Incomplete Epithelial–Mesenchymal Transition in p16-positive Squamous Cell Carcinoma Cells Correlates with β-Catenin Expression

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Abstract. *Background: The epithelial–mesenchymal transition (EMT) is suggested to be a crucial factor for the development of an invasive and metastatic cell phenotype, which is characterized by down-regulation of epithelial adhesive proteins (e.g. E-cadherin) and induction of mesenchymal proteins (e.g. vimentin). Therefore, there is a great clinical interest to specify this phenotype. Different growth factors induce EMT, such as epithelial growth factor (EGF) and transforming growth factor beta 1 (TGFβ1). The role of EMT in human papilloma virus (HPV)-positive squamous cell carcinoma (SCC) is still not understood. The aim of this study was to investigate the expression pattern in p16-positive and -negative SCC cells of vimentin, β-catenin and E-cadherin after stimulation with growth factors. Materials and Methods: We incubated the p16-positive CERV196 and p16-negative HNSCC22B SCC cell lines with EGF and EGF/TGFβ1 (10 ng/ml) and detected E-cadherin, vimentin and β-catenin by immunocytochemistry and enzyme-linked immunosorbent assay after 5, 24 and 96 h. Results: We found a low expression of vimentin in all studied tumor cell lines. The negative control of HNSCC22B cells showed a higher intrinsic level of membranous E-cadherin and β-catenin. We found statistically significant EGF/TGFβ1-induced expression of vimentin dependent on incubation time in p16-negative HNSCC22B cells. Particularly in the presence of EGF, we detected an increase of β-catenin and vimentin expression in p16-positive SCC tumor cell lines in addition to induced cell scattering and unexpected expression of E-cadherin. Conclusion: In conclusion, E-cadherin, β-catenin and vimentin expression are important features to characterize EMT-like events. We were able to show incomplete EGF-induced EMT with β-catenin expression in p16-positive SCC. Extended studies are required to investigate the mechanistic role of EMT markers, especially in p16-positive SCC, in order to develop new anti-SCC therapies to block EMT progression.*

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Head and neck squamous cell carcinoma (HNSCC) is the sixth leading cancer in incidence worldwide. More than 90% of these tumors are squamous cell carcinomas (SCC), representing 5-10% of all new cancer cases in the U.S. and Europe (1). The main risk factors for the development of HNSCC are alcohol and tobacco consumption. Clinical and pathological evidence suggest that viral oncogenic human papillomavirus (HPV) infection is another crucial aetiological factor (2). The epidemiological, genetic, molecular and clinical profile of HPV-associated HNSCC cells seems to differ from tobacco- and alcohol-induced HNSCC (non-HPV). HPV-positive oropharyngeal carcinomas are characterized by immundeficiency or immunosuppression in patients, a young age of onset, an increased number of intimate partners with a history of genital warts, and a strong association with sexual behaviour. Interestingly, both genders have the same risk of developing HPV-positive HNSCC (3). HPV prevalence in oropharyngeal SCCs was significantly higher in North America (47%) compared with Europe (28.2%) (4). The viral aetiology is linked to specific subtypes of HPV called high-risk types, such as HPV-16 and HPV-18, especially those occurring in SCC of the tonsils and the tongue base (5). The process of HPV-associated malignant transformation depends on the presence of the viral oncogenes *E6* and *E7*, which

inhibit, among others, two tumor-suppressor proteins: p53 and retinoblastoma protein (6).

Loss of cell adhesion and transformation of epithelial cells into a mesenchymal phenotype is described as epithelial–mesenchymal transition (EMT). EMT is suggested to be crucial for the development of a metastatic carcinoma cell phenotype and the facilitation of invasion. The process describes the development of non-motile, polarized epithelial cells into fibroblastoid, mesenchymal cells with a high ability to migrate. In p-16 negative oral squamous cell carcinoma (OSCC) the EMT is characterized by down-regulation of epithelial-specific adhesion proteins (*e.g.* tight and adherent junction proteins such as E-cadherin, cytokeratin, claudin, desmoplakin) and induction of mesenchymal proteins, such as vimentin, N-cadherin and fibronectin, as well as development of migratory attributes (*e.g.* cell scattering) (7-9). EMT can be induced by various growth factors, such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), transforming growth factor beta 1 (TGFβ1) and epithelial growth factor (EGF) (10-14).

One of the best-described cytokine inducers of EMT is TGFβ1, which can utilize various programs to promote metastasis of cancer cells through its effects on the tumor microenvironment, enhanced invasive properties, and inhibition of immune cell function (15). Different studies indicate an up-regulation of TGFβ1 in early stages of tumorigenesis to promote cancer suppression, while other studies detect an up-regulation of TGFβ1 at the latest stage of tumorigenesis to support tumor progression, metastasis and EMT (16, 17). The TGFβ1-mediated EMT was detected in various developmental processes and human cancer, including OSCC, bronchial, colonic, and breast cancer (18-21). In TGFβ1-associated EMT, it has been suggested that the transcription factors SNAIL and SLUG, which promote EMT by increasing expression of E-cadherin, are associated with aggressive tumor behaviour and poor clinical outcome in patients with cancer (22). EGF is also characterized as an inducer of EMT in different kinds of human cancer, such as esophageal cancer cells, OSCC cells, and ovarian cancer (10, 23-25). Stimulation with EGF promoted invasion, migration, and motility of SCC cells and they underwent EMT, with down-regulation of E-cadherin and up-regulation of N-Cadherin and vimentin (9). EGF/TGFβ1 co-stimulation induced EMT-like phenotype transition in OSCC cells, which is associated with a down-regulation of E-cadherin, up-regulation of laminin-332 (especially of the laminin γ2 chain), vimentin, matrix metalloproteinase-2 (MMP2) and MMP9 (18). The EMT process in p16-positive SCC cells is poorly-described. Rodrigues and colleagues have demonstrated that HPV-positive vulvar squamous cell carcinoma did not progress through EMT phenomenon, but had better prognosis (26). A study by Wakisaka and colleagues suggested that HPV-positive oropharyngeal squamous cell carcinoma tissues were significantly associated with EMT-induction, progression of lymph node metastasis and better clinical outcome than HPV-negative ones (27). In HPV-positive cervical cancer cells, exogenous EGF stimulation may enhance HPV-related cervical cancer cell proliferation by activating EGFR and cyclin D1, suggesting that the inhibitors of EGFR and cyclin D1 may be effective against cervical cancer cell proliferation (28). However, a more detailed investigation of HPV-associated EMT-induction is needed to clarify the mechanism leading to improve prognosis of HPV-positive SCC.

β-Catenin is a multi-functional protein, suggested to be one of the most important factors for decreasing cell–cell interactions in malignant epithelial cells (5, 29). However, β-catenin plays a crucial role in the development of head and neck cancer *via* a nuclear downstream effector of the canonical WNT-signalling cascade (9). By alteration of the degradation complex or destabilization of cell–cell adhesion and loss of E-cadherin expression, membranous β-catenin is released into the cytoplasm. The high accumulation of β-catenin in the cytoplasm leads to its nuclear translocation and acts as a co-factor of transcriptional regulators. It results in an up-regulation of various target genes such as *SLUG*, *vimentin* and *MMP9*, which are required for dysregulation of cell-cycle promotion, tumor progression, invasion and EMT (5, 8, 9). Interestingly, Rodrigues and colleagues reported that in vulvar squamous cell carcinoma besides loss of β-catenin being related with negativity for HPV infection, no nuclear β-catenin was detected at all, suggesting that other pathways apart from HPV-related WNT activation may be related with EMT-like evens (26).

The identification of tumor cell phenotypes responsible for malignant potential is of high clinical interest. Therefore, to induce the process of EMT, it is necessary to examine the phenotype of p16-positive and p16-negative SCC cells. The aim of the present study is to investigate the expression pattern of vimentin, β-catenin and E-cadherin quantitatively and qualitatively in HPV-negative and p16-positive SCC before and after stimulation with growth factors to evaluate the invasive metastatic cell phenotype and to detect differences between p16-positive and -negative SCC cells.

Materials and Methods

Cell lines and culture. The p16-negative HNSCC22B (UMSCC22B) was obtained from Dr. T. E. Carey (University of Michigan, Ann Arbor, MI, USA). They descended from human metastatic SCC of the *sinus piriformis* (hypopharynx). The p16-positive cell line CERV196 (CLS, Eppelheim, Germany) originated from a poorly-differentiated xenotransplanted cervical carcinoma MRI-H-196. Cell cultures were 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
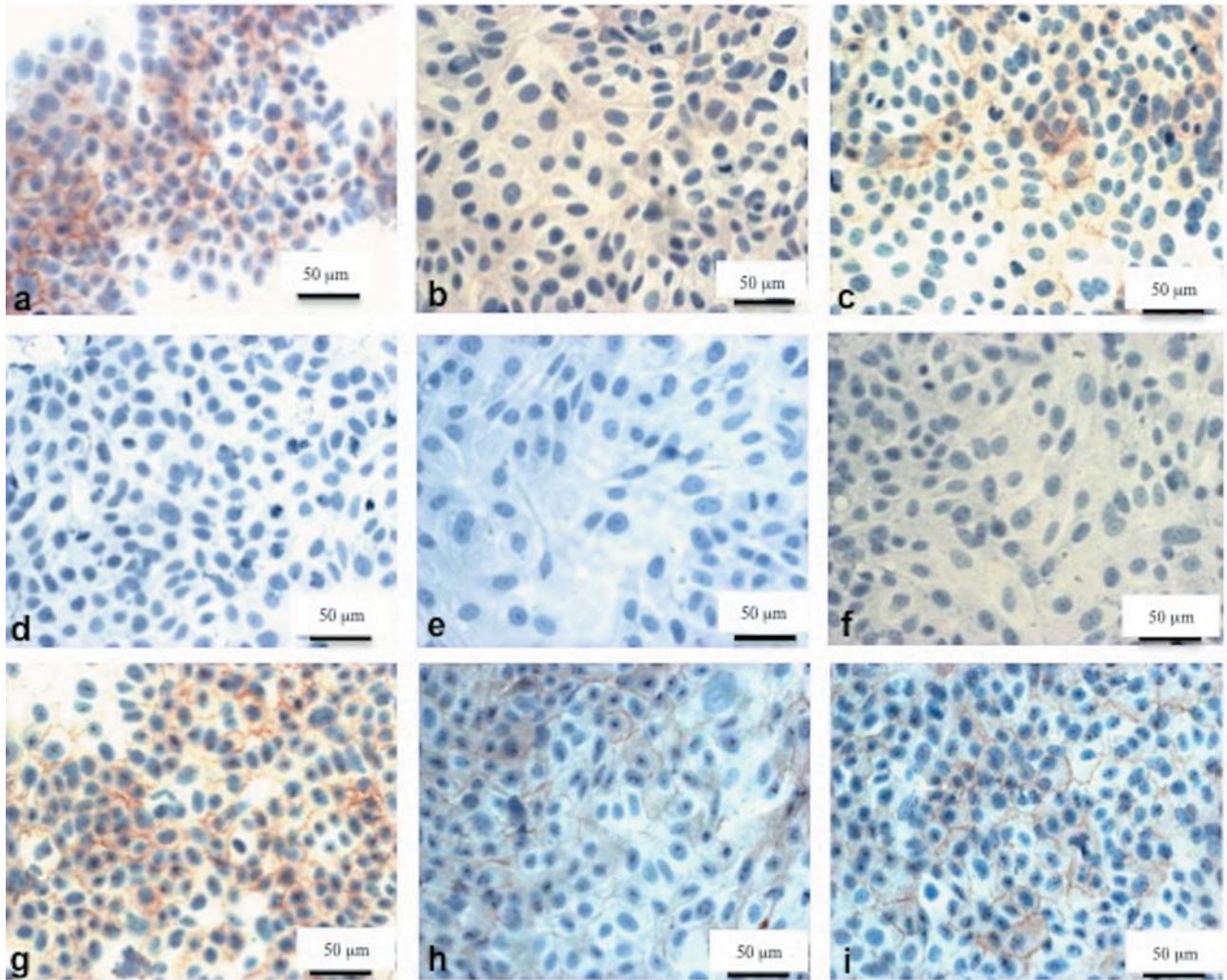
Figure 1. *Demonstration of growth behaviour for CERV196 cells and their protein expression of E-cadherin (a-c), vimentin (d-f) and β-catenin (g-i) in the negative control cells and after incubation with epidermal growth factor (EGF) and EGF/ transforming growth factor β1 (TGFβ1) (10 ng/ml) for 96 h (a-c). EGF causes cell scattering, down-regulation of membranous E-cadherin (b) and up-regulation of nuclear β-catenin (h) (bars: 50 μm).*

cells per well were seeded in eight-well cell culture slides (BD Biosciences, Heidelberg, Germany). When confluent, cells were starved using 0% FCS/DMEM for 5 h and then incubated for 5, 24 and 96 h with 10 ng/ml EGF (human, recombinant) or 10 ng/ml TGFβ1/EGF co-stimulation (TGFβ1: human, recombinant; EGF: human, recombinant) (Reprotech, Manufacturer of Quality Cytokine Products, Rocky Hill, NJ, USA) in 0% FCS/MEM. The stimulation was repeated every 24 h. Selection of the different drug concentrations and stimulation durations were defined after performing the alamarBlue (AbD Serotec, Oxford, UK) cell proliferation assay. After incubation, the supernatants were collected together in sterile tubes and stored at −20°C until further analysis.

Enzyme-linked immunosorbet assay (ELISA). β-Catenin (DuoSet mouse antihuman β-catenin, R&D Systems, Wiesbaden, Germany), E-cadherin (DuoSet mouse antihuman E-cadherin, R&D Systems,

Wiesbaden, Germany), vimentin (Path Scan total vimentin, mouse monoclonal antibody, wells coated, Cell Signaling, Boston, MA, USA) concentrations were determined by the ELISA technique (DMP2F0, R&D Systems, Wiesbaden, Germany). The system utilized a solid-phase monoclonal antibody and an enzyme-linked polyclonal antibody against β-catenin, E-cadherin and vimentin. The specificity of anti-human antibodies used in the ELISA kit were examined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis followed by western blotting. According to the manufacturer's instructions, the DuoSet IC ELISA (R&D Systems, Wiesbaden, Germany), each assay 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 β-catenin, E-cadherin and vimentin are reported as ng/ml and were defined after performing the alamarBlue cell proliferation assay, quantitatively

measuring proliferation of HNSCC tumour cell lines. After 0, 5, 24 and 96 h of incubation with 10 ng/ml EGF and EGF/TGFβ1 co-stimulation, the expression of β-catenin, E-cadherin and vimentin in the supernatants of the incubated cell cultures and untreated cell cultures was determined.

Immunocytochemistry. Immunocytochemical analysis was performed using an antibody directed against β-catenin (monoclonal rabbit antibody, 1:200, Abcam, Cambridge, UK), E-cadherin (monoclonal mouse antibody, 1:50, Abcam, Cambridge, UK) and vimentin (monoclonal mouse antibody, 1:50, Dako, Agilent Technologies Country, Glostrup, Denmark). 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 EGF and EGF/TGFβ1 co-stimulation 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).

The following steps were executed by the Dako TechMate 500 (Dako, Hamburg, Germany) automated staining system: Cells were incubated with a primary antibody solution for 30 min at room temperature using a working solution of antibody to cells of 1:300. The slices 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. For the negative controls, all reagents except the primary antibody were used. The sections underwent counterstaining by Harris haematoxylin for 30 s, followed by dehydration in graded ethanol and coverslipping. The immuno-histochemically demonstrated rates of β-catenin, E-cadherin and vimentin expression were determined.

Statistical analysis. Statistical analysis was performed in cooperation with PD 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 in β-catenin, E-cadherin and vimentin expression between incubated cultures and control cultures were analysed using Dunnett's test and a *t*-test, which are part of the general linear model procedures.

Results

Immunocytochemistry. We established several cell lines in our laboratory before starting cell stimulations. However, the p16-negative SCC cell line HNSCC22B (UMSCC22B) and p-16 positive SCC cell line CERV196 had the most pronounced epithelial phenotype (E-cadherin[+], N-cadherin[−], vimentin[−]) (data not shown) and were, therefore, ideal for EMT analysis.

Immuncytochemical studies showed that in negative controls, all tumor cell lines irrespective of HPV status expressed a similarly moderate level of membranous E-cadherin (Figure 1a) and β-catenin (Figure 1g). In the p16-positive CERV196 cell line, we detected a loss of membranous E-cadherin and a disaggregation of cells (scattering effect), especially after a prolonged incubation time with EGF stimulation (Figure 1b). In unstimulated cell lines, we detected no immuncytochemical positivity for vimentin. After incubation with EGF or EGF/TGFβ1, we also found scattering effect of cells, especially after EGF treatment, but no vimentin deposition (Figure 1d-f). However, stimulation with EGF and EGF/TGFβ1 co-stimulated cells showed qualitative differences in β-catenin deposition (Figure 1g-i). Whereas unstimulated cells preferentially exhibited membranous staining, EGF or EGF/TGFβ1 co-stimulated cells revealed a loss of membranous deposition and an increase in nuclear accumulation (Figure 1h-i). In comparison to unstimulated cells, EGF induced an increase and re-organization of nuclear deposited β-catenin in p16-positive SCC cell line (Figure 1h).

ELISA of total protein expression in HNSCC22B and CERV196. To analyze the effect of EGF and EGF/TGFβ1 on the HPV-negative HNSCC cell line and the HPV-positive cell line CERV196, we increased the stimulation time of 10 ng/ml of EGF and 10 ng/ml EGF/TGFβ1. In order to determine protein expression in the supernatant of the cell line, ELISA analyses were carried out 5, 24 and 96 h after the start of incubation. Compared to HPV-negative tumor cell lines, negative controls of CERV196 cells exhibited higher total protein expression levels. In contrast, we found high protein expression in CERV196 cells after EGF treatment, particularly after longer incubation periods (up to 96 h). Additionally, we also detected an increase of protein expression in HPV-negative cell lines, especially after 96 h of EGF stimulation, but the increase was less than that of the p16-positive SCC cells (Figure 2).

ELISA of E-cadherin and vimentin expression in HNSCC22B and CERV196 cells. In contrast to the low level of vimentin expression, a greater expression of E-cadherin was found in p16-positive (negative control=68.93 pg/ml) and p16–negative SCC cell lines (negative control=78.02 pg/ml). Interestingly, we found an increase of E-cadherin expression after prolonged treatment time with EGF and EGF/TGFβ1 in HNSCC22B and CERV196. After prolonged treatment with EGF/TGFβ1, we detected the strongest increase of E-cadherin in p16-positive SCC cell line in contrast to the results of the immunocytochemistry. In summary, we detected less expression of vimentin in unstimulated p16-negative and -positive cell lines. A significant time-dependent effect was
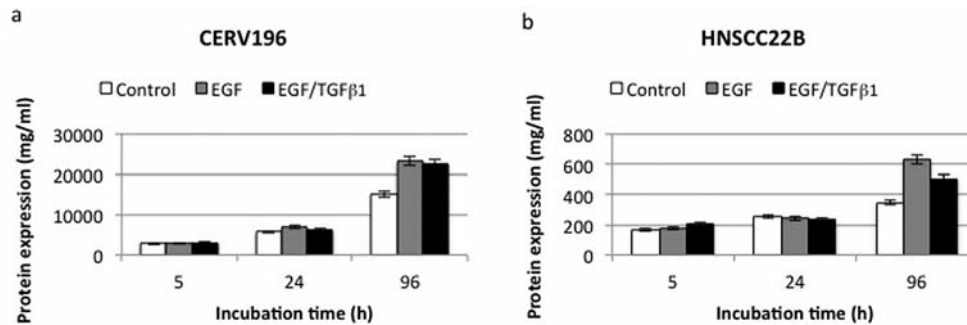
Figure 2. *Total protein expression in HNSCC22B (a) and CERV196 (b) cells after incubation with EGF (10 ng/ml) and EGF/TGFβ1 (10 ng/ml) for 5, 24, and 96 h.*

found in increased expression of vimentin in HNSCC22B cells when exposed to EGF/TGFβ1 co-stimulation (10 ng/ml) after 96 h (0.130 ng/ml, *p*<0.0100). In p16-positive SCC cells, the maximal expression of vimentin was measured after 24 h of EGF/TGFβ1 treatment (0.12 ng/ml, *p*=0.1159) (Figure 3) (Table II).

ELISA of β-catenin expression in HNSCC22B and CERV196 cells. Strong expression of β-catenin was detected in both SCC cell lines independently of HPV status. CERV196 and HNSCC22B cells showed a consistent trend towards an incubation time-dependent increase of β-catenin expression after incubation with EGF and EGF/TGFβ1. In HNSCC22B cells, we showed statistically significant expression after EGF/TGFβ1 co-stimulation in the 5 h (3,956.34 ng/ml, *p*=0.0349) to 24 h (7,539.83 ng/ml, *p*=0.0702) timeframe. We also found a statistically significant impact on β-catenin expression in HNSCC22B cells after 96 h (20,485.27 ng/ml *p*=0.0359) of EGF/TGFβ1 co-stimulation. Interestingly, we detected a statistically significant impact on β-catenin expression in p16-positive SCC cells after 24 h (6,925.89 ng/ml, *p*=0.0508) and 96 h of EGF (2,3301.3 ng/ml, *p*<0.0001), but we also found a significant time-dependent increase of β-catenin levels in p16-positive SCC cells when exposed to EGF/TGFβ1 after 96 h (22,461.9, *p*<0.0001) (Figure 3) (Table I).

Discussion

HNSCC is the sixth most common cancer in incidence worldwide. Presently, the identified risk factors are tobacco and alcohol consumption, which seem to have a synergistic effect. In addition, a subgroup of HNSCC is caused by infection with high-risk types of HPV, especially cancer of the oropharynx (30). Poor survival can be ascribed to the high frequency of local invasion, cervical lymph node dissemination, distant metastasis, and second primary cancers (31). However, HNSCC has a high propensity to develop local recurrence after treatment (32). To improve the poor

prognosis for patients with these neoplasias, a combination of chemo- and radiotherapy was elaborated for advanced stages of HNSCC stages. A concomitant treatment of chemoradiotherapy enhanced the overall and five-year survival rate of patients with advanced stages, and even the locoregional control rate improved (5). To further improve the survival rate, particularly for unresectable HNSCC, innovative strategies and targeted-therapies have to be analyzed (33). However, HNSCC arises from the accumulation of epigenetic and genetic events, including the ability to resist apoptosis, insensitivity to anti-growth signals, abnormalities in cancer-associated signaling pathways, limitless replicative potential, self-sufficiency in growth signals, increased angiogenesis, metastasis and invasion (32). Although the tumorigenic pathways and the molecular aetiologies of HNSCC have been studied extensively, there exist only a few diagnostic clinical applications in practice today.

To improve the survival rate, it is important to investigate phenotypic changes in HNSCC and to examine which HNSCC cells destroy the basement membrane, invade and metastasize. There has been an increased interest in the EMT and its part in HNSCC progression. Identification of a tumor cell phenotype responsible for malignant potential in HPV-positive and -negative SCC is of high clinical interest. Richter and Umbreit demonstrated complete EMT in OSCC cells with an epithelial phenotype after prolonged co-stimulation with EGF and TGFβ1. Complete EMT includes down-regulation of E-cadherin, up-regulation of vimentin, and detection of cell scattering effect in cell growth (18).

The armadillo protein β-catenin is the central denominator of WNT signalling. The WNT/β-catenin signaling pathway regulates the expression of a number of genes essential for cell proliferation and differentiation. During the EMT, the adherent junctions destabilize and are cleaved at the plasma membrane and subsequently degraded. Therefore, β-catenin can no longer interact with E-cadherin and is translocated to the nucleus or also degraded (34). After nuclear translocation, β-catenin can lead to an up-regulation of transcription genes,
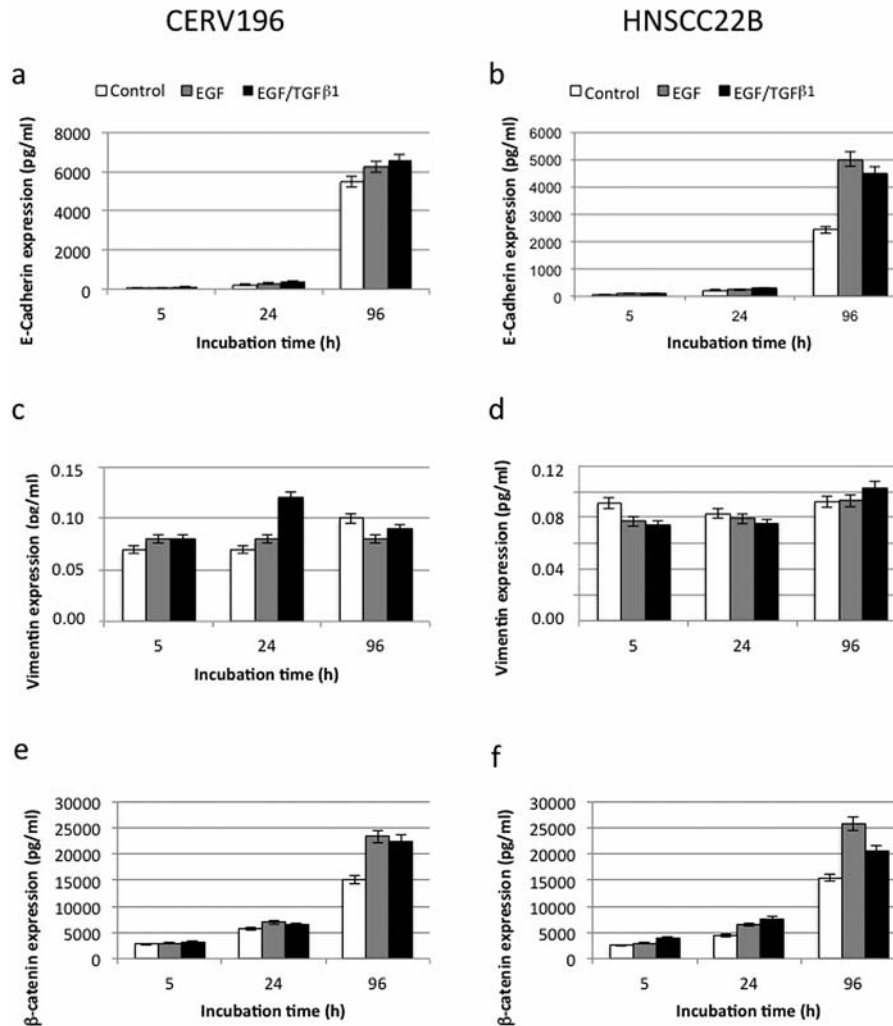
CERV196

HNSCC22B

Figure 3. *Expression of E-cadherin (a, b), vimentin (c, d) and β-catenin (e, f) in HNSCC22B and CERV196 cells after incubation with EGF (10 ng/ml) and EGF/TGFβ1 (10 ng/ml) for 5, 24, and 96 h.*

which promote cytoplasmatic degradation and cancer progression, having implications in EMT progression (35). The metastatic process of epithelial tumors is characterized by alteration in E-cadherin and β-catenin expression and seems to occur at different points in time, depending on the patient's HPV status (26). Our data showed strong expression of β-catenin in both SCC cell lines, independently of HPV status. CERV196 and HNSCC22B cells showed a consistent trend towards an incubation time-dependent increase of β-catenin expression after incubation with EGF and EGF/TGFβ1. However, we only detected a statistically significant impact on the β-catenin expression in p16-positive SCC cells after 24 h (p=0.0508) and 96 h after of EGF stimulation (p<0.0001). It is known that over 90% of HNSCCs demonstrate an overexpression of EGFR at the

protein level (36). Local or regional recurrence and overall reduced survival have been consistent with up-regulation of EGFR expression (37, 38). The aggressive nature of the p16-positive HNSCC cell line is associated with the expression of EGFR (39). Recently, Mirghani and colleagues reported an inverse correlation of HPV status for oropharyngeal SCC and suggested that the role of the EGFR signalling pathway in the majority of HPV-positive oropharyngeal SCC is less important than in its HPV-negative counterparts (40).

An overexpression of E6/E7 in HPV-positive HNSCC cells has been found, and ErbB-2 activation has been shown to down-regulate expressions of E-cadherin and membranous β-catenin. On the other hand, a nuclear translocation of β-catenin with subsequent up-regulation of oncoproteins responsible for tumor progression has been detected (41).

Table I. *Enzyme-linked immunosorbent assay for β-catenin expression in HNSCC22B and CERV196 cells after incubation with EGF and EGF/TGFβ1 for 5, 24, and 96 h. In particuar, β-catenin expression in the p16-positive CERV196 cells was statistically significantly increased after prolonged treatment with EGF. Mean values and statistical correlation compared to the negative control (p-value, ±standard deviation, Dunnet's test, n=3) are shown. Bold text indicates statistically significant differences.*

Mean β-catenin expression, pg/ml (*p*-value compared to the control)						
Incubation time (h)	Control	±SD	EGF (10 ng/ml)	±SD	EGF/TGFβ1 (10 ng/ml)	±SD
HNSCC22B						
5	2,547.88	800.73	2,909.26 (0.6539)	420.36	**3,956.34 (0.0349)**	288.22
24	4,354.37	2,182.16	6,497.27 (0.2163)	1,078.01	**7,539.83 (0.0702)**	908.92
96	15,469.02	6,825.31	**20,485.27 (0.0359)**	74.41	20,485.3 (0.2915)	1,588.66
CERV196						
5	2,723.47	322.28	2,888.44 (0.8234)	88.29	3,082.55 (0.4549)	570.76
24	5,712.67	397.23	**6,925.89 (0.0508)**	784.51	6,357.53 (0.2927)	204.86
96	15, 064.97	260.06	**2,3301.3 (<0.0001)**	616.89	**22,461.9 (<0.0001)**	123.09

Table II. *Enzyme-linked immunosorbent assay for vimentin expression in HNSCC22B and CERV196 cells after incubation with EGF and EGF/TGFβ1 for 5, 24, and 96 h. In particuar, vimentin expression in UMSCC22B cells was statistically significantly increased after 96 h of EGF/TGFβ1 stimulation. In contrast, vimentin expression in CERV196 was statistically significantly reduced after 96 h of stimulation with EGF. Mean values and statistical correlation compared to the negative control (p-value, ±standard deviation, Dunnet's test, n=3) are shown. Bold text indicates statistically significant differences.*

Mean vimentin expression, pg/ml (*p*-value compared to the control)						
Incubation time (h)	Control	±SD	EGF (10 ng/ml)	±SD	EGF/TGFβ1 (10 ng/ml)	±SD
HNSCC22B						
5	0.091	0.003	0.077(0.2784)	0.017	0.074 (0.1804)	0.007
24	0.083	0.015	0.079 (0.7884)	0.003	0.075 (0.4650)	0.004
96	0.092	0.001	0.093 (0.8338)	0.003	**0.103 (0.0100)**	0.005
CERV196						
5	0.07	0.006	0.08 (0.5435)	0.010	0.08 (0.8756)	0.003
24	0.07	0.002	0.08 (0.9663)	0.005	0.12 (0.1159)	0.042
96	0.10	0.004	**0.08 (0.0161)**	0.001	0.09 (0.3350)	0.005

Furthermore, in unstimulated cell lines we detected no positivity for vimentin immuncytochemically. After incubation with EGF or EGF/ TGFβ1, we also showed a scattering effect of cells–especially of p16-positive SCC cells after EGF treatment–but no vimentin deposition (Figure 1d-f). Boyer and colleagues have described the process of EMT as a morphological change from epithelial-like sheets of tumor cells to scattered, fibroblast-like cells competent of invading the basement membrane (42). EMT in cell culture can either be stable in that the mesenchymal phenotype of the cells is sustained by a promotory stimulus, or the EMT can be reversible in that the cells are transformed or experience a mesenchymal–epithelial transition when the stimulus which promoting EMT is stopped. Experimental studies that quantitatively define the

transient and incomplete modification of phenotype features often observed in cultured tumour cells offer insight into the dynamic role EMT might have in the cancer process (22).

The EMT is characterised by the expression of mesenchymal markers such as fibronectin, N-cadherin, and vimentin (22). Vimentin is another protein up-regulated in the aggressive tumor phenotype related to the EMT phenomenon (19). Our results did not indicate a significant association between vimentin expression and HPV infection, but we found an up-regulation of vimentin in p16-negative HNSCC22B cells after 96 h of EGF/TGFβ1 co-stimulation. In contrast, we detected a statistically significant impact on vimentin expression in p16-positive CERV196 cells after 96 h of treatment with EGF.

Acquisition of EMT features has also been associated with chemoresistance acquired after standard chemotherapy (43). However, the process of EMT is suggested to confer resistance to targeted agents. In certain studies, lung cancer cell lines that have undergone EMT, expressing vimentin/fibronectin, demonstrated resistance to the growth inhibitory effects of EGFR kinase inhibition (erlotinib) *in vitro* and in xenografts, as well as to other EGFR inhibitors such as gefitinib and cetuximab (44-47).

Cell contact also seems to play an important role in epithelial–myofibroblast transition regulation. Initial injury of cell–cell junctions is necessary for TGFβ1-induced transdifferentiation of kidney tubular cells into α-smooth muscle actin-expressing myofibroblasts (48). However, injury or absence of intercellular contacts promotes a potentiating effect on the transdifferentiation of epithelial cells to myofibroblasts. In our study, SCC cells were stimulated with EGF or EGF/TGFβ1 after the cells grew confluently. However, the effect of EGF/TGFβ1-induced EMT may be partially inhibited by up-regulating cell contacts. The relationship between growth factor-induced EMT and cell density should be examined in future investigations.

The mechanistic role of the EMT markers dependant on cell–cell contact that have been associated with p16-positive HNSCC should be more clearly defined in order to develop new anti-HNSCC therapies that block EMT progression. In p16-positive, vimentin-down-regulated and E-caderin-up-regulated SCC cells, we showed incomplete EMT with significantly up-regulated expression of β-catenin and cell scattering, but only a small increase in expression of vimentin and E-cadherin expression, particularly after prolonged treatment with EGF. Future studies examining the molecular interaction of EMT and its potential role in the development of p16-positive and -negative SCC are needed for a better understanding of drug-induced EMT and of its resistance to targeted therapy.

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