Conclusion: The selective drug treatment options significantly influenced the expression of CD44 and AREG in HPV$^-$ and HPV$^+$ tumor cells, constituting the need for personalized treatment options.*

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The rate of human papillomavirus (HPV) association with squamous cell carcinoma (SCC) has dramatically increased over the past decades (1). Since 2015, the most common HPV-associated cancer in Western countries such as the United States is represented by oropharyngeal SCC (2). For the oropharynx, researchers and clinicians have the possibility to investigate SCC with distinct immunological characteristics, depending on the tumorigenesis. Patients with HPV-associated tumors demonstrate improved therapeutic response rates leading to a better clinical outcome than patients with non-HPV-associated SCC (3). To date, the reasons for this difference are not fully understood and are investigated in the molecular mechanisms of the underlying oncogenic processes. One of the key features in both HPV$^+$ and HPV$^-$ SCC is the overexpression of epidermal growth factor receptor (EGFR), a member of the receptor tyrosine kinase family. Several investigations have confirmed that overexpression of EGFR is a negative predictor for clinical outcome (4, 5).

EGFR signaling results in inhibition of the signal transducer and activator of transcription gene 1 (STAT1) and inhibits the cellular antigen-processing machinery, providing an escape mechanism for tumor cells from immunosurveillance (6).

Despite these findings, EGFR overexpression has not been shown to correlate with therapeutic response to receptor blockade with the EGFR-specific monoclonal antibody cetuximab (7, 8), and reported therapeutic response rates are low (around 20%) (9). The reasons for therapeutic resistance are manifold, including mutations in EGFR downstream signaling, especially rat sarcoma gene (*RAS)* and b-rapidly accelerated fibrosarcoma gene (*BRAF)* mutations, or activation of the phosphoinositide 3-kinase (PIK3CA)/phosphatase and tensin homolog (PTEN) and Janus kinase (JAK)/signal transducer and activator of transcription protein (STAT)

signaling pathway and epithelial-to-mesenchymal transition of cancer cells (10).

CD44 is a multifunctional surface protein, involved in the regulation of cell differentiation, proliferation and survival, and has been linked to cells with cancer stem cell characteristics (11). In head and neck (HN) SCC, CD44 expression was linked to poor clinical outcome due to advanced tumor stage, metastasis, therapeutic resistance and invasion (12). Additionally, specific CD44 isoforms act as co-receptors to tyrosine kinase signaling, including the EGFR signaling cascade (13).

Amphiregulin (AREG) is a transmembrane glycoprotein from the EGFR family, interacting with EGFR, and regulating cellular growth and proliferation (14). AREG engages adjacent cells *via* juxtacrine signaling. After processing *via* proteolytic membrane proteases, AREG also functions *via* autocrine and paracrine signaling. Elevated AREG expression is associated with chronic inflammation and tumor growth (15).

In contrast to monoclonal antibodies that block the human epidermal growth factor receptor (HER) tyrosine kinases directly (*e.g.* cetuximab for HER1, trastuzumab for HER2), selective tyrosine kinase inhibitors (TKIs) inhibit the intracellular receptor signaling cascade by inhibiting phosphorylation thereby preventing activation. This inhibition can occur through competition with the substrate, adenosine triphosphate (ATP), inhibition of the phosphorylating enzyme or by deactivating it through conformational change (16).

Erlotinib and gefitinib belong to the first generation of TKI. Erlotinib is approved for the treatment of non-small cell lung cancer (NSCLC) and pancreatic cancer, with significant improvement of therapeutic overall response rates (17). Gefitinib is approved in the therapy of NSCLC and under investigation for different solid cancer types with TKI mutations such as breast cancer. An important aspect of therapy with TKI is the development of therapeutic resistance. Amplification of the proto-oncogene hepatocyte growth factor receptor (*MET)* leading to activation of HER3 signaling and *EGFR* T790M mutations have been identified as important mechanisms of therapeutic resistance to TKI therapy (18, 19). Afatinib is a member of the irreversible second-generation TKIs of the HER family (20). The down-regulation of HER signaling is achieved through covalent binding to kinase domains, resulting in irreversible inhibition of autophosphorylation (21). Afatinib is approved in the therapy of metastatic NSCLC with *EGFR* mutations/deletions (22). Dasatinib and nilotinib are small-molecule TKIs, acting through competitive binding of ATP-binding sites, resulting in dysregulation of tyrosine kinase enzymatic activity. Dasatinib and nilotinib have been investigated in hematopoietic malignancies and inhibit platelet-derived growth factor-β receptor signaling, ephrin receptor kinases and mast/stem cell growth factor receptor (23). Dasatinib has also been shown to inhibit sarcoma tyrosine kinase (SRC) family kinases, a major means of resistance to anti-HER2 therapy in patients with breast cancer (24-26).

As both surface proteins AREG and CD44 have a strong association with EGFR signaling pathways influencing tumor progression and therapeutic response, we aimed to investigate the effect of different selective TKIs on the expression of CD44 and AREG in HPV^+ and HPV^- SCC.

Materials and Methods

Cell lines, drugs and study design. The HPV^- UMSCC cell lines were kindly provided by T.E. Carey, Ph.D. University of Michigan, Ann Arbor, MI, USA. UMSCC-11A cell line originated from a primary squamous cell carcinoma of the epiglottis, whereas UMSCC-14C originated from a skin metastasis of an oral SCC after radiation, chemotherapy and surgery. The CERV-196 cell line is positive for HPV16 and was provided from poorly differentiated SCC of the uterine cervix and acquired from Cell Lines Service GmbH, Eppelheim, Germany.

HPV^- cells were cultured with Eagle's minimum essential medium (Gibco, Life Technologies, Carlsbad, CA, USA) and supplemented with 2 mM of L-glutamine, 10% fetal calf serum and Pen-Strep (Gibco, Life Technologies). Cultured HPV^+ cells were supplemented with 2 mM L-glutamine, 1.0 g/l sodium bicarbonate, 1.0 g/l sodium pyruvate, 0.1 mM non-essential amino acids and 10% of fetal bovine serum (Gibco, Life Technologies). Cell cultures were grown under standardized conditions (37˚C, 5% CO_2, 95% humidity). For subcultures, 0.05% trypsin/0.02% EDTA solution (Sigma Aldrich, St. Louis, MO, USA) was added for 5 min at 37˚C. Incubation time ranged from 24 to 96 hours. Nilotinib, dasatinib, gefitinib, erlotinib and afatinib were provided by the Oncological Department, University Hospital Mannheim GmbH. The drugs were dissolved in dimethylsulfoxide at a concentration of 20 μmol/l. Cell proliferation assay was performed in 96-well microtiter plates (alamarBlue©, AbD Serotec, Oxford, UK).

Enzyme-linked immunosorbent assay (ELISA) for CD44 and AREG. To determine the protein concentrations of CD44 and AREG in treated and untreated cells, a sandwich ELISA technique was applied. For both proteins, DuoSet ELISA development kits (R&D Systems, Inc., Minneapolis, MN, USA; and Bio-Techne GmbH, Wiesbaden, Germany) were used (DY7045-05 for CD44, and DY989 for AREG) and performed in accordance with the manufacturer's instructions. The optical density was measured at a wavelength of 450 nm with wavelength correction set to 540 nm with an MRX Microplate Reader (DYNEX Technologies, Chantilly, VA, USA). Concentrations were determined in pg/ml and the detection range was 7.8-1,500 pg/ml for CD44 and 15.6-1,000 pg/ml for AREG. The inter-assay coefficient of variation reported by the manufacturer was <10%.

Statistical analysis. The statistical analysis was performed using the mean values for each experiment. Each experiment was independently performed three times. The means were compared to the mean values of the negative control using the two- coefficient variance test to assess statistical significance (SAS Statistics software, version 9.3; SAS Institute, Inc., Cary, NC, USA). The resulting *p*-values were adjusted using Dunnett's test. For all analyses, a value of $p \leq 0.05$ was defined as statistically significant.

Table I. *Enzyme-linked immunosorbent assay of cluster of differentiation 44 expression (pg/ml) in UMSCC-11A, 14C and CERV-196 after incubation with 20 μmol/l afatinib, dasatinib, erlotinib, gefitinib or nilotinib compared to the negative control.*

	Incubation time (h)	Negative control Mean	Afatinib		Dasatinib		Erlotinib		Gefitinib		Nilotinib	
			Mean	*p*-Value	Mean	*p*-Value	Mean	*p*-Value	Mean	*p*-Value	Mean	*p*-Value
UMSCC-11A	24	9.5	6.2	**0.004**	11.3	**0.022**	8.3	0.474	8.6	0.836	13.8	0.052
	48	25.4	27.5	0.772	29.5	0.314	13.0	**0.004**	26.0	0.995	26.0	0.992
	72	82.6	26.4	**<0.001**	84.0	0.991	28.9	**<0.001**	42.6	**<0.001**	87.5	0.896
	96	92.5	41.6	**0.004**	118.1	0.139	46.2	**0.001**	82.1	0.513	115.1	0.091
UMSCC-14C	24	14.4	25.1	**0.019**	16.0	0.667	15.6	0.910	14.6	>0.999	35.9	**<0.001**
	48	32.1	30.5	0.904	24.4	**0.004**	17.6	**<0.001**	20.7	**0.006**	72.4	**<0.001**
	72	49.4	28.6	**<0.001**	19.2	**0.001**	11.7	**<0.001**	24.6	**0.002**	144.6	**<0.001**
	96	31.2	44.6	0.117	29.9	0.938	20.1	**0.007**	34.9	0.415	158.0	**<0.001**
CERV-196	24	14.4	21.3	**<0.001**	14.7	0.972	13.0	0.862	18.1	0.337	17.6	0.229
	48	43.8	32.1	**0.002**	37.0	0.127	30.3	**0.002**	30.2	**<0.001**	52.2	0.077
	72	339.2	59.1	**<0.001**	170.0	**<0.001**	49.3	**<0.001**	66.3	**<0.001**	266.1	0.060
	96	747.3	54.6	**<0.001**	186.3	<0.001	101.2	**<0.001**	89.7	**<0.001**	708.1	0.891

Bold values indicate statistical significance ($p<0.05$).

Table II. *Enzyme-linked immunosorbent assay of amphiregulin expression (pg/ml) in UMSCC-11A, 14C and CERV-196 after incubation with 20 μmol/l afatinib, dasatinib, erlotinib, gefitinib or nilotinib compared to the negative control.*

	Incubation time (h)	Negative control Mean	Afatinib		Dasatinib		Erlotinib		Gefitinib		Nilotinib	
			Mean	*p*-Value	Mean	*p*-Value	Mean	*p*-Value	Mean	*p*-Value	Mean	*p*-Value
UMSCC-11A	24	823.3	584.0	**<0.001**	512.7	**<0.001**	339.0	**<0.001**	325.7	**<0.001**	804.0	0.800
	48	900.7	780.7	**<0.001**	756.7	**<0.001**	500.0	**<0.001**	540.0	**<0.001**	887.3	0.464
	72	910.7	838.3	**0.003**	753.0	**<0.001**	552.7	**<0.001**	655.7	**<0.001**	901.7	0.670
	96	934.0	808.7	**<0.001**	860.7	**0.001**	636.7	**<0.001**	784.3	**<0.001**	887.3	0.067
UMSCC-14C	24	273.7	173.7	**<0.001**	28.7	**<0.001**	44.7	**<0.001**	22.0	**<0.001**	103.0	**<0.001**
	48	413.3	212.7	**<0.001**	30.3	**<0.001**	48.0	**<0.001**	25.7	**<0.001**	152.7	**<0.001**
	72	449.3	414.3	0.349	39.3	**<0.001**	79.7	**<0.001**	43.7	**<0.001**	228.0	**<0.001**
	96	480.3	466.3	0.760	63.0	**<0.001**	82.0	**<0.001**	51.3	**<0.001**	289.3	**<0.001**
CERV-196	24	19.0	20.3	0.864	11.0	**<0.001**	12.0	**<0.001**	12.0	**<0.001**	27.0	**0.001**
	48	41.0	22.3	**<0.001**	13.7	**<0.001**	13.7	**<0.001**	9.7	**<0.001**	73.3	**<0.001**
	72	56.3	24.3	**<0.001**	20.3	**<0.001**	15.3	**<0.001**	14.0	**<0.001**	132.0	**<0.001**
	96	62.3	25.3	**<0.001**	18.3	**<0.001**	15.7	**<0.001**	15.7	**<0.001**	186.3	**<0.001**

Bold values indicate statistical significance ($p<0.05$).

The statistical analysis was performed in collaboration with Professor Dr. C. Weiss, Institute of Biomathematics, Medical Faculty Mannheim, University of Heidelberg, Germany.

Results

CD44 expression levels in UMSCC-11A, UMSCC-14C and CERV-196 cells. CD44 expression was observed in every tested cell line (Table I). Expression levels were nearly constant in all three cell lines at the beginning of incubation. After 96 hours of incubation, CERV-196 cells displayed the highest level of CD44 expression in the untreated (negative control) group in comparison to UMSCC-11A and UMSCC-14C cells. We observed a statistically significant decrease of CD44 expression in UMSCC-11A cells after treatment with afatinib, and with erlotinib after 96 hours (p=0.004; p=0.001).

For UMSCC-14C cells, erlotinib treatment significantly reduced the CD44 expression from 48 hours on (p=0.007). Gefitinib led to a significant reduction of CD44 expression after 48 and 72 hours (p=0.006 and p=0.002, respectively). Dasatinib led to a significant decrease of CD44 after 48 and 72 hours (p=0.001). Interestingly, nilotinib increased the CD44 expression in the UMSCC-14C cell line dramatically ($p<0.001$).

For CERV-196 cells, nilotinib was the only drug tested that showed no significant reduction of CD44 expression after 96 hours (p=0.891). Contrary to this, afatinib, dasatinib, erlotinib and gefitinib showed a significant effect on the reduction of CD44 expression in CERV-196 cells (p<0.001).

AREG expression levels in UMSCC-11A, 14C and CERV-196 cells. AREG expression was observed in every tested cell line (Table II). Highest expression levels were observed in UMSCC-11A cells.

Afatinib, dasatinib, erlotinib and gefitinib significantly reduced expression of AREG in both HPV$^-$ cell lines (p≤0.001). Interestingly, nilotinib was not able to reduce the AREG expression level significantly even after 96 hours in UMSCC-11A cells (p=0.067). In UMSCC-14C cells, dasatinib, erlotinib, gefitinib and nilotinib treatment led to a decrease in AREG expression (p<0.001). We observed similar reductions after 24- and 48-hour treatment with afatinib (p<0.001), yet these effects were not maintained after 72 and 96 hours (p=0.349 and p=0.760, respectively). Nilotinib led to a significant decrease of AREG expression in UMSCC-14C (p<0.001).

In HPV$^+$ CERV-196 cells, afatinib, dasatinib, erlotinib, and gefitinib treatment showed consistent reduction of AREG expression with increasing incubation despite the level increasing in the negative control group (p<0.001). In contrast, AREG expression dramatically increased after nilotinib treatment for any duration (p<0.001).

Discussion

Together with technological advances, individualized therapeutic options are increasing in response to emerging drug resistance to TKI therapy. In an interesting publication from Xu and colleagues, novel druggable targets in head and neck cancer were identified with high-throughput phenotyping (27). They used different HNSCC cell lines, including UMSCC-14C, and screened target genes for cancer specificity and potential therapeutic drug response, including small-molecule TKIs such as dasatinib. Approaches like these might help to identify candidate targets and potentially effective drugs. Our findings of distinct responses of HPV$^+$ and HPV$^-$ cell lines to different selective TKIs support this notion. In our investigation, we focused on two essential molecules responsible for cellular proliferation, differentiation and cell survival, influencing tumor progression and therapeutic resistance: CD44 and AREG.

This is one of the first *in vitro* studies to investigate the impact of afatinib, dasatinib, erlotinib, gefitinib and nilotinib on the expression of CD44 and AREG in HPV$^+$ and HPV$^-$ SCC. Our results showed both CD44 and AREG to be expressed in all three cell lines studies here.

CD44 is a marker widely used to identify cancer stem cells (28). Cancer stem cells are considered to be responsible for increased renewal capacity and tumor heterogeneity, and therefore, immune evasion, as well as therapy resistance (29). One of the main escape mechanisms is the epithelial-to-mesenchymal transition of tumor cells, which is closely associated to their CD44 expression level (30). High CD44 expression and low expression of epithelial cell adhesion molecule (EpCAM) have been associated with migratory cancer stem cells with increased risk for metastasis (31). Hufbauer and colleagues report a two-fold increase in CD44^{high} and EpCAM^{low} cell fractions from HPV16-E6E7-positive tumors (31). These findings may in part contribute to the clinical characteristics of early metastasis of HPV$^+$ tumor in patients (32). In accordance with this, the HPV$^+$ CERV-196 cell line in our untreated control group presented the highest CD44 expression level. As patients with HPV$^+$ and HPV$^-$ tumors also differ in therapeutic response rates, we investigated the effect of TKI therapy on CD44 expression. Here, we observed distinct changes in CD44 expression level, depending on HPV status, tumor cell line and TKI used. Interestingly, Nilotinib did not reduce the CD44 expression level after 96 hours for all cell lines, but in fact led to a significant increase in expression in the UMSCC-14C cell line. In contrast, treatment with afatinib, dasatinib, erlotinib and gefitinib significantly reduced the CD44 expression. These findings are in accordance with those of Abhold and colleagues, who observed reduced induction of genes responsible for metastasis, tumorigenesis, cell proliferation and drug resistance, including stromal cell-derived factor 1, transcription factor NANOG and CD44 after treatment with gefitinib (33). The clinical relevance of CD44 expression level was – amongst others – assessed by Nasman and colleagues, who also observed improved survival in patients with low CD44 expression and HPV$^+$ status (34). Furthermore, CD44 was shown to be associated with the important WNT/β-catenin signaling cascade, with a key role in carcinogenesis and therapeutic resistance (35). Roy and colleagues demonstrated that CD44 inhibition sensitized cisplatin-resistant HNSCC cells (35). Additionally, others have investigated the important link between CD44 expression and EGFR signaling, where high CD44 expression was found to be associated with p16$^-$ tumors and with higher EGFR expression (36).

In order to resolve the conundrum of contradictory higher CD44 expression levels in HPV$^+$ tumors, the association of CD44 expression level with therapeutic resistance yet better clinical outcome and therapeutic response rates of patients with HPV$^+$ tumors, Modur and colleagues offer an explanation: Lower EGFR expression in HPV$^+$ tumors result in a lack of association of CD44 with EGFR in the cancer stem cell compartment, with loss of activation of downstream extracellular signal-regulated kinase (ERK)1-ERK2 signaling (37, 38).

Another important molecule for EGFR signaling with an prognostic impact is its ligand AREG (39). Overexpression

of AREG has been linked to therapeutic resistance (40). In a multivariate analysis of EGFR ligand expression and its impact on prognosis of patients with HNSCC, AREG was found to be the dominant predictor (41). Furthermore, Chang and colleagues identified AREG expression as a potential biomarker for TKI therapy (42). In HNSCC, Kogashiwa and colleagues observed a better clinical outcome in patients with higher AREG gene expression and cetuximab therapy in comparison to patients with lower AREG expression (43). In our investigations, we found lower AREG expression in the HPV^+ CERV-196 cell line in comparison to UMSCC-11A and UMSCC-14C tumor cells. AREG expression was significantly reduced in UMSCC-11A and UMSCC-14C after dasatinib, erlotinib and gefitinib treatment. Zhang and colleagues reported augmented antitumor effects of erlotinib after knockdown of phosphoinositide-dependent kinase 1, a key kinase of the EGFR AREG signaling cascade (44). Interestingly, UMSCC-14C was the only cell line in our experiments in which nilotinib treatment significantly lowered AREG expression. As the UMSCC-14C cell line was derived from a patient with recurrent disease, a plausible explanation may be due to therapeutic changes in AREG signaling pathways. In UMSCC-11A, the reduction was not significant, yet in CERV-196 cells, we observed a drastic increase of AREG expression upon treatment with nilotinib. The reasons for this observed up-regulation in the HPV^+ cell line remain unclear; in a previous study, we found higher SRC expression in HPV^+ cell lines upon nilotinib treatment (45). Others have shown SRC-dependent induction of nuclear EGFR translocation (46), which might have contributed to our observed results. Summarizing, these results support the need for a more sophisticated, patient- (and tumor-) dependent analysis in order to identify the optimal use of anti-EGFR therapies and improve patient selection for individualized therapy, as resistance mechanisms include AREG signaling pathways. Hsu and colleagues identified a mechanism of TKI resistance to erlotinib treatment through the YES-associated protein (YAP)-dependent up-regulation of AREG expression (47). After inhibition of YAP by small-interfering RNA, down-stream signaling of AREG expression reduced significantly and sensitivity erlotinib was restored, followed by reduced migration, invasion and tumor sphere formation (47).

These results demonstrate that interactions between selected TKIs and expression levels of key molecules in cancer signaling and progression are distinct and depend on the TKI used as well as the tumor's HPV status. Therefore, individualized, targeted cancer therapy needs to take these findings into consideration in order to avoid or overcome therapeutic resistance. Future therapeutic approaches are required to consider and monitor the expression of different molecular key factors in order to adjust the (TKI) treatment to the patient's individual needs.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

Benjamin Kansy: Writing of the article, generation of tables, data analysis, conception of the study. Christoph Aderhold: Performance of experiments, data analysis. Lena Huber: Performance of experiments, data analysis. Sonja Ludwig: Data analysis, writing of the article. Richard Birk: Performance of experiments, data analysis. Anne Lammert: Performance of experiments, data analysis. Stephan Lang: Providing conceptional design of the study, data analysis. Nicole Rotter: Providing conceptional design of the study, data analysis. Benedikt Kramer: Writing of the article, generation of tables, data analysis, conception of the study. The article was critically reviewed by all Authors

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