MMP9, Cyclin D1 and β-Catenin Are Useful Markers of p16-positive Squamous Cell Carcinoma in Therapeutic EGFR Inhibition *In Vitro*

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Abstract. *Background/Aim: In the United States 53,640 new cases of head and neck cancer were estimated in 2013. Over 95% of these cases were evaluated as squamous cell carcinoma (SCC). At present, smoking, drinking alcohol, chewing betel and infection with high-risk types of human papilloma virus (HPV) are classified as risk factors of oropharyngeal squamous cancer cell carcinoma (OPSCC). It could be suggested that patients with HPV-positive OPSCC have a better response to chemoradiotherapy than patients without. In many studies, there was observed an inverse correlation between epithelial growth factor receptor (EGFR) expression and HPV status in p16-positive SCC. Therefore, it is of great clinical interest to specify the phenotype of cancer cells in order to further individualize treatment modalities. The aim of the study was to investigate the expression pattern of specific markers in p16-positive SCC cells after stimulation with lapatinib and gefitinib. Materials and Methods: We incubated p16-positive CERV196 cells with lapatinib and gefitinib (2 μg/ml) and after 5, 24 and 96 h determined E-cadherin, vimentin, matrix metalloproteinase-9 (MMP9), cyclin D1 and β-catenin by immunocytochemistry, enzyme-linked immunosorbent assay and quantitative polymerase chain reaction (PCR). Results: We found an increase of E-cadherin and a decrease of vimentin in unstimulated cells. We detected an alteration of expression of vimentin and E-cadherin level after treatment with lapatinib and gefitinib. We demonstrated a statistically significant lapatinib- and gefitinib-induced repression of cyclin D1, MMP9 and β-catenin in CERV196 cells dependent on incubation time. Conclusion: Cyclin D1 and MMP9 expression profiles may represent an early measure of sensitivity and level of response to lapatinib and gefitinib. The presented cell culture model is, therefore, well-suited for further study of epigenetic regulation of molecular targeted-therapy by EGFR inhibition and prevention of mesenchymal transition in p16-positive SCC cells.*

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The main risk factors for the development of head and neck squamous cancer cells (HNSCC) are alcohol and tobacco consumption. Clinical and pathological evidence suggests that viral oncogenic human papillomavirus (HPV) infection is another crucial etiological factor (1). The epidemiological, genetic, molecular and clinical profile of HPV-associated HNSCC cells seems to differ from that of tobacco- and alcohol-induced HNSCC (non-HPV). HPV-positive oropharyngeal carcinoma cells are characterized by being found in immunodeficient or immunosuppressed patients, those with a young age of HNSCC onset, those with a higher number of intimate partners with a history of genital warts and a strong association with sexual behavior (2). Epidermal growth factor receptor (EGFR), a transmembrane tyrosine kinase receptor, is normally expressed at low levels on the surface of most normal cells. In a variety of tissues, the main function of EGFR is to induce cell growth, proliferation, survival and de-differentiation. EGFR is also a member of the human epidermal growth factor (HER) family, and is overexpressed in more than 90% of HNSCCs, a high level of EGFR being associated with a worse clinical outcome (3). Aberrant expression of type I receptor tyrosine kinases such as EGFR (or c-ERBB1 to c-ERBB4) is frequently associated with the progression and genesis of human cancer (4).

Concurrent chemoradiation has been associated with improvement in locoregional control and organ preservation but at the cost of significant acute and chronic toxicity. HPV-positive oropharyngeal SCC (OPSCC) responds much better to treatment than HPV-negative OPSCC. However, many patients with HPV-positive OPSCC may not need the intensive chemoradiotherapy protocol, which is associated with serious side-effects. It may, thus, be concluded that HPV-preventative and individualized therapy are essential in treatment of this type of tumor (5). Biological agents targeting EGFR as a potential modality in combination with chemoradiotherapy or radiotherapy are currently under evaluation in clinical trials (6, 7). The development of monoclonal antibodies or agents that inhibit the receptor or the receptor tyrosine kinase are of high clinical interest.

Gefitinib (Iressa®; AstraZeneca) is a monoclonal antibody, which inhibits the ERBB1 tyrosine kinase and has shown anti-tumorous activity against small cell lung cancer and head and neck cancer (8, 9). Altered E-cadherin/β-catenin expression in non-small cell lung cancer harboring EGFR mutations was associated with poor response to EGFR tyrosine kinase (10). Lapatinib is a novel synthetic small-molecule inhibitor of epithelial growth factor-1 (EGF1) and human ERBB2 (HER2) tyrosine kinases. It is used in the form of lapatinib ditosylate (Tyverb®; GlaxoSmithKline), which showed high cell potency. It is an anti-EGFR-targeted treatment for breast tumors and other types of solid cancer (8). Fumagalli and colleagues demonstrated that lapatinib efficacy is restricted to the HPV-positive HNSCC cell lines (11).

A crucial process for the development of a metastatic carcinoma cell phenotype and the potential for invasion is epithelial-mesenchymal transition (EMT). In oral squamous cell carcinoma (OSCC), this process is defined by a decrease of epithelial-specific adhesion proteins (*e.g.* tight and adherens junction proteins such as cytokeratin, E-cadherin, desmoplakin, and claudin). Furthermore EMT, which can be activated by various growth factors, such as EGF and transforming growth factor beta 1, induces expression of mesenchymal proteins, such as N-cadherin, fibronectin and vimentin. It also induces the development of migratory attributes and changes in morphology (*e.g.* cell scattering) (12-16). Other EMT regulators that seem to control tumorigenesis are matrix metalloproteinases (*e.g.* MMP3 and MMP9) (17). MMPs as gelatinases play an important role in degrading the extracellular matrix (ECM). These enzymes are also associated with morphogenesis, organization, bone development, wound healing and cancer development (18-21). MMPs play a key role in tumor cell invasion of the basement membrane and stroma, blood vessel penetration, metastasis and tumor promotion (22). Autocrine or paracrine signaling through EGFR potentiates the invasive potential of HNSCC *via* selective up-regulation and activation of MMP9 (23).

Recently, in p16-positive, vimentin-down-regulated and E-cadherin-up-regulated SCC cells we showed incomplete EMT, with a significant up-regulation of β-catenin and cell scattering, with only a minor increase in expression of vimentin and decrease in E-cadherin expression, particularly after prolonged incubation with EGF (24). Cell proliferation and differentiation are regulated by the WNT (wingless gene and INT-1 gene) /β-catenin signaling pathway. The armadillo protein β-catenin is the central denominator of WNT signaling. However, during EMT, the interaction between β-catenin and E-cadherin are disconnected and β-catenin is translocated to the nucleus or also degraded (25). In other studies, in vulvar cancer, it was demonstrated that an alteration of E-cadherin and β-catenin expression characterizes the metastatic development of epithelial tumors, which occurs at different points in time depending on the patient's HPV status. HPV-related tumors are associated with better prognosis and outcome and do not progress through EMT characteristics (26). Cells can also undergo the reverse process, mesenchymal-epithelial transition (MET), to continue their differentiation. These dynamic EMT and MET events highlight the great flexibility of differentiated cells during morphogenesis and carcinogenesis (27). Most metastases of colorectal adenocarcinoma cells are able to change from the differentiation and morphology of the primary tumor and exhibit organized epithelial structures, despite the straightforward EMT-associated alteration in the phenotype at the invasive front. This phenomenon reflects re-differentiation in the metastases (28, 29).

The cell-cycle control protein cyclin D1 promotes control over G_1 and S phase transition, which alters cell proliferation rates. Overexpression of cyclin D1 leads to shortening of the G_1 phase resulting in an abnormally high cell proliferation (30). An increased level of cyclin D1 expression has been reported in a number of malignancies including esophageal, ovarian, breast, uterine, colon, lung, prostate, lymphoma, as well as head and neck cancer. However, *in vivo* and *in vitro* experiments demonstrated a correlation between cyclin D1 up-regulation and tumor growth, lymphangiogenesis and distant metastasis (31). It is suggested that HPV-positive SCC cells are associated with repression of cyclin D1 expression (32). In contrast to these results, in head and neck cancer not typically related to HPV, cyclin D1 up-regulation has been associated with poor outcome (33, 34). However, cyclin D1 might also be a potential prognostic factor to predict worse survival and outcome in cancer.

The aim of the present study was to investigate the expression pattern of vimentin, E-cadherin, MMP9 while adding of β-catenin and cyclin D1 quantitatively and qualitatively in HPV-positive SCC cells before and after stimulation with lapatinib and gefitinib in order to evaluate the outcome after different kinds of EGFR inhibition, and determine the alteration of potential prognostic markers,

Table I. *Sequence of polymerase chain reaction (PCR) primers used in the mRNA expression analysis.*

Gene	Nucleotide sequence of sense (s) and antisense (as) primers	Annealing temperature (˚C)	Reference/Barcode
E-Cadherin	AAG GTG ACA GAG CCT CTG GAT (s)	59.8	MALDI/017968410
	CGT CTG TGG CTG TGA CCT (as)	58.2	MALDI/017968411
Vimentin	TCT ACG AGG AGG AGA TGC GG (s)	61.4	MALDI/017968414
	GGT CAA GAC GTG CCA GAG AC (as)	61.4	MALDI/017968415
Cyclin D1	ATG CCA ACC TCC TCA ACG AC (s)	59.4	MALDI/017968418
	GGC TCT TTT TCA CGG GCT CC (as)	61.4	MALDI/017968419
β-Catenin	TGG ATG GGC TGC CTC CA (s)	67.8	MALDI/0179684412
	ACC AGC CCA CCC CTC GA (as)	69.6	MALDI/0179684413
GAPDH	GGT GGA GGT CGG AGT CAA CGG A (s)	65.8	MALDI/018348211
	GAG GGA TCT CGC TCC TGG AGG A (as)	65.8	MALDI/018348212

GAPDH: Glycerinaldehyde 3-phospate dehydrogenase. MALDI: Matrix-assisted laser desorption/ionization.

especially MMP9 and cyclin D1, after treatment with lapatinib and gefitinib in HPV-associated SCC cells *in vitro*.

Materials and Methods

Cell lines and culture. The p16-positive SCC cell line CERV196 (CLS, Eppelheim, Germany) originated from a poorly-differentiated xenotransplanted cervical carcinoma MRI-H-196. Cell culture was carried-out at 37˚C in a fully humidified atmosphere with 5% CO_2 using Dulbecco's modified minimum essential medium (DMEM; Fisher Scientific Co., Pittsburgh, PA, USA) supplemented with 10% fetal calf serum (FCS) and antibiotics (Life Technologies Inc., Gainthersburg, MD, USA). For immunocytochemistry, 1×10^4 cells per well were seeded in 8-well cell culture slides (BD Biosciences, Heidelberg, Germany). Lapatinib (Tyverb®; GlaxoSmithKline GmbH & Co, Heidelberg, Germany) and gefitinib (Iressa®; AstraZeneca, Wedel, Germany) were stored at 4˚C and dissolved in 0.5% FCS/DMEM at the time of application. When sub-confluent, cells were starved using 0% FCS/DMEM for 5 h and then incubated for 5, 24 and 96 h with lapatinib (2 μg/ml) or gefitinib (2 μg/ml) in 0.5% FCS/DMEM. Treatment with lapatinib (2 μg/ml) or gefitinib (2 μg/ml) was repeated every 24 h. Selection of the different drug concentrations and durations were defined after performing the alamarBlue (AbD Serotec, Oxford, UK) cell proliferation assay. After incubation, cells were centrifuged and the supernatants were collected together in sterile tubes and stored at −20˚C until further analysis.

Enzyme-linked immunosorbent assay (ELISA). Concentrations of E-cadherin (DuoSet mouse antihuman E-cadherin; R&D Systems, Wiesbaden, Germany), vimentin (Path Scan® total vimentin, sandwich ELISA kit, mouse monoclonal antibody-coated wells; Cell Signaling, Boston, MA, USA), MMP9 (DuoSet recombinant mouse, DY911; R&D Systems, Abingdon, UK), cyclin D1 (Path Scan® total cyclin D1 sandwich ELISA kit, human antibody; Cell Signaling) and β-catenin (DuoSet mouse antihuman β-catenin; R&D Systems, Wiesbaden, Germany) in the supernatants of the cell cultures and untreated cell cultures were determined by the ELISA technique (DMP2F0; R&D Systems, Wiesbaden, Germany). The system utilized a solid-phase monoclonal antibody and enzyme-linked polyclonal antibodies against E-cadherin, vimentin, MMP9, cyclin D1 and β-catenin. The specificity of antihuman antibodies used in the ELISA kit (DuoSet IC ELISA; R&D Systems, Wiesbaden, Germany) was examined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis followed by western blotting. According to the manufacturer's instructions, each analyte was measured in 100 μl of supernatant. All analyses and calibrations were performed in triplicate. The optical density was detected using a microplate reader at a wavelength of 540 nm. Concentrations of E-cadherin, MMP9, and β-catenin are reported as pg/ml. The coucentration of vimentin is given as μg/ml and of cyclin D1 in absorbance of 450 nm.

Immunocytochemistry. Immunocytochemical analysis was performed using an antibody directed against E-cadherin (ab 1416, monoclonal mouse antibody, 1:50; Abcam, Cambridge, UK), vimentin (M 0725, monoclonal mouse antibody, 1:50, Fa; Dako, Agilent Technologies, Glostrup, Denmark), MMP9 (ab 38898, polyclonal rabbit anti-human antibody, 1:100; Abcam), cyclin D1 (ab 6152, monoclonal mouse antibody, 1:100; Abcam) and β-catenin (monoclonal rabbit antibody, 1:200; Abcam). Immunostaining was performed using the streptavidin-biotin complex immunostaining method. Before performing immunocytochemistry, the SCC cells were cultured in 8-well chambers overnight. While growing to confluency, cells were exposed to different concentrations of lapatinib or gefitinib for different incubation periods (0, 5, 24, 96 h). Subsequently, they underwent fixation with acetone and alcohol (2:1) and were washed with phosphate-buffered saline (PBS) (Buffer kit; Dako, Hamburg, Germany). The following steps were executed by Dako TechMate 500 (Dako, Hamburg, Germany) automated staining system: Cells were incubated with primary antibody solution for 30 min at room temperature using a working solution of antibody to cells of 1:300. The slides were washed three times with PBS for 5 min each time (Buffer kit; Dako, Hamburg, Germany). Immunoreaction was shown with the Dako ChemMate Detection kit according to the guidelines of the manufacturer (APAAP, mouse, no. K5000; Dako, Hamburg, Germany). Cells were incubated in sheep serum. Immunoreaction was demonstrated with the monoclonal antibodies previously described. Incubation was followed by the addition of a specific biotinylated secondary antibody and streptavidin-biotin horseradish peroxidase complex (Amersham, Freiburg, Germany). To perform the peroxidase reaction, aminoethylcarbazol as chromogen was used. Before washing the cells several times, endogenous peroxidase was blocked using Dako REAL™ Levamisole (Bottle G, APAAP, mouse, no. K5000; Dako Hamburg, Germany). For the negative controls, all reagents except the primary antibody were

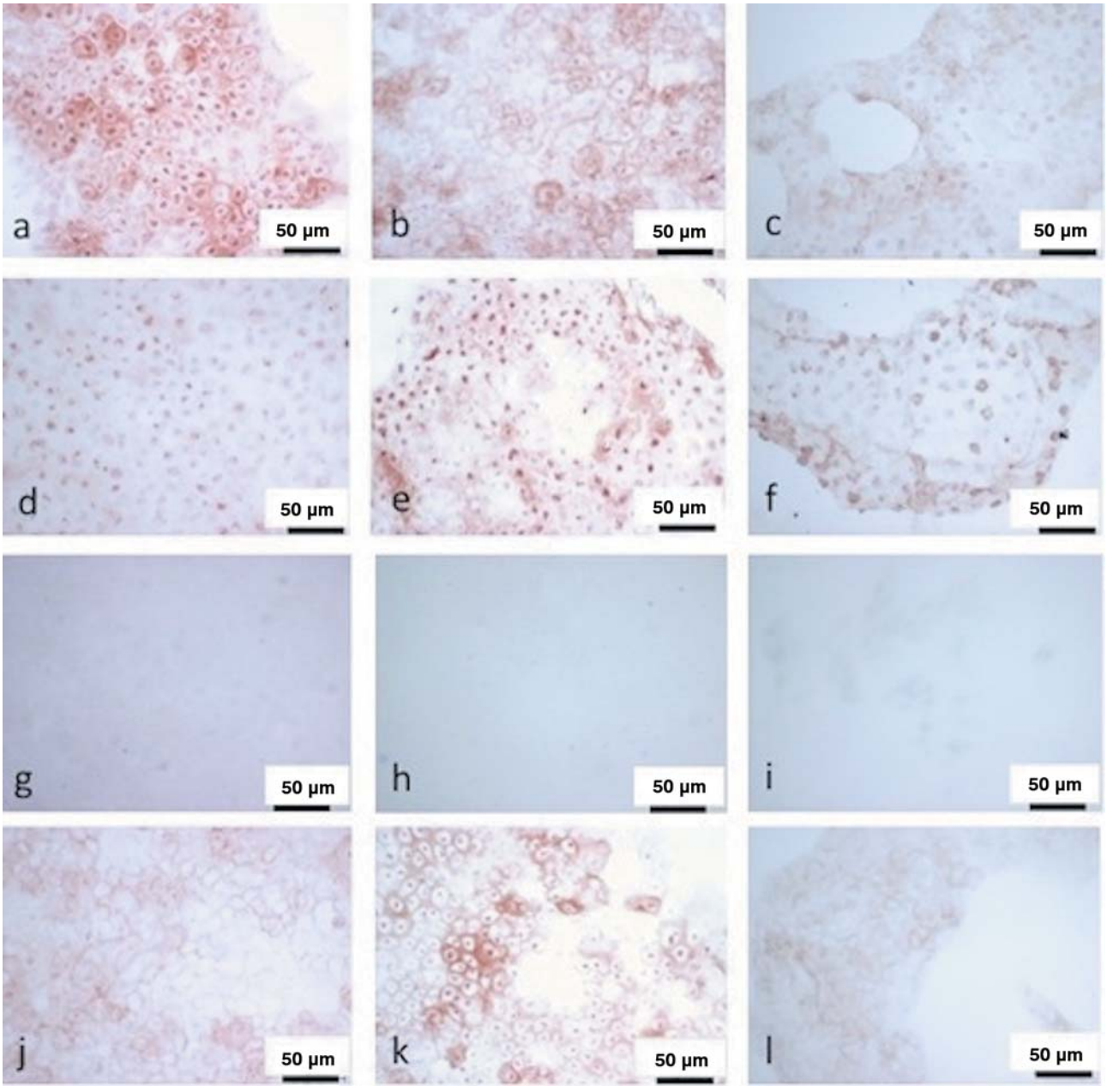

Figure 1. *Negative control, demonstration of growth behaviour and declining immunohistochemical reactivity for E-cadherin (a-c), matrix metalloproteinase 9 (MMP9) (d-f), cyclin D1 (g-i) and β-catenin (j-l) in CERV196 cells after incubation with lapatinib (2 µg/ml) for 24 and 96 h.*

used. The sections underwent counterstaining with Harris hematoxylin for 30 s, followed by dehydration in graded ethanol and coverslipping. The rates of E-cadherin, vimentin, MMP9, cyclin D1 and β-catenin expression demonstrated immunohistochemically were determined. The immunopositivity of the cells was analyzed using an Axio Visio Scan Scope scanning system (microscope Axiophot, and Axio Visio Scan Scope 4.8.2 software; Zeiss, Oberkochen, Germany). The staining intensity was evaluated as follows: strong reactivity, >80% of the cells stained positively; moderate reactivity, 50-80% of the cells stained positively); and no positive cells.

cDNA synthesis and quantitative real-time polymerase chain reaction (PCR). Expression of glycerinaldehyd 3-phosphate dehydrogenase (GAPDH), E-cadherin, vimentin, cyclin D1 and β-catenin mRNA was analyzed by quantitative real-time PCR. Total RNA was isolated with the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer´s instructions and stored at −80˚C. An aliquot of 0.5 µg total RNA was treated with 1 unit DNAse (Fermentas, St. Leon-Rot, Germany) for 30 min at 37˚C. Reverse transcription of RNA (0.5 µg) was performed with oligo (dT) 18-26 primer and 200 units of SUPERSCRIPT II (Invitrogen, Karlsruhe, Germany) and 24 units of

Ribo Lock™ RNAse inhibitors (Fermentas) for 1 h at 42˚C. The cDNA was used for PCR analysis. Primer sequences and annealing temperature are presented in Table I. The QuantiTect/primer assays were purchased from QIAGEN GmbH (Hilden, Germany). cDNAs were amplified with Taq Man fast advanced SYBR® Green QRT-PCR Master Mix (Stratagene-Agilent Technologies, Waldbronn, Germany). The following cycle profile was used: one cycle at 95˚C for 2 s followed by 50 cycles at 95˚C for 3 s, and 60˚C for 30 s. Amplification was performed using the M×3005P™ QPCR System (Stratagene-Agilent Technologies, Waldbronn, Germany). For relative quantification, a standard curve was generated for every individual run. The data were analyzed using the relative standard curve method. To check the specificity of the amplification products, the dissociation-curve method was used. Data were analyzed using M×3005P™ analysis software (Stratagene-Agilent Technologies). The threshold method was used for quantification of the mRNA level. Cycle threshold change (ΔCT) values were calculated based on the internal standard *GAPDH* (35). Results are expressed as percentages of the non-stimulated controls ($2^{-\Delta\Delta Ct}$ data analysis method). Experiments were performed in triplicate.

Statistical analysis. Statistical analysis was performed in cooperation with Professor Dr. C. Weiss, Institute of Biomathematics, Faculty of Medicine, Mannheim, Germany. All data were subjected to the means procedure. A *p*-value of 0.05 or less was considered statistically significant. The differences ELISA in E-cadherin, vimentin, MMP9, and cyclin D1 expression between test cultures and control cultures were analyzed using Dunnett´s test and the Kruskal-Wallis one-way analysis of varience by ranks, which are part of the general linear model procedures.

Table II. *Grading of immunostaining for E-cadherin, vimentin, MMP9, cyclin D1 and β-catenin in p16-positive CERV196 cells after incubation with 2 μg/ml gefitinib and with 2 μg/ml lapatinib.*

	Immunostaining at		
	5 h	24 h	96 h
Control group			
E-Cadherin	++–+++	+–++	++
Vimentin	0	0	0
MMP9	+–++	+–++	+–++
Cyclin D1	0	0	0
β-Catenin	++	++–+++	++
Gefitinib			
E-Cadherin	+–++	++	+
Vimentin	0	0	0
MMP9	+–++	+	+
Cyclin D1	0	0	+
β-Catenin	++	+	+
Lapatinib			
E-Cadherin	++	++	+–++
Vimentin	0	0	0
MMP9	++	+–++	+–++
Cyclin D1	0	0	0
β-Catenin	++	+–++	+–++

0, No positive cells; +, weak immunostaining; ++, moderate immunostaining; +++, strong immunostaining.

Results

Immunocytochemistry of E-cadherin, vimentin, MMP9, cyclin D1 and β-catenin in CERV196. We established several cell lines in our laboratory before starting stimulation. The cells were assessed for morphological changes, and evaluated for the expression of key EMT markers. The p16-positive SCC cell line CERV196 had the most pronounced epithelial phenotype (scattering$^-$, E-cadherin$^+$, vimentin$^-$). Immunocytochemical studies showed that in negative controls, CERV196 cells expressed a moderate level of membranous β-catenin and a low level of nuclear MMP9. A strong level of membranous and a low level of nuclear E-cadherin was detected in control CERV196 cells. No positivity for vimentin and cyclin D1 was detected in the control (Figure 1 and Table II). After incubation with gefitinib, we detected down-regulation of E-cadherin, particularly after 96 h of gefitinib treatment. Less expression of E-cadherin was also found after 96 h of lapatinib treatment. Gefitinib induced a decrease of MMP9, particularly after 24 and 96 h incubation with gefitinib. After 24 h incubation with lapatinib, there were quantitative differences in MMP9 expression in p16-positive CERV196 cells (Figure 1 d-f). No expression of cyclin D1 and vimentin after treatment with lapatinib and gefitinib was apparent. In contrast to this finding, in cells treated for 24 h with lapatinib, there was an increase of membranous of β-catenin and its nuclear accumulation. After 96 h of gefitinib treatment, we detected a reduction of β-catenin staining (Figure 1 j-l). Additionally, gefitinib induced reduction of β-catenin (Table II).

ELISA of E-cadherin, vimentin, MMP9, cyclin D1 and β-catenin expression in CERV196 cells. In p16-positive CERV196 cells, a strong expression of E-cadherin was determined in the control group (5 h=4,315 pg/mL). P16-positive CERV196 cells showed a consistent trend towards an incubation time-dependent decrease of E-cadherin after incubation with lapatinib, especially after 96 h of incubation. We identified a statistically significant increase of E-cadherin expression after 96-h treatment with gefitinib at 2 μg/ml (7,571.99 pg/ml, $p<0.0001$). Statistically significantly increased E-cadherin expression compared to the control was also found after 5 h of incubation with lapatinib at 2 μg/ml (5,465.28 pg/ml, $p=0.0447$) (Table III).

We found a low level of vimentin expression in untreated p16-positive CERV196 cells (5.62 μg/ml). Vimentin expression increased after treatment with gefitinib, especially after 96 h. The level of vimentin expression did not change significantly from 5 h to 96 h after incubation with lapatinib (Table III). In untreated p16-positive CERV196 cells, MMP9 expression increased in a time-dependent manner. Additionally, there was significant down-regulation of MMP9

Table III. *Enzyme-linked immunosorbent assay for MMP9, cyclin D1, β-catenin, E-cadherin and vimentin expression in p16-positive CERV196 cells after incubation with lapatinib and gefitinib.*

	Mean expression (*p*-Value)					
Incubation time (h)	Control	±SD	Lapatinib (2 μg/ml)	±SD	Gefitinib (2 μg/ml)	±SD
MMP9, pg/ml						
5	262.11	7.811	9.89 (<0.0001)	2.659	9.02 (<0.0001)	7.735
24	1,225.38	60.038	7.48 (<0.0001)	34.02	9.13 (<0.0001)	14.447
96	1,687.88	28.72	10.01 (<0.0001)	0.85	10.04 (<0.0001)	18.032
Cyclin D1, absorbance 450 nm						
5	3.41	0.059	0.71 (0.0495)	0.107	0.72 (0.0495)	0.085
24	3.26	0.178	0.39 (0.0495)	0.249	0.49 (0.0495)	0.017
96	3.5	0.064	0.27 (0.0369)	0.046	0.33 (0.0339)	0.064
β-Catenin, pg/ml						
5	34,940.67	2,079.382	10,626 (<0.001)	1,878.945	11,414 (<0.001)	1,694.983
24	27,977.33	2,694.199	6,691.67 (0.166)	4,620.862	10,852.33 (0.0405)	1,327.045
96	34,749	2,088.372	4,007.33 (<0.001)	4,617.068	17,761.67 (<0.0001)	2,402.354
E-Cadherin, pg/ml						
5	4,315	360.263	5,465.28 (0.0447)	564.695	5,147.39 (0.1300)	485.254
24	3,377.52	1,093.095	4,907.69 (0.5945)	259.686	3,994.3 (0.9093)	3,406.694
96	2,755.3	262.912	2,720.05 (0.9901)	156.779	7,571.99 (<0.0001)	156.779
Vimentin, μg/ml						
5	5.62	1.186	5.6 (0.9997)	0.957	4.78 (0.5198)	0.824
24	7.24	2.258	4.68 (0.1093)	0,723	3.73 (0.0366)	0.307
96	4.58	1.067	4.37 (0.9989)	0.346	11.01 (0.4336)	11.376

Data are mean values and statistical significance compared to the negative control. p-value by Dunnett´s test, n=3.

expression after incubation with gefitinib for 5 h (9.02 pg/ml, $p<0.0001$) to 96 h (10.04 pg/ml, $p<0.001$). MMP9 expression also statistically significantly differed from that of the control after 5 h (9.89 pg/ml, $p<0.001$), 24 h (7.48 pg/ml, $p<0.001$) and 96 h (10.01 pg/ml, $p>0.001$) incubation with lapatinib.

In summary, we found a lower expression level of cyclin D1 in the control group of p16-positive CERV196 cells (Table III). A strong statistically significant decrease of cyclin D1 expression was found in p16-positive CERV196 cells after 5 (0.71, $p=0.0495$), 24 (0.39, $p=0.0495$) and 96 h (0.27, $p=0.0369$) incubation with lapatinib. Incubation with gefitinib had a significant impact on cyclin D1 repression after 5 (0.72, $p=0.0495$), 24 (0.49, $p=0.0495$) and 96 h (0.33, $p=0.0339$) incubation. The maximal statistically significant repression of cyclin D1 was measured after 96 h of incubation with lapatinib (0.27, $p=0.0369$). In untreated p16-positive CERV196 cells, we revealed a high level of β-catenin expression, especially after 5 and 96 h. We identified a statistically significant decrease of β-catenin expression after incubation with gefitinib in a time-dependent manner ($p<0.041$). Additionally, after incubation with lapatinib, β-catenin expression was also significantly down-regulated time-dependently, but compared to incubation with gefitinib, stronger repression after lapatinib treatment was found, especially after 96 h (4,007.33, $p<0.001$) (Table III).

mRNA expression of E-cadherin, vimentin, cyclin D1 and MMP9 in CERV196 cells. To obtain information on E-cadherin, vimentin, cyclin D1 and *MMP9* expression during lapatinib and gefitinib treatment, mRNA was analyzed by quantitative real-time PCR after 5, 24 and 96 h lapatinib and gefitinib treatment of p16-positive CERV196 cells. Results are presented in Figure 2. We detected a time-dependent up-regulation of E-cadherin mRNA expression after lapatinib and gefitinib treatment. The strongest increase of mRNA expression of E-cadherin was found after 5 h incubation with lapatinib. In contrast, it was observed that lapatinib and gefitinib caused a time-dependent down-regulation of mRNA for vimentin and cyclin D1. The most prominent effect was seen for cyclin D1 mRNA after 96 h incubation with gefitinib (Figure 2).

Discussion

A sub-group of HNSCC is caused by infection with high-risk types of HPV, especially cancer of the oropharynx (36). In 2007, HPV, particularly HPV 16, was recognized by the International Agency for Research on Cancer as a risk factor for OPSCC (5). In many studies, it was described that patients with HPV-positive OPSCC had a much better response to chemoradiotherapy than patients with HPV-

negative OPSCC (5). It is also described that patients with HPV-positive SCC are younger and have a better prognosis than patients with HPV-negative SCC (37). Therefore, most patients with HPV-positive OPSCC may not require the intensive chemoradiotherapy regimen administered today to most patients and which is associated with serious side-effects and increased costs for society. As a result, it is essential to identify which patients should and which should not receive intensified therapy. Consequently, both HPV prevention and individualized therapy are important issues for this type of cancer. To improve individualized treatment, more molecular and immunological knowledge is necessary to determine the efficacy of different treatments according to HPV status and to define predictive biomarkers for identifying more appropriate treatment.

The cyclin D1 protein has been extensively examined in cancer development and is seen as an important regulator of the G_1-to-S-phase transition in the cell cycle and might be shown to mediate DNA repair (31). In an other meta-analysis, cyclin D1 overexpression was significantly associated with poor overall survival in patients with colorectal cancer (38). Contrastingly, Ren and colleagues in their meta-analysis showed an immunhistochemical up-regulation of cyclin D1 in bladder cancer in association with a good progression-free survival (39). Two viral oncoproteins of high-risk HPV types, E6 and E7, support tumor progression by inactivating the *p53* and retinoblastoma (*Rb*) tumor-suppressor gene products (37). Mutations of *Rb* are rare, but expression of upstream regulators of Rb function, such as cyclin D1, is commonly altered in HNSCC (40).

Aberrant EGFR signaling can often be observed in HNSCC during the oncogenic process. Overexpression of EGFR correlates with tumor spread and poor prognosis (41). Interestingly, it is suggested that EGFR alterations, defined as high gene copy numbers and overexpression, are inversely correlated to HPV-positive SCC (42, 43). However, a subgroup of HPV-positive tumors expressed a high level of EGFR (44, 45). Will this subgroup of patients benefit from drug targeting? The molecular target therapies specifically directed to EGFR might improve the outcomes and reduce toxicities (7). Identification of the tumor cell phenotype responsible for malignant potential is therefore of high clinical interest. The aim of this study was to investigate the expression pattern of vimentin, E-cadherin, β-catenin, MMP9 and cyclin D1 quantitatively and qualitatively in HPV-positive SCC cells before and after incubation with lapatinib and gefitinib. Our data showed stronger expression of E-cadherin, down-regulation of vimentin expression in untreated p16-positive CERV196 cells. After incubation with lapatinib, and gefitinib, we demonstrated an up-regulation of E-cadherin and down-regulation of vimentin, especially after 24 h incubation. Less up-regulation of vimentin protein was detected after 96 h of gefitinib treatment.

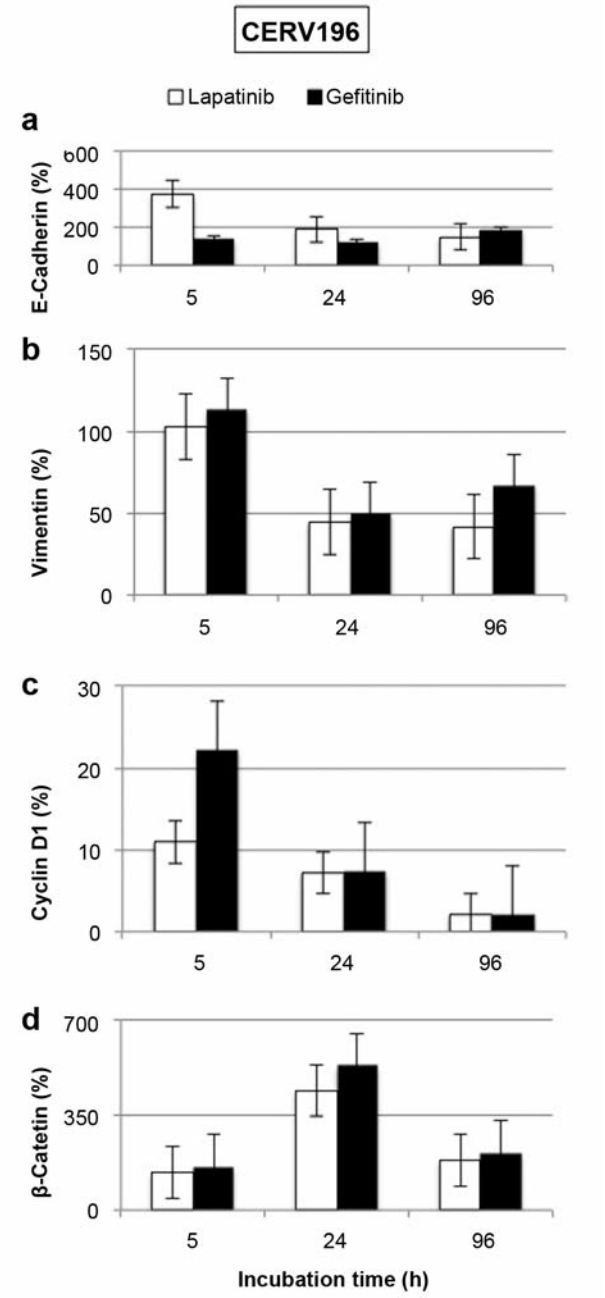

Figure 2. *Graphical presentation of changes in mRNA expression of E-cadherin (a), vimentin (b), cyclin D1 (c), and β-catenin (d) in p16-positive CERV196 cells after incubation with lapatinib (2 μg/ml) and gefitinib (2 μg/ml). Experiments were performed in triplicate; columns show the mean value and standard error of the mean as a percentage of the untreated control.*

Maseki and colleagues found that the EMT in gefitinib-resistant cells of HNSCC is mediated by the down-regulation of EGFR and compensatory activation of downstream protein kinase B/glycogen synthase kinase-beta-3 pathway (46).

Despite an initial response to EGFR tyrosine kinase inhibitors (TKI) in patients with EGFR-mutant cancer, most eventually show resistance and this results in treatment failure. EMT is associated with drug resistance and act as a determinant of insensitivity to EGFR-TKI (47).

The EMT of tumor cells is associated with nuclear accumulation of the transcriptional activator β-catenin (28). Interestingly, we quantitatively found less up-regulation of mRNA expression of β-catenin, particularly after 24 h treatment with gefitinib. After prolonged treatment with gefitinib and with lapatinib, a decrease in mRNA expression of β-catenin was detected. In contrast to this, the protein expression of β-catenin was statistically significantly time-dependently down-regulated. We also found an increase in nuclear and membranous staining of β-catenin after 24 h treatment with gefitinib. Interestingly, aberrant nuclear expression of β-catenin can also confer EMT and stem cell formation of tumor cells, thereby driving malignant tumor progression (28).

In quantitative real-time PCR, there was a remarkable increase of E-cadherin gene expression and a decrease of vimentin gene expression, as well as of cyclin D1 in a time-dependent manner, supporting the hypothesis of preventing EMT by EGFR inhibition. EGF/ transforming growth factor beta 1 (TGFβ1)-driven EMT in OSCC indicated a most extensive up-regulation of *MMP2* and *MMP9* (48). Additionally, we noted strong significant down-regulation of MMP9 expression after gefitinib and lapatinib treatment in a time-dependent manner. The EMT is a complex process mainly characterized by decreasing expression of epithelial markers (*e.g.* E-cadherin), and up-regulation of mesenchymal markers (*e.g.* vimentin). La Monica and colleagues studying EGFR-mutant non-small cell lung cancer cells demonstrated that continuous exposure to gefitinib prevented the EMT, with increased E-cadherin expression and down-regulation of N-cadherin and vimentin (49). This further supports our hypothesis that gefitinib is capable of inhibiting phenotypic changes associated with the EMT. Interestingly, we found high expression of cyclin D1 in the control group of p16-positive CERV196 cell. Li and colleagues demonstrated down-regulation of cyclin D1 expression in the presence of HPV in SCC of the tonsils. In recent studies, overexpression of cyclin D1 was associated with poor clinicopathological outcome and survival in OSCC and colorectal cancer (34, 38, 50). Cyclin D1 and MMP9 may be prognostic markers in clinical practice. After incubation with lapatinib and gefitinib, we found a strong significant repression of cyclin D1 and MMP9 in p16-positive SCC cells. O'Neill and colleagues also showed a strong down-regulation of cyclin D1 in lapatinib-sensitive breast cancer cells following 12-h treatment (51). Cyclin D1 and MMP9 expression profiles may be suggested as an early measure of sensitivity and the level of response to gefitinib and lapatinib. Several factors during EMT play significant roles in the development of drug resistance, but these are dependent on the metastatic grade of the tumor, which is defined as the level of de-differentiation and degree of EMT. The mechanistic role of EMT markers dependent on cell-cell contact that have been associated with HNSCC cells should be characterized more clearly in order to develop new anti-HNSCC therapies that prevent EMT progression. In our study, the cells were grown to sub-confluence before starting treatments. Cell-cell contact is also suggested to play an important role in regulation of EMT. In TGFβ1-induced transdifferentiation of kidney cells into α-smooth muscle actin-expressing myofibroblasts, cell-cell junctions are essential (52).

In summary, we showed a strong significant cyclin D1 and MMP9 repression induced by lapatinib and gefitinib in a p16-positive CERV196 squamous cell line. Despite this, overexpression and activity of MMP9 was demonstrated to have prognostic significance in the progression of SCC. Furthermore, it was proven how MMPs are linked to tumor progression and may improve therapeutic strategies. The cyclin D1 and MMP9 expression profile may be suggested to represent an early measure of sensitivity and level of response to lapatinib and gefitinib. The presented cell culture model is, therefore, well-suited to further study the epigenetic regulation of molecular targeted-therapy by anti-EGFR treatment and in preventing EMT in p16-positive SCC.

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