Unexpected Alteration of β-Catenin and c-KIT Expression by 5-FU and Docetaxel in p16-positive Squamous Cell Carcinoma Compared to HPV-negative HNSCC Cells *In Vitro*

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Abstract. *Background: Head and neck squamous cell carcinoma (HNSCC) is the sixth most common type of cancer worldwide. In several tumour entities, the tyrosine kinase receptor c-KIT is associated with tumour transformation in the epithelial tissue in cases of aberrant expression. Furthermore, tumour development and dissemination are a result of dysregulated cellular pathways such as the WNT/β-catenin pathway. β-Catenin is a multifunctional protein within the canonical WNT signalling pathway and a pivotal factor for the stabilization of cell-cell interactions. In malignant tissues, β-catenin triggers tumour proliferation and progression. The aim of this study is to investigate the expression patterns of c-KIT and β-catenin in human papillomavirus-negative and p16-positive SCC and to evaluate the chemosensitivity of the tumour cells to the chemotherapeutical agents docetaxel and 5-fluorouracil (5-FU). Materials and Methods: We incubated the tumour cell lines with docetaxel (5 μmol/ml) and 5-FU (1 μmol/ml) and detected β-catenin and c-KIT by immunohistochemistry and enzyme-linked immunosorbent assay (ELISA) after 48, 72, 120, 192 and 240 h. Results: We found a reliable trend towards decreased β-catenin expression levels in p16-positive and p16-negative tumour cell lines when incubated with docetaxel, in addition to induced apoptotic effect. At best, 5-FU had a slight influence on the alteration of the expression of β-catenin. Dose escalation of docetaxel and 5-FU had no statistically significant effect on the expression of β-catenin or c-KIT. In HPV-negative HNSCC, a reduced expression level of β-catenin and c-KIT was detected in an incubation period-dependent manner. p16-transformed SCC (CERV196) cells were characterized by a reduced susceptibility to docetaxel induced alteration of β-catenin expression. Conclusion: We were unable to confirm the clinically-substantiated increased chemosensitivity of p16-positive tumour cells in vitro. Extended studies and clinical trials are needed to investigate these findings further in HPV-associated HNSCC.*

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common type of cancer worldwide, with approximately 600,000 new cases diagnosed every year (1, 2). More than 90% of head and neck carcinomas are squamous cell carcinomas (SCC). Head and neck cancer (HNSCC) is a heterogeneous group of malignant entities that differ greatly in tumour aggressiveness and response to treatment. Chemotherapy and radiation treatment are used as an alternative to surgery to eliminate most of the proliferating cancer cells. The outcome of head and neck squamous cancer is still poor due to the development of local recurrence in approximately 10-30% of all cases (2). Therefore, the overall 5-year survival rate for patients with head and neck squamous cancer has not improved in recent years (1). Alcohol and tobacco consumption have been identified as the main risk factors for the development of HNSCC. However, up to 25% of patients have no history of alcohol or tobacco abuse (3). Clinical and pathological evidence suggest that viral oncogenic human papillomavirus (HPV) infection is another crucial etiological factor (4). In various neoplasms, such as bronchial cancer, breast cancer,

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cervical carcinoma, prostate carcinoma, colorectal cancer and recently HNSCC, an association with HPV infection was demonstrated in recent decades (4-9). HPV-associated HNSCC seemed to differ from tobacco- and alcohol-induced HNSCC (non-HPV) in its epidemiological, genetic, molecular and clinical profile. Oropharyngeal HPV-associated carcinomas are characterized by a younger age of patients at onset, a male predominance, and a strong association with sexual behaviour (10, 11). However, patients with HPV-positive oropharyngeal cancer have substantially improved outcomes, such as a 28- 80% lower risk of death compared to HPV-negative patients (12). The higher survival rate for patients with HPV-positive oropharyngeal cancer was attributed to the younger age at onset, good performance status and the presence of functionally intact p53-positive (13). Furthermore, the viral aetiology seems to be linked to specific subtypes of HPV, such as HPV-16 and HPV-18, especially those arising from SCC of the tonsils and the tongue base (14). HPV belongs to the family of Papillomaviridae, a small DNA virus with a preference for squamous epithelia. Typical sites of HPV infection are the proliferative, basal compartment of stratified squamous epithithelium or the basal layer (15). Over 200 different HPV types have been discovered to date (16). High-risk HPV types such as HPV-16 and HPV-18 may lead to malignant transformation of epithelial tissue. This process depends on the presence of the viral oncogenes E6 and E7. The expression of E6 and E7 inactivates two tumour suppressor proteins: p53 and retinoblastoma protein (pRb). The loss of several key regulatory proteins induces cell-cycle progression, de-differentiation and proliferation of HPV-infected epithelial cells, and facilitates the induction of the transformed phenotype, with a high frequency of mutation and chromosomal instability (11, 17). Loss of cell adhesion and transformation of epithelial cells in a mesenchymal phenotype is described as epithelial mesenchymal-transition (EMT). EMT is one of the fundamental steps inducing invasion and progression. It is characterized by down-regulation of epithelial-specific adhesive proteins of epithelial cells (*e.g.* of tight and adherent junction proteins such as E-cadherin), and induction of mesenchymal proteins such as vimentin, as well as development of migratory attributes (18). Vincan and Barker found that both β-catenin-dependent and -independent-WNT signalling was implicated in EMT during colorectal cancer progression (19). Interestingly, data from Stenner and collegues showed an up-regulation of β-catenin and down-regulation of E-cadherin expression in primary HPV-positive tonsillar carcinomas, which might play an important role during tumour progression and metastasis (20).

β-Catenin is a multifunctional protein, suggested to be one of the most important factors for reducing cell-cell interactions in malignant transformed epithelial cells. Adherent cell-cell contacts are dependent on β-catenin/α-catenin binding and α-catein/cadherin connection (11, 21). However, β-Catenin plays a crucial role in the development of head and neck cancer *via* a nuclear downstream effector of the canonical WNT signaling cascade (22, 23). After 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. β-Catenin acts as a cofactor of transcriptional regulators and increases, for instance, the effect of T-cell factor/lymphocyte enhancing factor (TCF/LEF). This pathway results in up-regulation of various target genes, such as the zinc-finger protein SLUG, vimentin, and matrix metalloproteinase-9, which are required for dysregulation of cell-cycle progression, tumour progression, migration and invasion (11, 23, 24). Under normal conditions, cytosolic β-catenin is phosphorylated at multiple serine and threonine sites by glycogen synthase kinase 3β (GSK3β) and thereby degraded. Abnormal WNT/β-catenin signalling is associated with many human diseases, such as degenerative disorders, osteoporosis, aging and the development of tumourigenesis. The multiprotein degradation complex of β-catenin and TCF regulate the initiation of WNT (25-27).

c-KIT is a member of the receptor tyrosine kinase family and structurally related to the platelet derived growth factor (PDGF) receptor. The ligand of c-KIT is also known as stem cell factor or Steele factor (SCF) and is a cell-surface protein with two isoforms. Various studies have detected the association of dysregulated or ectopic expression of c-KIT and malignant transformation, especially in epithelial tissue (28, 29). c-KIT functions as an oncogene in several tumour types, in particular gastrointestinal stromal tumours (GIST), mastocytosis, and melanoma. It activates mutations in the extra- or intracellular domain (30, 31). Pharyngeal HNSCC exhibits higher c-KIT expression in contrast to neoplasia of the larynx (32). Binding of SCF to c-KIT initiates a conformational change, which leads to dimerization of the receptor and activation of its intrinsic tyrosine kinase and autophosphorylation on key tyrosine residues that served as docking sites for signal transduction molecules (33). Thereby, multiple downstream signaling pathways can be triggered, including the rat sarcoma (RAS)/extracellular-signal regulated-kinases (ERK), phosphatidylinositol 3-kinase (PI3-K), Src kinases and janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathways, which modulate proliferation, cellular motility and cell survival (34). However, both β-catenin and c-KIT seem to play a crucial role in cancer development and progression. Thus, a reduction of the expression pattern of β-catenin and c-KIT or the inhibition of their biological function by chemotherapeutic agents could lead to progress in the treatment of HNSCC.

Docetaxel and 5-fluorouracil (5-FU) are important anticancer agents that are widely used in the treatment of a variety of cancer types, including HNSCC. Docetaxel is mainly utilised for the treatment of non-small cell lung (NSCLC), gastric, breast, and prostate cancer to stop cancer progression, or to achieve cancer remission (35-38). Docetaxel belongs to the chemotherapy drug class of the taxanes. Its cytotoxic activity is based on stabilization of microtubule assembly, while physiological microtubule disassembly was forestalled in the absence of guanosine-5'-triphosphate (GTP) (35, 39). In the presence of docetaxel, microtubules accumulate inside the cell and cause initiation of apoptosis (40). *In vitro* and *in vivo* studies demonstrated the anti-neoplastic activity of docetaxel for a wide range of known cancer cells. In combination with other anti-neoplastic agents, docetaxel demonstrates a synergistic effect and often increased cytotoxic activity, possibly due to its more rapid intracellular uptake (35). 5-FU is an analogue of pyrimidine nucleosides that blocks the synthesis of deoxythymidylic acid by thymidylate synthetase and disrupts normal DNA and RNA function. This chemotherapeutic agent is used in the treatment of colonic, breast, ovarian, and prostatic cancer (41, 42).

The purpose of this study was to evaluate the expression pattern of nuclear β-catenin and c-KIT in p16-positive SCC and HPV-negative HNSCC tumour cells. Furthermore, the chemosensitivity of p16-positive SCC cells were compared to non-HPV tumour cell lines after single-drug treatment with 5-FU and docetaxel as established anticancer regimes for head and neck tumours. To our knowledge, this is the first study to compare expression patterns of effector molecules of the WNT pathway and c-KIT expression in viral-transformed squamous neoplasm after treatment with 5-FU and docetaxel.

Materials and Methods

Cell lines and culture. Two different HNSCC cell lines 11A and 14C were obtained from Dr. T. E. Carey (University of Michigan, MI, USA). These cell lines originated from human HNSCC of the larynx and oropharynx.

The CERV196 cell line (CLS, Eppelheim, Germany) was descended from a poorly-differentiated SCC of the cervix with HPV-16 positivity. The CERV196 tumour originated *in vitro* from a xenotransplanted cervical carcinoma MRI-H-196. The CERV196 cells were cultured in Eagle's minimum essential medium with Earle's BSS and 2mM L-glutamine containing 10% fetal calf serum (FCS), 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate and 1.5g/l sodium bicarbonate. Cell cultures were carried out at 37˚C in a fully-humidified atmosphere with 5% CO_2. We utilised Dulbecco's modified minimum essential medium (DMEM) (Fisher Scientific Co., Pittsburgh, PA, USA) with 10% FCS and antibiotics (Life Technologies Inc., Gainthersburg, MD, USA) for UMSCC11A, UMSCC14C and CERV196. The supplies of 5-FU and docetaxel were stored at 4˚C and lysed in sterile water at the time of application. For incubation with HNSCC cell lines, different concentrations of 5-FU (1 μmol/ml) and docetaxel (5 μmol/ml) were defined and used for stimulation up to 48, 72, 120, 192 and 240 h. Selection of the different drug concentrations and times of stimulation were defined after performing the alamarBlue (AbD Serotec, Oxford, UK) cell proliferation assay, establishing the relative cytotoxicity of the chemotherapeutic drugs and quantitatively measuring the proliferation of HNSCC tumour cells. After incubation, the supernatants were pooled together in sterile tubes and stored at –20˚C until further analysis.

Immunohistochemistry for c-KIT. Before performing immunohistochemistry, HNSCC cells were cultured on glass coverslips overnight. When the cells grew to confluency, they were exposed to different concentrations of 5-FU and for different incubation periods (0, 72 and 192 h). Subsequently, they underwent fixation with acetone and alcohol (2:1) and were then washed with phosphate-buffered salt solution (PBS). Immunohistochemical analysis was performed using a polyclonal rabbit antibody to human c-KIT (sc-5535, dilution 1:200, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The following steps were executed by an automated staining system, Dako TechMate 500 (Dako, Hamburg, Germany): Adjacent cells were then incubated with perioxidase block for 30 min. The cells were washed once for three times with PBS for 5 min each time (Buffer kit; Dako). Immunoreaction was shown with the Dako ChemMate Detection kit according to the guidelines of the manufacturer (APAAP, mouse, no. K5000; Dako): After incubation with 10% rabbitserum 30 min cells were exposed to the rabbit monoclonal antibody for c-KIT as the primary solution for 30 min at room temperature, taking a working dilution of antibody cells. The incubated cell lines were refrigerated overnight. Afterwards, the cells were washed three times with PBS and incubated with secondary antibody. Cells were treated with the chromogen alkaline phosphatase substrate (Neufuchsin; Dako) for 20 min at room temperature. For negative controls all reagents except for the primary antibody were used. The sections were incubated by Mayer's hematoxylin for 3min, followed by dehydration in graded ethanol and coverslipping. The immunohistochemically demonstrated rates of c-KIT expression were assayed semiquantitatively.

Enzyme-linked immunosorbent assay (ELISA). Cell cultures were incubated in 6-well chambers with different chemotherapeutic drug concentrations and washed with PBS. Then 350 μl per well of lysis buffer was added. Afterwards, gently agitating the lysed cells with a vortex at 2-8˚C for 30 min and micro-centrifuging (14,000 g for 5 min). Concentrations of β-catenin and c-KIT in cell culture supernatants were measured by an enzyme linked immunosorbent assay (ELISA) technique (R&D Systems, Wiesbaden, Germany). The system utilised a solid-phase monoclonal antibody and an enzyme linked polyclonal antibody raised against human β-catenin and c-KIT. The specificity of the β-catenin and c-KIT antibodies which were used in the ELISA kit was tested by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. According to the guidelines of the manufacturer, each ELISA was performed on a volume of 100 μl of supernatant. After 48, 72, 120, 192 and 240 h of incubation with docetaxel (5 μmol/ml) or 5-FU (1 μmol/ml), the protein expression of β-catenin and c-KIT of the treated and untreated cells were assayed. All analyses and calibrations were performed in duplicate.

The calibrations on each microtitre plate included the β-catenin and c-KIT standards provided in the kit. The optical density was detected using a microplate reader at a wavelength of 450 nm. Wavelength correction was defined to 540 nm and concentrations are reported in pg/ml.

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 was considered statistically significant. The statistical tests performed were the two-coefficient variance analyses (SAS Statistics, Cary, NC, USA) and Dunnett's test.

Results

Immunohistochemistry for c-KIT in HNSCC14C, 11A, and CERV196 cell. Immunohistochemical studies for c-KIT illustrated that all tumour cell lines, irrespective of HPV status, expressed similarly low levels of c-KIT compared to the chemonaive controls. Furthermore, a decreased reactivity for c-KIT expression was detected in an incubation period-dependent manner up to 192 h with 5-FU for HNSCC11A, 14C and CERV196 cell lines. Interestingly, we detected an increased reactivity for c-KIT after 72 h of 5-FU treatment, especially in HNSCC11A and CERV196 cells. Under prolonged incubation, a decreased immunoreactivity for c-KIT was detected. We also showed a slightly increased reactivity for c-KIT after 72 h of docetaxel treatment, particulary in CERV196 cells (data not shown). Dose escalation of these chemotherapeutic agents had only a slight influence on c-KIT expression by immunohistochemistry (Figure 1).

ELISA of total protein expression in HNSCC14C, 11A and CERV196 cells. Compared to HPV-negative tumour cell lines, negative controls of CERV196 cells exhibited higher total protein expression levels. The HPV16-positive SCC line seemed to be less vulnerable towards 5-FU and docetaxel therapy, particularly after shorter incubation periods (up to 72 h). Between 72 and 240 h, there was a significant reduction of total protein expression in CERV196 cells after incubation with docetaxel ($p<0.0008$). The level of total protein in HPV16-positive CERV196 cells only showed a significant alteration after 72 h and 240 h of 5-FU treatment ($p<0.02$). In non-HPV-associated HNSCC, a consistent and statistically significant suppression of total protein was measured after treatment with 5-FU between 72 and 192 h ($p\leq0.016$). For HNSCC11A and 14C cells, there was a significant reduction of total protein expression after exposure to docetaxel in an incubation time-dependent manner by 72–240 h when compared to the negative control ($p<0.0001$). In HNSCC14C, a maximal reduction of total protein was detected after 240 h of docetaxel treatment ($p<0.0001$). The drug concentration had no significant influence on the expression of total protein (Table I).

Table I. *Enzyme linked immunosorbet assay (ELISA) for total protein expression in HNSCC11A, 14C, and CERV196 cells*.

		Total protein expression, pg/ml (*p*-value)	
Incubation time (h)	Control Mean value	5-FU (1 μmol/ml) Mean value/ (*p*-value)	docetaxel (5 μmol/ml) Mean value/ (*p*-value)
HNSCC11A			
48	995	**646 (<0.0001)**	**299 (<0.0001)**
72	917	**718 (<0.0001)**	**317 (<0.0001)**
120	1074	**773 (0.0007)**	**254 (<0.0001)**
192	1132	**623 (<0.0001)**	**160 (<0.0001)**
240	928	909 (0.9919)	**221 (<0.0001)**
HNSCC14C			
48	806	683 (0.4247)	**516 (0.0016)**
72	1004	**585 (<0.0001)**	**444 (<0.0001)**
120	959	**788 (0.0152)**	**240 (<0.0001)**
192	1035	**667 (0.0021)**	**146 (<0.0001)**
240	1070	**794 (0.0043)**	**140 (<0.0001)**
CERV196			
48	1949	1968 (0.9439)	1829 (0.3888)
72	2114	**1824 (0.0185)**	**1736 (0.0003)**
120	1901	1853 (0.3145)	**1293 (0.0007)**
192	1921	1677 (0.0757)	**593 (<0.0001)**
240	2013	**771 (<0.0001)**	**174 (<0.0001)**

Mean values and statistical correlation compared to the negative control (*p*-value, Dunnett's test, n=3) are shown. Bold indicates statistically significant differences.

ELISA of β-catenin expression in HNSCC14C, 11A and CERV196 cells. Compared to HPV-negative tumour cell lines, negative controls of CERV196 showed lower intrinsic expression of secreted β-catenin. The suppression of secreted cytosolic β-catenin in HPV16-positive SCC showed no significance after treatment with 5-FU or docetaxel. The HPV-associated SCC cell carcinoma line exhibited a low vulnerability to doxetacel and 5-FU therapy until 120 h of treatment. In CERV196 cells we found a maximal reduction of β-catenin after treatment with docetaxel after 240 h ($p=0.1351$). Between 48 and 192 h, a time-dependent reduction of expression of β-catenin was measured in HNSCC14 cells after incubation with docetaxel in HNSCC14C. For HNSCC11A, there was no significant reduction of β-catenin expression levels by 5-FU irrespective of the applied drug concentration or incubation time when compared to the negative control. β-Catenin in HNSCC14C and CERV196 cells showed a trend towards decreased expression after incubation with doctetaxel after an extended incubation time. In HNSCC11A, under docetaxel β-catenin expression was significantly reduced in HNSCC11A within 72–240 h ($p\leq0.007$) (data not shown).

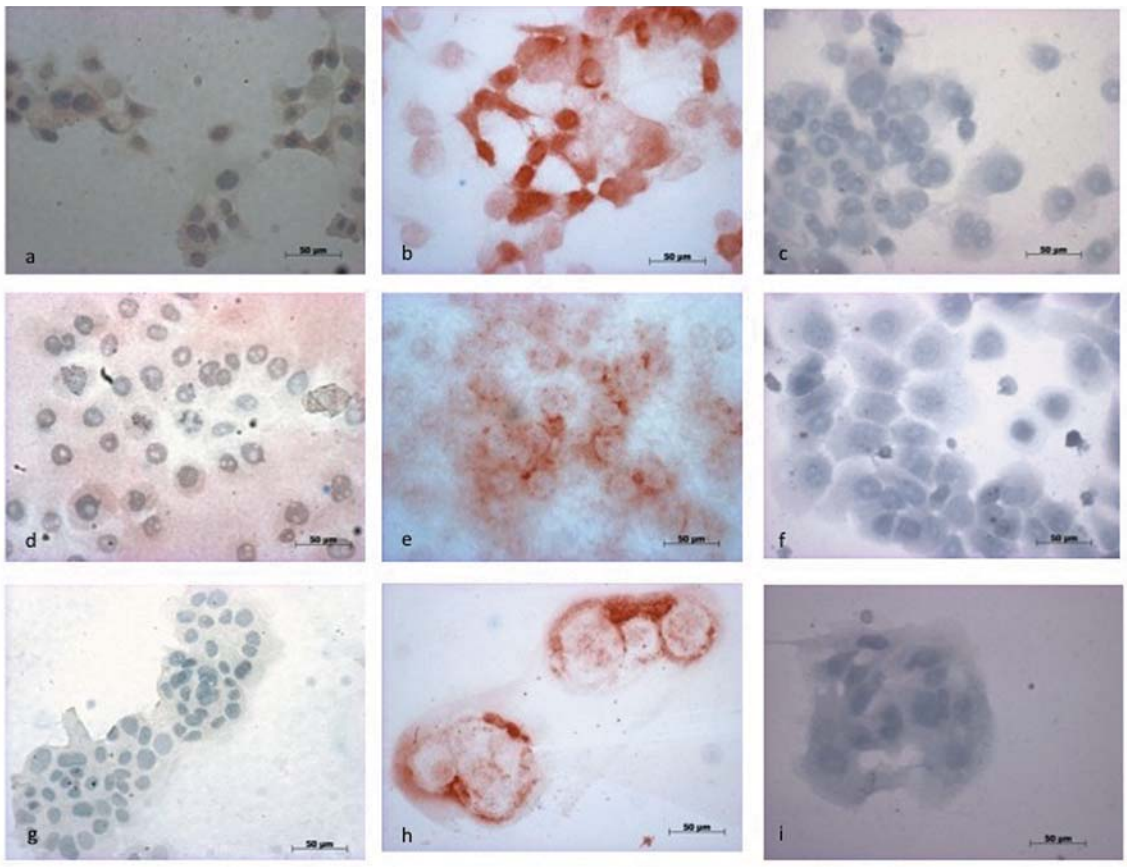

Figure 1. *Negative control and declining immunohistochemical reactivity for c-KIT after incubation with 5-FU (1 μmol/ml) for 72 and 192 h for the following: HNSCC11A (a-c), HNSCC14C (d-f), CERV196 (g-i). Decreasing immunoreactivity was detected with a prolonged incubation period (192 h) with 5-FU (1 μmol/ml).*

Unlike the treatment with 5-FU, only HNSCC11A cells, which were exposed to docetaxel consistently, exhibited a significant reduction of cytoplasmatic β-catenin expression levels in an incubation period-dependent manner, when compared to the negative control. The expression of β-catenin after treatment with docetaxel in HNSCC14C and HPV16-positive CERV196 cells showed no significant alteration. An increase of the concentration had no additional effect in reducing the expression of β-catenin (Figure 2).

ELISA of c-KIT expression in HNSCC14C, 11A and CERV196 cells. In summary, we detected less expression of c-KIT in all three cell lines. We found a distinct trend towards reduction of c-KIT expression after prolonged treatment time. In CERV196 cells, maximal reduction of c-KIT was measured after 120 h of 5-FU treatment. We found a consistant trend towards incubation period-dependent reduction of c-KIT level in CERV196 cells when exposed to 5-FU and docetaxel within the 48–192 h timeframe. Docetaxel suppressed c-KIT expression in HNSCC11A between 48 and 240 h of treatment in addition to the anticipated cellular effect of docetaxel. To 48–120 h, we found a smaller reduction in expression of c-KIT after incubation with 5-FU in HNSCC11A cells. In HNSCC14C cells, only a long incubation period (240 h) with 5-FU and docetaxel led to a greater reduction of expression of c-KIT. Compared to CERV196 cells, HNSCC14C cells exhibited a relatively constant expression of c-KIT levels after 192 h of incubation with 5-FU. The concentration of the chemotherapeutics had no statistically significant impact on the c-KIT expression (Figure 2).

Discussion

The purpose of this study was to investigate the effects of 5-FU and docetaxel on HPV-16-associated HNSCC compared to non-HPV-induced HNSCC and their impact on the expression of β-catenin and c-KIT. We found that compared to HPV-negative tumour cell lines, negative controls of CERV196 cells exhibited higher expression of total protein. However, p16-positive tumour cell lines seemed to have a

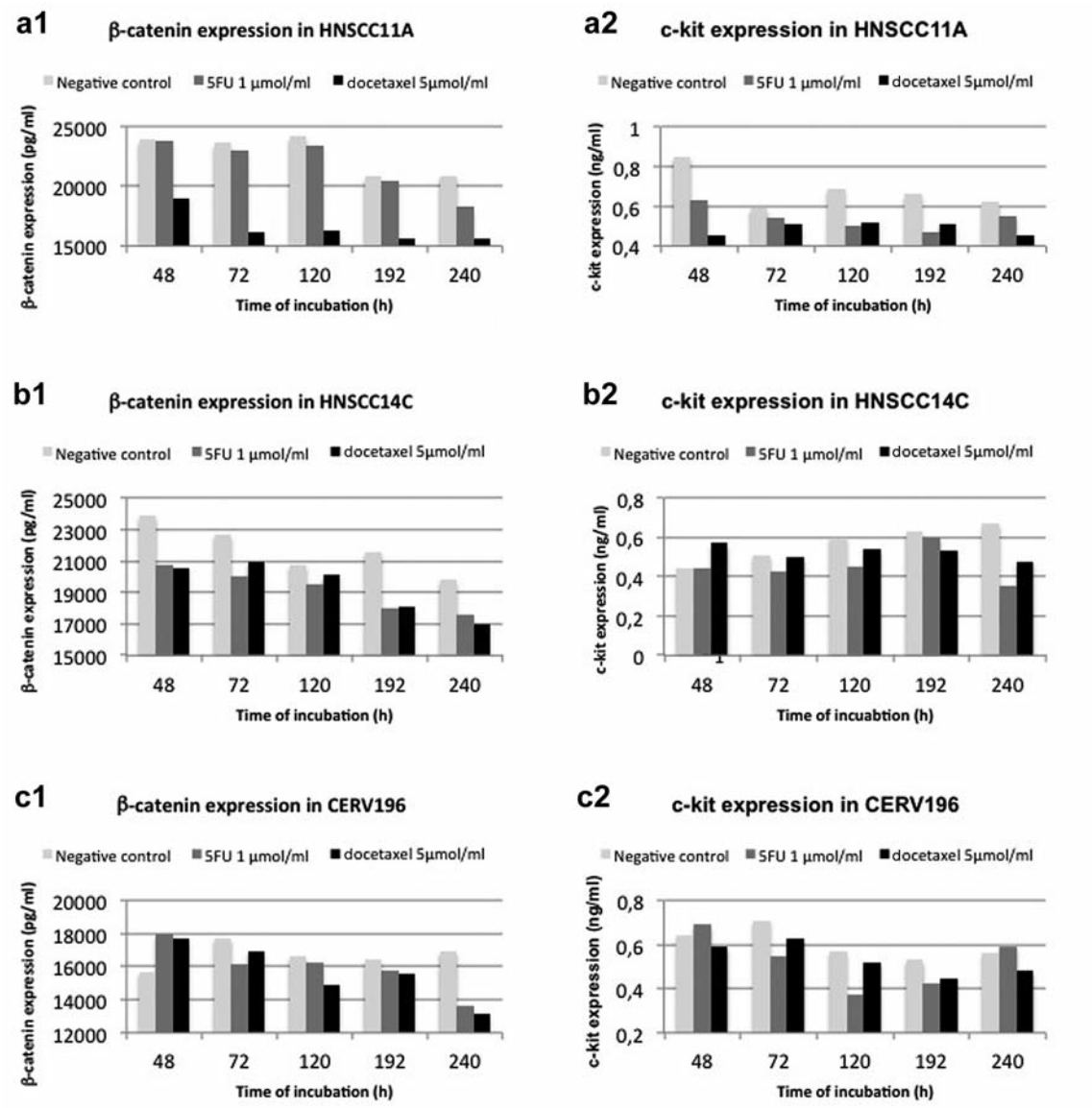

Figure 2. *Expression of c-KIT and β-catenin in head and neck squamous cancer and CERV196 after incubation with 5-FU and docetaxel. β-catenin and c-kit expression in HNSCC11A (a1+a2), 14C (b1+b2) and CERV196 (c1+c2) cells and incubation with 5-FU and docetaxel. In HNSCC11A, a reduced expression level of β−catenin and c-KIT was detected in an incubation period-dependent manner. The CERV196 cells were characterized by a reduced susceptibility to docetaxel induced alteration of the β-catenin expression.*

stronger basal metabolism in general. In contrast, we demonstrated a lower intrinsic expression of nuclear β-catenin in negative controls of CERV196 cells compared to HPV-negative tumour cell lines. 5-FU had no, or at best, only a slight influence on the alteration of the expression of β-catenin. Furthermore, we showed that dose escalation of docetaxel or 5-FU had no statistically significant effect on the expression of β-catenin or c-KIT, thus for simplification, only the initial dose of 5-FU (1 µmol/ml) and docetaxel (5 µmol/ml) is described. We showed a reliable trend towards a significant reduction of β-catenin expression levels in p16-positive SCC and non-HPV HNSCC cells when incubated with doxetacel in an incubation period-dependant manner, independent of the apoptotic process and a reduced cell count. This observation was quite unexpected when related to the molecular mechanism of the chemotherapeutic agent.

A poor outcome in a variety of malignancies, such as breast, gastric, non-small cell lung, colorectal, and hepatocellular carcinoma is associated with an increase of cytosolic and nuclear β-catenin expression levels (43-47). Interestingly, Pukkila and colleagues showed cytoplasmic augmentation and nuclear translocation of β-catenin in

correlation with a poor outcome of oropharyngeal and hypopharyngeal squamous cell carcinoma. Thus expression of nuclear β-catenin is an independent predictor of lower overall survival in head and neck cancer (48). Further data by Rampias and colleagues showed that viral oncogenes E6 and E7 participate in nuclear accumulation of β-catenin and induction of WNT pathway in HPV-associated cancer compared to non-HPV-induced cancer in oropharyngeal and cervical cancer cell lines (26). They suggested that repression of E6 and E7 levels caused down-regulation of nuclear β-catenin, changing the level of free cytoplasmatic E-cadherin (26). The HPV16-positive squamous carcinoma cell line seemed to be less vulnerable towards 5-FU and docetaxel therapy, particularly after short incubation periods. Ding and colleagues detected that cells were increasingly resistant to treatment with cisplatin in HPV16-associated endocervical cell model for SCC (49). Whether this effect was associated with an accumulation of viral oncogene E6/E7 and WNT activation or lower vulnerability of HPV-16-associated HNSCC needs to be evaluated. On the other hand, it was demonstrated that oncoprotein expression of E6 and E7 did not prevent 5-FU mediated G_1/S arrest and apoptosis in 5-FU-resistant carcinoma cell lines (50). Liu and collegues found that 5-FU presented some inhibitory effects on the E6 and E7 oncoproteins of HPV16 in laryngeal cancer cells. However, the mechanism of the antiviral effect of 5-FU is still unclear (51). Dihydropyrimidine dehydrogenase (DPD) is a natural enzyme that influences pyrimidine degradation. DPD, expressed in different kinds of tumours, is the enzyme for the rate-limiting step of 5-FU catabolism that accounts for more than 80% of its elimination (52-55). Thus, carcinomas with a high DPD level are more resistant to 5-FU, in contrast to carcinomas with low levels of DPD. Several strategies have been developed to inhibit DPD-mediated degradation of 5-FU. Some DPD inhibitors were detected and prospected, such as eniluracil and 5-chlorodihydropyrimidine. Eniluracil improved the tumour response rate to 5-FU from 0-88% in a rat model of colorectal carcinoma cells. The rats were divided into 3 groups: group A was not treated with eniluracil, group B was treated with eniluracil adequate, and group C was treated with eniluracil excess. Spector and Cao exhibited that the antitumor activity of 5-FU was significantly diminished when eniluracil dose is in 5-fold excess to 5-FU (56). Creation of 5-FU pro-drugs that inhibit DPD-mediated degradation in the liver was also attempted (41, 42). Until the present study, the relationship between sensitivity of 5-FU and DPD expression and HPV-status had not been examined as far as we are aware. Furthermore, our data suggests that all tumour cell lines, irrespective of HPV status, express low levels of c-KIT. Interestingly, increased concentration of docetaxel or 5-FU only had a mild influence on c-KIT expression levels in immunohistochemistry without statistical significance. Treatment with 5-FU in HPV-associated HNSCC showed a maximal decrease of expression of c-KIT level at 120 h of incubation; from this time on an increased expression level of c-KIT was detected. Shorter incubation periods with 5-FU were necessary to reduce c-KIT levels. Interestingly, the immunohistochemistry data showed decreased immunoreactivity against c-KIT only under prolonged incubation time. The mechanism of this effect is still unclear and should be investigated.

When analyzing the effect of docetaxel on the expression of c-KIT in CERV196 cell line, a slight trend towards suppressed c-KIT expression was detected after a longer incubation period. However, the large number of studies investigating the expression of the c-KIT receptor and its ligand showed a potential association of their coexpression with neoplastic transformation, primarily in epithelial tissues (28, 29, 57). Analysis of KIT mutations in individual patients is important to ensure that the right kinase inhibitors are used for therapy to avoid drug resistance (58). In different tumour cells, the use of c-kit inhibitor STI571 as small molecule-targeted therapy was associated with a decrease in tumour growth (59, 60).

This study could not confirm the clinically substantiated increased chemosensitivity of p16-positive tumour cells, but we could detect differences in the alteration of expression of β-catenin and c-KIT after treatment with the several chemotherapeutic agents. However, for therapeutic treatment of HNSCC, knowledge of the HPV-status is still an important factor. Additional studies designed to explore the inclusion of DPD or c-KIT inhibitors in the treatment of p16-positive HNSCC might enhance the efficacy of standard chemotherapeutic agents and eventually improve the clinical outcome of patients with HNSCC, depending on HPV status.

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