Chemotherapeutic Alteration of VEGF, PDGF and PDGFRα/β Expression Under 5-FU *vs*. Docetaxel in HPV-transformed Squamous Cell Carcinoma Compared To HPV-negative HNSCC *In Vitro*

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Abstract. *Background: Head and neck squamous cell carcinoma (HNSCC) is the most common malignant epithelial tumor in the upper aerodigestive tract. The incidence of HNSCC induced by the oncogenic human papilloma virus (HPV) is rising, indicating a growing importance of the viral etiology. Cell proliferation, migration and tumor vascularization are regulated by a set of angiogenic peptides such as PDGF (platelet-derived growth factor), PDGFRα/β (platelet-derived growth factor receptor α/β) and VEGF (vascular endothelial growth factor). In locally advanced HNSCC docetaxel is used for induction chemotherapy (ICT) combined with platinum-based chemotherapy and 5-fluorouracil (5-FU). This study sought to evaluate the expression of angiogenic factors (VEGF, PDGF and PDGFRα/β) in HPV-positive (CERV196) and HPV-negative squamous cell carcinoma (HNSCC 11A and 14C) and the efficacy of chemotherapy with docetaxel as a potential treatment modality, compared to 5-FU as a single-drug application. Materials and Methods: Tumor cell lines were incubated with 5-FU or docetaxel at a concentration of 1.0 and 5.0 μmol/ml. Enzyme-linked immunosorbent assay (ELISA) and immunohistochemical analyses were carried out after 48, 72, 120, 192 and 240 hours, in order to identify changes in protein expression of VEGF, PDGF and PDGFRα/β. Results: We demonstrated a significant reduction of VEGF and PDGFRβ expression after incubation with docetaxel by ELISA and of PDGF by immunohistochemistry, irrespective of the HPV status, whereas the application of 5-FU had a significantly weaker impact on the expression of angiogenic peptides. HPV-positive CERV196 cells were characterized by a reduced susceptibility to a docetaxel-altered expression. Conclusion: Although neither of the applied drugs are selective anti-angiogenic agents, docetaxel surprisingly was demonstrated to cause a significant decrease of angiogenic factors in this study.*

Head and neck squamous cell carcinoma (HNSCC) has a global incidence of approximately 631,000 cases and approximately 352,000 associated deaths and is the sixth most common cancer worldwide (1). Its clinical appearance and progress are very heterogeneous. Despite improvements in surgical techniques, radiation and chemotherapy during the past decades, the five-year survival rate has remained almost unchanged (2). New predictive markers are necessary to optimize treatment.

The incidence of oropharyngeal cancer, especially in the region of the tonsils and the tongue base, has increased steadily over the past 20 years in fairly young patients, at an age of 20 to 44 years (3). In contrast, the incidence of HNSCC has decreased overall, which is believed to be due to a reduced prevalence of smoking (4-6). Abuse of tobacco and alcohol remain the most important risk factors for head and neck tumors overall (7). Tobacco and alcohol appear to have a synergistic effect on the mucosal surfaces in the process of field cancerization (8).

Risk factors for human papilloma virus (HPV) infection are indeed promiscuity and the practice of unsafe sex. HPV infection has been associated with anogenital carcinomas and

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more recently oral and oropharyngeal cancer (9). In contrast to a decreasing incidence of HNSCC overall, the incidence of HPV-associated oropharyngeal cancer is rising, indicating the importance of the viral etiology (3, 10, 11). In the USA, 40-80% of oropharyngeal malignancies are associated with HPV, whereas data from Europe vary from 20% in countries with high tobacco and alcohol consumption to 85% in Sweden (12). This suggests that HPV is now the primary cause of tonsillar carcinoma in North America and parts of Europe (12-14). The International Agency for Research on Cancer (IARC) designates HPV as a risk factor for oropharyngeal cancerogenesis (15-18).

HPV-positive HNSCC seems to be different to tobacco- and alcohol-induced HNSCC (HPV-negative) in its genetic, molecular and clinical profile. HPV-positive HNSCCs occur more often in younger patients with lower tobacco exposure but greater exposure to marijuana, oral sex or multiple sexual partners, which is consistent with the predominant sexual transmission of HPV (19-22).

HPV is a DNA virus with a circular, double-stranded genome. The viral genome encodes three oncoproteins (E5, E6, and E7) (12). Today the family of *Papillomaviridae* contains more than 100 subtypes, which can be classified into low- and high-risk sub-populations (13). Manifestation of HPV infections range from benign papillomas to invasive cervical, vulvar, vaginal, anal and penile carcinomas as well as HNSCC (20, 23, 24). Viral oncogenes of HPV suppress pro-inflammatory signaling and prevent eradication by the immune system of the host (25, 26). The expression of the oncogenes mentioned before and loss of regulatory proteins lead to cell-cycle progression, proliferation and dedifferentiation of the epithelial cells (27-29). These oncoproteins degrade and de-stabilize two major tumor suppressor proteins, p53 and Rb (found to be mutated in many other forms of human cancer). This is contrary to HNSCC induced by alcohol and tobacco exposure, which show mutational loss of *Rb* and *TP53*, leading to uncontrolled cellular growth in up to 80% of HNSCCs (30-33). *TP53* positively regulates the expression of angiogenic inhibitors and suppresses pro-angiogenic factors (34-36).

Besides the differences in etiology, HPV-positive carcinomas differ from HPV-negative ones in their response to treatment and survival. HPV positivity is considered to be a favourable prognostic biomarker as patients with HPV-positive oropharyngeal cancer have higher response rates to radiation and chemotherapy, and therefore have a significantly better survival rate and local disease control (32, 37-39). The literature offers different explanations for the clinical advantages of patients with HPV-associated HNSCC, such as lack of tobacco and alcohol abuse and presence of functional TP53 (37).

The different cell types of the tumor stroma, like endothelium, cancer-associated-fibroblasts (CAFs), pericytes and the infiltrating inflammatory cells form the extracellular matrix (ECM) and have been attributed with important functions in the progression of the disease (40). Cells of the ECM provide proliferative, anti-apoptotic and angiogenic factors (41-45). These factors empower the tumor to interact widely with its microenvironment (46-48). Several functions such as endothelial cell migration, proliferation and capillary tubule formation, are enabled by angiogenic peptides. Vascular endothelial growth factor (VEGF) induces angiogenesis *in vivo* and stimulates vascular permeability as a potent endothelial mitogenic factor. Its expression is associated with an increase of tumor growth and angiogenesis *in vivo* in a mouse model (49-51). Cell proliferation, differentiation and migration of vascular cells are promoted by VEGF. Enhanced expression of VEGF has been detected in a number of malignant tumors, including HNSCC (52, 53). Its expression in HNSCC is strongly correlated with tumor angiogenesis and is inversely correlated with apoptosis, showing the anti-apoptotic potential of VEGF (54, 55). For instance, VEGF expression can be induced by loss or inactivation of tumor suppressor genes or overexpression of oncogenes. Several studies demonstrated that HPV-16 oncoprotein E7 strongly stimulates various angiogenic factors, including VEGF (25). Tumor angiogenesis, microvessel density and VEGF expression have been associated with poor prognosis of HNSCC and are considered to be predictive markers (53-55).

Platelet-derived growth factor (PDGF) plays an important role in cellular interaction and phenotypic tissue architecture. The different PDGF isoforms and the corresponding receptors are involved in the regulation of cell proliferation, chemotaxis and survival in normal as well as in tumor cells. The biological activity of PDGF is linked to tyrosine kinase receptors, named platelet-derived growth factor receptor α and β (PDGFRα and β). Binding of the ligand to its receptor leads to activation of the tyrosine kinase and autophosphorylation. This induces a cascade of signaling molecules that are responsible for the different biological effects of PDGF (56-58). Previous studies showed the increased expression of PDGF and its associated receptors in various types of malignant human tumor (59, 60). PDGF helps to establish a well-vascularized stroma and tumor proliferation by stimulating the process of angiogenesis in fibrosarcomas, breast carcinoma, melanoma and HNSCC (58-65).

Inhibition of angiogenesis by blocking angiogenic cytokines or their pathways has, thus, become a major target also in experimental cancer therapies (66, 67).

Docetaxel is a taxane and a semi-synthetic analog of paclitaxel, an extract from the bark of the Pacific yew tree. Docetaxel is a well-established chemotherapeutic agent, which interferes with microtubules by binding reversibly to tubulin. This prevents depolymerization of the microtubules.

As a result, mitosis is inhibited between metaphase and anaphase. Docetaxel does not prevent disassembly of interphase microtubules and does not prevent entry into the mitotic cycle. It has also been shown to induce apoptosis of tumor cells and to have anti angiogenic and immunostimulating properties (68). Docetaxel *in vitro* reduces murine and human tumor cell survival by 50% at concentrations of 4-35 ng/ml *in vitro* (69). Over 80% of murine transplantable tumors were found to be very sensitive to docetaxel, with complete regression of advanced-stage tumors. Activity was also observed in >90% of advanced-stage human tumor xenografts in mice (69). In combination therapy studies, synergism with 5-fluorouracil (5-FU), cyclophosphamide, etoposide and anti-angiogenic drugs was observed *in vivo*. In toxicological studies in mice and dogs, docetaxel produced haematological, gastrointestinal and neuromotoric toxicity (68, 69). Docetaxel is used in the treatment of breast, ovarian, prostate, and non-small cell lung cancer. In the treatment of locally advanced HNSCC, docetaxel is part of induction chemotherapy (ICT) in combination with platinum-based chemotherapeutic drugs and 5-FU (70, 71). It could be shown that the combination with docetaxel leads to significantly longer survival compared to patients who received cisplatin and fluorouracil ICT plus radiotherapy (72, 73).

5-FU is a pyrimidine analog and works as a suicide inhibitor by irreversible inhibition of thymidylate synthase. Thymidylate synthase methylates deoxyuridine monophosphate (dUMP) into deoxythymidine monophosphate (dTMP). Administration of 5-FU, thus, causes a lack of dTMP. Thymidine is a nucleoside required for DNA replication. As a pyrimidine analog, 5-FU is transformed inside the cell into different cytotoxic metabolites which are incorporated into DNA, finally inducing cell-cycle arrest and apoptosis by inhibiting the cell's ability to synthesize DNA. It can also be incorporated into RNA, leading to interference with the maturation of nuclear RNA. However, its conversion to 5-fluoro-2'deoxy-5'monophosphate leading to inhibition of thymidylate synthase, and subsequently of DNA synthesis, is considered to be its main mechanism of action. Resistance to 5-FU is mainly attributed to aberrations in its metabolism or to alterations of thymidylate synthase, gene amplification and altered kinetics in respect to nucleotides or folates. Biochemical modulation of 5-FU metabolism can be applied to overcome resistance to 5-FU (74). The parenteral preparation for intravenous infusion is the major means of application (75). 5-FU is primarily eliminated in the liver by the enzyme dihydropyrimidine dehydrogenase (DPD). About 3% of patients exhibit a partial DPD deficiency indicating an increased risk for developing 5-FU-related toxic side-effects. Although resistance to 5-FU is multi-factorial, DPD activity in tumor cells (*in vitro* and *in vivo*) is significantly related to 5-FU sensitivity. Lower DPD activity leads to increased 5-FU efficacy (76).

The aim of this study was to evaluate the impact of docetaxel on the expression levels of certain angiogenic factors in HPV-positive and -negative squamous cell carcinomas in comparison to 5-FU as a standard chemotherapeutic drug in the treatment of HNSCC.

Materials and Methods

Cell lines. The two different HNSCC cell lines 11A and 14C were obtained from Dr T.E. Carey (University of Michigan, MI, USA). These cell lines originate from human HNSCC of the oropharynx and larynx. The CERV196 cell line was established from a poorly-differentiated squamous cell carcinoma of the cervix and is HPV-16 positive (CLS, Eppelheim, Germany). The CERV196 cells were cultured in Eagle's minimum essential medium with 2 mM L-glutamine and Earle's balanced salt solution (BSS) adjusted to contain 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate and 10% fetal bovine serum. Cell cultures were carried out at 37˚C in a fully-humidified atmosphere with 5% CO_2 using Dulbecco's modified essential medium (DMEM) (Fisher Scientific and Co., Pittsburgh, PA, USA), supplemented with 10% fetal calf serum (FCS) and antibiotics (Life Technologies Inc., Gaithersburg, MD, USA). Docetaxel and 5-FU were stored at 4˚C and dissolved in sterile water at the time of use. The HNSCC cell lines were incubated with different concentrations of docetaxel (1.0 and 5.0 μmol/ml) or 5-FU (1.0 and 5.0 μmol/ml) for 2, 3, 5, 8 and up to 10 days. These different drug concentrations were selected after performing the alamarBlue (AbD Serotec, Oxford, UK) cell proliferation assay, quantitatively measuring proliferation of HNSCC tumor cell lines and establishing the relative cytotoxicity of the chemotherapeutic drugs examined. After the defined incubation time and centrifugation, the supernatants were collected in sterile tubes and stored at –20˚C until further analysis.

Immunohistochemistry for PDGFα/β. Immunohistochemical studies were performed using a monoclonal rabbit antibody directed against human PDGFα/β (ACRIS Antibodies, Herford, Germany). Cells were cultured overnight on glass coverslips (Nunc, Wiesbaden, Germany) before immunohistochemistry was performed. When 50% confluent, cells were exposed to different chemotherapeutic drug concentrations and different incubation periods (48, 120 and 240 h) and underwent fixation with acetone and alcohol (2:1). Afterwards, cells were washed with phosphate-buffered saline (PBS). Adjacent cells were then incubated with peroxidase block (Dako, Hamburg, Germany) for 30 min. Cells were then washed three times with PBS for 5 min each time. This was followed by incubation with 10% sheep serum for 30 min. Cells were exposed to the rabbit monoclonal antibody against PDGFα/β as the primary antibody solution for 30 min at room temperature, using a working dilution of antibody to cells of 1:100. The incubated cell lines were then refrigerated overnight. Afterwards, cells were washed three times with PBS and incubated with a secondary antibody (antirabbit; Amersham, Freiburg, Germany) in a 1:100 solution for 45 min at room temperature. Following incubation, cells were washed three times. Subsequently, cells were exposed to an enzyme suspension [AEC, aminoethylcarbazole (red) or DAB, diaminobenzidine (brown)] for 5-15 min. The sections underwent a counterstaining with Mayer's hematoxylin for 30 s. This procedure was followed by dehydration in graded ethanol and coverslipping.

The immunohistochemical PDGF expression was determined semi-quantitatively. The staining intensity was described as follows (after counting 100 cells per slide): strong reactivity, >80% of the cells were positive (indicated as +++); moderate reactivity, 50-80% of the cells stained positive (indicated as ++); weak reactivity, <50% of the cells were positive (indicated as +); negative immunostained cells, 0% reactivity (indicated as 0).

Enzyme-linked immunosorbent assay (ELISA) for total PDGFRα/β and VEGF. After incubation in 6-well chambers with different chemotherapeutic drug concentrations, cells were rinsed with PBS and then 350 μl per well of lysis buffer was added. After gently agitating lysed cells with a vortex at 2-8˚C for 30 min and micro-centrifuging at 14,000 ×g for 5 min, the supernatant was transferred into a clean tube.

VEGF concentrations were determined by ELISA (R&D Systems, Wiesbaden, Germany). The system used a solid-phase monoclonal antibody and an enzyme-linked polyclonal antibody against recombinant VEGF165. The specificity of antibodies to human VEGF used in the ELISA kit was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blotting. According to the manufacturer's directions, each ELISA was performed on 100 μl of supernatant. All analyses and calibrations were carried out in duplicate. The calibrations on each microtiter plate included recombinant human VEGF standards that were provided in the kit. Optical density was determined using a microplate reader at a wavelength of 450 nm. Wavelength correction was set to 540 nm and concentrations are reported as pg/ml. The interassay coefficient of variation reported by the manufacturer varied from 6.2 to 8.8%, range of detection was 62.4 to 4,000 pg/ml.

The DuoSet IC ELISA (R&D Systems, Wiesbaden, Germany) measures human PDGFR with an immobilized capture antibody specific for human PDGFR, which binds both tyrosine-phosphorylated and unphosphorylated PDGFR. After washing away the unbound material, a biotinylated detection antibody specific for total human PDGFR is used to detect tyrosine-phosphorylated and unphosphorylated PDGFR, utilizing a standard streptavidin-horseradish peroxidase (HRP) format.

The capture antibody was diluted 1:180 (4 μg/ml). The diluted capture antibody was added at 100 μl per well and the plate was sealed and incubated overnight. Afterwards, the contents of each well were aspirated and the wells were washed three times with 400 μl Tween wash buffer. The plates were blocked by adding 300 μl blocking buffer to each well and incubation at room temperature for 1-2 h. According to the manufacturer's directions, each ELISA was carried out with 100 μl of supernatant of the sample. Washing with Tween buffer followed, as described. After diluting the detection antibody to a concentration of 500 ng/ml, the detection antibody (100 μl) was added to each well for an incubation period of 2 h. Again cells were washed. Adding 100 μg of streptavidin-HRP to each well was followed by incubation for 20 min at room temperature. Afterwards, 100 μl of substrate solution was added to each well for 20 min followed by 50 μl of stop solution. Optical density was determined using a microplate reader, settings as described above. The inter-assay coefficient of variation reported by the manufacturer was below 10%, range of detection for PDGFRα was 312 to 20,000 pg/ml and for PDGFRβ 250 to 16,000 pg/ml. All analyses and calibrations were carried out three times.

Table I. *Grading of immunostaining for platelet-derived growth factor (PDGF) α/β: 5-FU (5-fluorouracil), HNSCC (head and neck squamous cell carcinoma) 11A and 14C.*

	Immunostaining index after		
	48 h	120 h	240 h
Negative control			
HNSCC 11A	+++	++	+++
HNSCC 14C	++	+++	++
CERV196	+++	+++	+++
5-FU (5 μmol/ml)			
HNSCC 11A	+++	+++	++
HNSCC 14C	++	+	++
CERV196	+++	++	+++
Docetaxel (5 μmol/ml)			
HNSCC 11A	++	+	0
HNSCC 14C	++	++	+
CERV196	+++	+++	+

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 means procedure. A p-value ≤0.05 was considered statistically significant. The performed statistical tests were the two-coefficient variance test (SAS Statistics, Cary, NC, USA) and the Dunnett's test.

Results

Immunohistochemistry for platelet-derived growth factor (PDGF) α/β in HNSCC 11A, 14C and CERV196 cells. Two independent observers assessed the immunohistochemical staining for PDGFα/β in order to estimate the rates of protein expression. All tumor cell lines, regardless of HPV status, expressed PDGFα/β to a similar extent. The controls showed high reactivity for PDGFα/β. Furthermore, HNSCC 11A and 14C cells presented a lower reactivity of PDGFα/β expression, dependent on an extended incubation period with docetaxel.

HPV-positive CERV196 cells exhibited reduction of PDGFα/β expression under incubation with docetaxel. Incubation with 5-FU showed no clear reduction in the expression of PDGFα/β. In both cases (5-FU and docetaxel) increased drug concentrations had no effect on an altering PDGFα/β expression. Immunostaining was localized in the cytoplasm of the cells (Figure 1, Table I).

ELISA for VEGF expression in HNSCC 11A, 14C and CERV196 cells. VEGF was expressed in all evaluated cell lines and expression levels of VEGF in HPV-negative and -positive SCCs were rather similar. There was a clear trend towards decreased levels when cells were incubated with chemotherapeutic drugs. This trend can be seen more

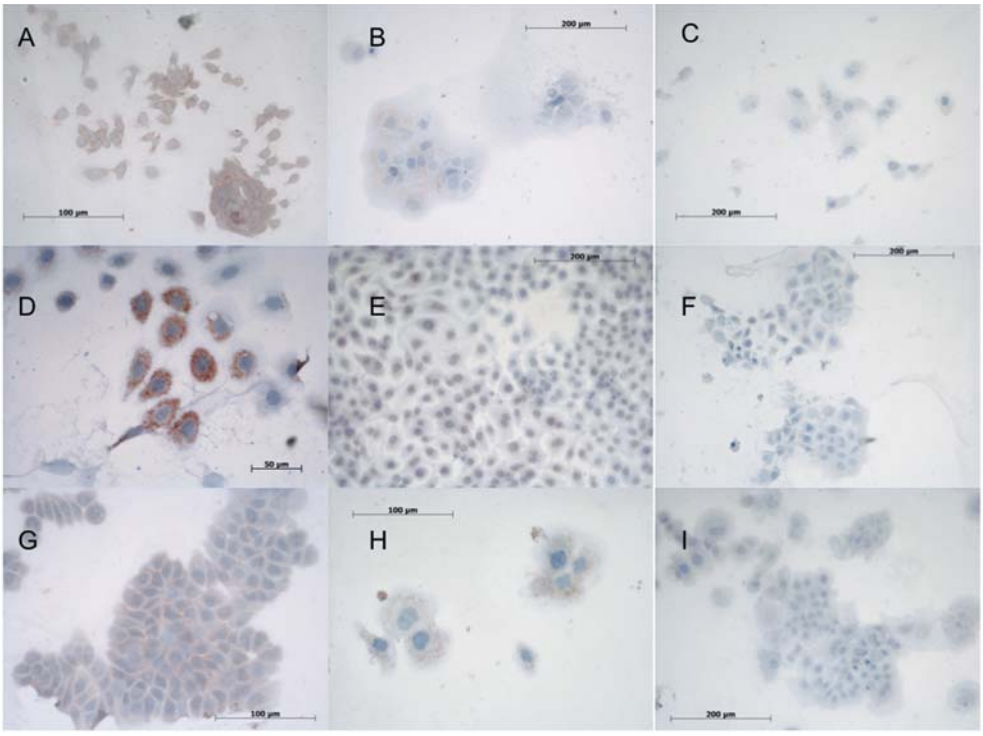

Figure 1. *Immunohistochemical staining for platelet-derived growth factor (PDGF) α/β: Head and neck squamous cell carcinoma (HNSCC) 11A negative control with positive reactivity after 48 h of incubation (A), and after incubation with docetaxel (5.0 μmol/ml) for 120 h (B) and 240 h (C). HNSCC 14C negative control with positive reactivity after 48 h of incubation (D), and after incubation with docetaxel (5.0 μmol/ml) for 120 h (E) and 240 h (F). CERV196 negative control of CERV196 with positive reactivity and typical growth pattern in tumor cell colonies after 48h of incubation (G), and after incubation with docetaxel (5.0 μmol/ml) for 120 h (H) and 240 h (I).*

clearly after longer incubation (see Figure 2), whereas different drug concentrations had no statistically significant effects on the expression of VEGF. Therefore only the data for 5-FU and docetaxel at a concentration of 5.0 μmol/ml are shown. 5-FU led to a statistically significant reduction of the expression of VEGF for HNSCC 11A cells, after five days (p-value=0.0069) and for HNSCC 14C after three days of incubation (p-value=0.0027) when compared to the negative control, but had no significant effect on the VEGF expression in CERV196 cells when compared to the negative control.

Docetaxel induced a significant reduction of the VEGF expression for HNSCC 11A with a p-value of <0.0001, with one exception after eight days of incubation (p-value=0.0009). For HNSCC 14C cells there was a statistically significant reduction in VEGF expression after five and eight days of incubation with docetaxel, p-values being 0.0098 and 0.0009, respectively. For CERV196 cells there was a significant decrease of VEGF expression after 10 days of incubation with a p-value of 0.0242 (Table II, Figure 2).

Comparing the impact of 5-FU with docetaxel concerning the reduction of VEGF expression, there was a statistically highly significant difference for HNSCC 11A cells, regardless of the time of incubation (p-value=0.0001). For HNSCC 14C cells significant differences between 5-FU and docetaxel were detected after three and eight days of incubation (p-values=0.0085 and 0.0051). In CERV196 cells there was a statistically significant difference between the effect of 5-FU and docetaxel after 10 days of incubation (p-value=0.0361).

ELISA for PDGFRα/β expression in HNSCC 11A, 14C and CERV196 cells. Expression of PDGFRα can be detected in all studied cell lines. Expression levels in CERV196 cells were almost twice as high as in the HPV-negative SCC cell lines. No clear trend towards an alteration of PDGFRα expression by 5-FU or docetaxel was detected. Concerning PDGFRα there was no statistically significant reduction in expression irrespective of the applied chemotherapeutic drug, the concentration used or the incubation time when compared to the negative control (Table III. and Figure 3).

PDGFRβ was expressed at lower levels in all cell lines compared to the levels measured for PDGFRα and VEGF in negative control.

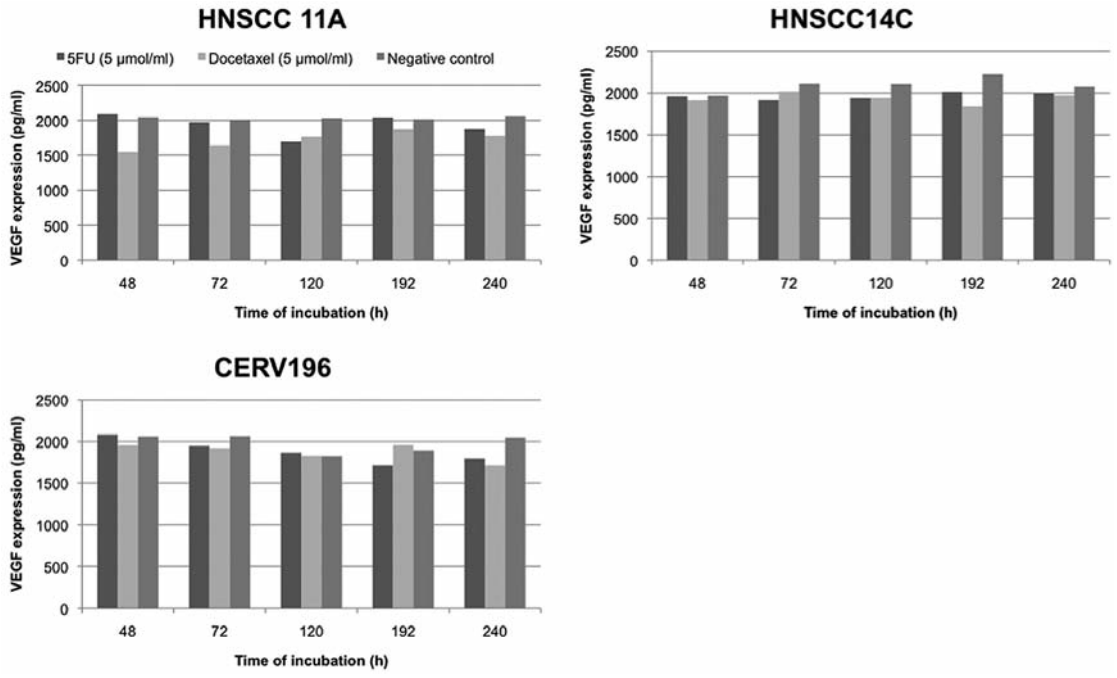

Figure 2. *Vascular endothelial growth factor (VEGF) expression in HNSCC 11A, 14C and CERV196 cells under treatment with 5-FU and docetaxel compared to the negative control. Data are mean values.*

5-FU had a statistically significant effect on the expression of PDGFRβ in HNSCC 14C cells after five and eight days of incubation, with *p*-values of 0.0373 and 0.0001 respectively, and for CERV196 cells after 10 days of incubation (*p*-value=0.0346).

Similar results were found for docetaxel. There was a statistically significant alteration of PDGFRβ levels in HNSCC 14C cells after eight days of incubation (*p*-value <0.0001) and in CERV196 cells after 10 days of incubation (*p*-value=0.0038).

Neither drug had a statistically significant effect on the expression of PDGFRβ in HNSCC 11A cells (Table IV. and Figure 4).

In the comparison of 5-FU with docetaxel, there was a statistically significant difference for HNSCC 14C cells after eight days of incubation (*p*-value=0.0035). For HNSCC 11A and CERV196 cells, there was no statistically significant difference in the effect of 5-FU and docetaxel at all.

Discussion

Although docetaxel is not an anti-angiogenic drug, our study showed it to cause a significant reduction of the analyzed angiogenic factors PDGFR and VEGF, irrespective of the pro-apoptotic potential. Previously published data showed a significant impact of imatinib, a specific anti-angiogenic

Table II. *Vascular endothelial growth factor (VEGF) expression (pg/ml) by enzyme-linked immunosorbent assay (ELISA) in HNSCC 11A, 14C and CERV196 cells under treatment with 5-fluorouracil (5-FU) and docetaxel, compared to the negative control. Data are mean values and p-values. Statistical significance is shown in bold.*

Time of incubation (h)	Negative control	5-FU (5 µmol/ml)		Docetaxel (5 µmol/ml)	
	Mean value	Mean value	*p*-Value	Mean value	*p*-Value
HNSCC 11A					
48	2044.5	2087.667	0.1245	1546.667	**<0.0001**
72	2003.667	1973	0.3879	1644	**<0.0001**
120	2030	1696	**0.0069**	1767.667	**<0.0001**
197	2014.5	2041.333	0.9081	1871	**0.0009**
240	2055.333	1873	0.3372	1779.667	**<0.0001**
HNSCC 14C					
48	1969	1961.333	0.9030	1913.333	0.9370
72	2111	1915.333	**0.0027**	2019.5	0.3495
120	2108	1940	0.2402	1942.333	**0.0098**
197	2224.5	2014.667	0.2098	1843.333	**0.0009**
240	2075.5	1996.333	0.5306	1974	0.1409
CERV196					
48	2060.667	2084	0.9528	1957.333	0.8268
72	2064.667	1947.667	0.6023	1922	0.8606
120	1823.667	1866.333	0.6701	1827.333	0.9239
197	1893.667	1717.667	0.8091	1959.333	0.9815
240	2048.333	1794.667	0.6082	1717.667	**0.0242**

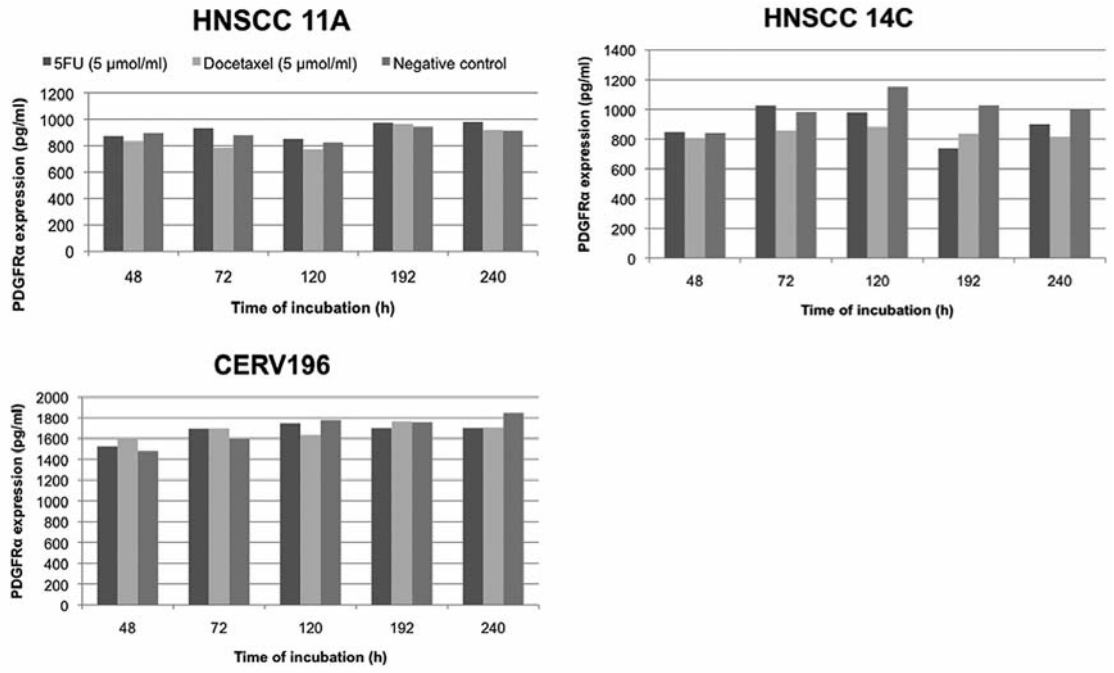

Figure 3. *Platelet-derived growth factor receptor (PDGFR)-α expression in HNSCC 11A, 14C and CERV196 cells under treatment with 5-fluorouracil (5-FU) and docetaxel compared to the negative control. Data are mean values.*

Table III. *Platelet-derived growth factor receptor (PDGFR) α expression (pg/ml) by enzyme linked immunosorbent assay (ELISA) in HNSCC 11A, 14C and CERV196 cells under treatment with 5-fluorouracil (5-FU) and docetaxel compared to the negative control. Data are mean values and p-values. Statistical significance is shown in bold.*

Time of incubation (h)	Negative control	5-FU (5 µmol/ml)		Docetaxel (5 µmol/ml)	
	Mean value	Mean value	*p*-value	Mean value	*p*-value
HNSCC 11A					
48	895.667	871.5	0.9786	834.333	0.9403
72	878.2	934.0	0.9998	783.667	0.9842
120	825.367	848.767	0.9382	772.3	0.9969
197	941.333	972.667	0.9908	963.333	0.9841
240	914.333	979.667	1.000	920.0	0.9968
HNSCC 14C					
48	842.133	848.667	0.9815	807.0	0.8102
72	981.033	1026.567	0.9806	859.333	0.1174
120	1150.667	978.333	0.1522	882.333	0.1009
197	1028.0	738.333	0.1535	836.667	0.0523
240	1000.333	899.333	0.2253	815.667	0.1358
CERV196					
48	1477.95	1523.533	0.9514	1594.867	0.3115
72	1602.467	1694.667	0.5508	1697.5	0.8833
120	1779.033	1743.333	0.6340	1632.667	0.6216
197	1756.015	1700.667	0.9042	1767.033	0.6579
240	1852.333	1701.667	0.3385	1707.2	0.1951

Table IV. *Platelet-derived growth factor receptor (PDGFR) β expression (pg/ml) by enzyme linked immunosorbent assay (ELISA) in HNSCC 11A, 14C and CERV196 cells under treatment with 5-FU and docetaxel compared to the negative control. Data are mean-values and p-values. Statistical significance is shown in bold.*

Time of incubation (h)	Negative control	5-FU (5 µmol/ml)		Docetaxel (5 µmol/ml)	
	Mean value	Mean value	*p*-value	Mean value	*p*-value
HNSCC 11A					
48	80.707	69.967	0.8182	75.233	0.9919
72	85.977	59.973	0.2339	71.647	0.1030
120	101.77	80.677	0.6754	71.1	0.1669
197	78.48	78.837	0.9906	67.223	0.9174
240	101.66	57.673	0.0583	67.623	0.0669
HNSCC 14C					
48	51.267	36.013	0.0771	43.75	0.0666
72	50.5	41.633	0.2234	47.027	0.7380
120	70.773	60.77	**0.0373**	66.133	0.3327
197	96.88	70.857	**0.0001**	49.327	**<0.0001**
240	75.953	45.35	0.0750	50.463	0.1264
CERV196					
48	71.667	84.0	0.0266	95.7	0.0036
72	102.817	85.8	0.1731	93.067	0.5147
120	103.507	87.333	0.6074	79.553	0.1436
197	101.133	89.64	0.6264	80.723	0.2312
240	126.4	84.233	**0.0346**	76.75	**0.0038**

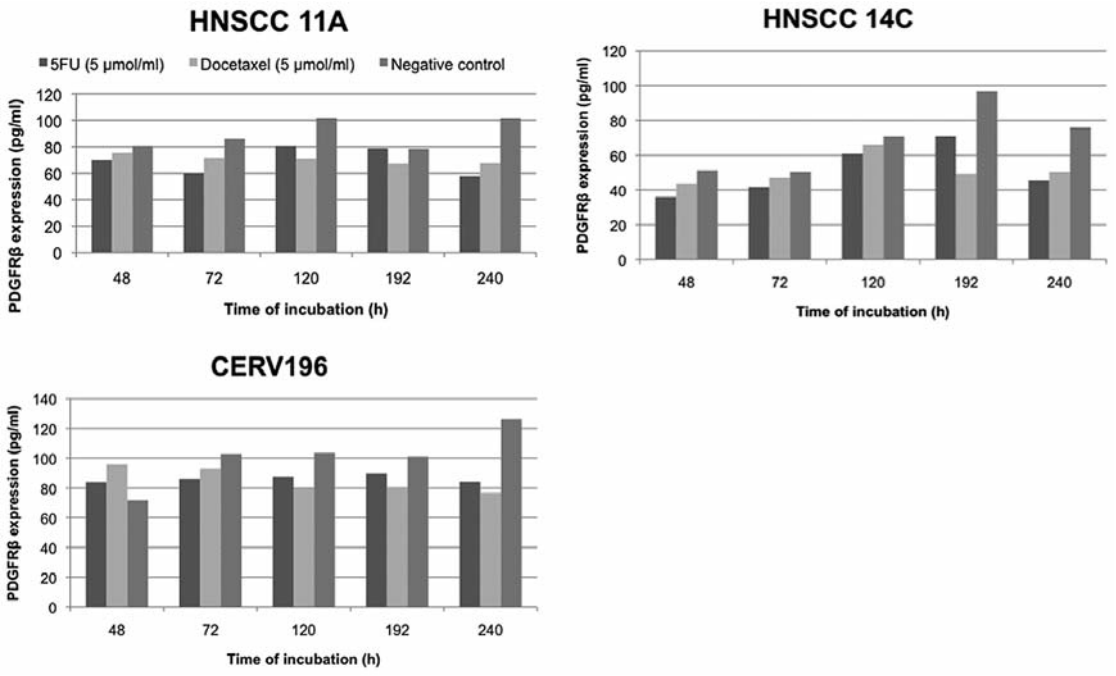

Figure 4. *Platelet-derived growth factor receptor (PDGFR)-β expression in HNSCC 11A, 14C and CERV196 cells under treatment with 5-FU and docetaxel compared to the negative control. Data are mean values.*

drug, on the expression levels of PDGFR and VEGF, indicating the possible use of this agent for targeted therapy, especially because dysregulation of PDGF/PDGFR and VEGF signaling is a known functional element in the tumor biology of HNSCC and correlates with poor clinical outcome for the patient (53-55, 57, 59, 60, 77, 78).

In our study we showed that VEGF is expressed at similar levels in all the studied cell types. 5-FU appears to be less effective at altering the expression of VEGF in all cell lines compared to docetaxel. A significant effect could be observed after two and three days in HPV-negative HNSCC but this effect could not be maintained during prolonged incubation. 5-FU had no impact on VEGF expression in CERV196 cells at all. Docetaxel, on the contrary, led to a highly significant reduction of VEGF expression in HNSCC 11A cells independently of the period of incubation. In 14C cells a significant alteration after five and eight days of incubation was detected. However, HPV-positive SCCs, like CERV196 cells seem to be less susceptible for docetaxel-related VEGF suppression than HPV-negative SCCs. In the context of its anti-mitotic effect by disturbing the function of spindle fibers, docetaxel appears to be surprisingly effective in HNSCC cells at reducing expression of angiogenic factors. Docetaxel is known to be more cytotoxic than 5-FU by directly affecting mitosis and the spindle fibers. Again CERV196 cells seem to be less vulnerable towards the applied chemotherapeutic drug. It has been shown that VEGF expression in HNSCC tumors strongly correlates with tumor angiogenesis and inversely correlates with apoptosis, which confirms an anti-apoptotic potential of VEGF (54, 55).

For PDGFRα, no significant decrease of expression was detected in this study independently of the drug, concentration or time of incubation used. Expression levels of PDGFRα were lower compared to VEGF levels. Although no statistically significant data were collected, *p*-values fell after a prolonged incubation time in the case of HNSCC 14C and CERV196 cells indicating the need for extended observation. PDGFRβ was expressed at lower levels compared to PDGFRα and VEGF in all cell lines. Interestingly no significant alteration of expression was detected in HNSCC 11A. In HNSCC 14C cells we showed a decrease of expression by 5-FU after five and eight days and by docetaxel after 10 days. In the case of CERV196 there was only a significant reduction after 10 days of incubation with 5-FU and docetaxel. These data suggest that PDGFR reduction is possible but requires a longer period of incubation with the drugs applied in this study.

The results of immunohistochemistry for PDGF support the findings of ELISA showing a lack of alteration of PDGF expression by 5-FU, and a considerable impact of docetaxel on HPV-negative HNSCC compared to HPV-positive CERV196 cells.

Neither 5-FU nor docetaxel were originally designed for targeting VEGF or PDGFR. So specifically designed inhibitors in a targeted therapy are a promising opportunity to reduce tumor progression. A study by Fujita *et al*. demonstrated a significant anti-proliferative effect of bevacizumab (Avastin®), a monoclonal antibody against VEGF, *in vivo*. Interestingly its combination with paclitaxel, another taxane like docetaxel, had synergistic therapy effects, with reduced blood vessel density and an increased apoptotic index (79). Furthermore, a study by Schultz *et al*. showed that targeted therapy with imatinib, a tyrosine kinase inhibitor, leads to a highly significant reduction of PDGFR and VEGF expression and revealed synergistic effects with standard chemotherapeutic drugs such as carboplatin (77, 78). Thus the use of a targeted antiangiogenic therapy in combination with taxanes and/or platinum-based chemotherapeutic drugs in multimodal chemotherapy could help to improve the therapeutic effect and eventually the outcome for the patient (70, 72, 73, 78, 79).

In summary the inclusion of a substance specifically targeting angiogenic factors in therapy regimes with platinum-based chemotherapeutic drugs or taxanes in the treatment of HNSCC might enhance the efficacy of standard medication and eventually improve the outcome and long-term survival of patients with oropharyngeal cancer. It is, therefore, important to see HPV-positive SCC as a distinct tumor entity of oropharyngeal cancer.

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References

1 Ferlay J, Shin HR, Bray F, Forman D, Mathers C and Parkin DM: Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer *127*: 2893-2917, 2010.

2 Edwards BK, Brown ML, Wingo PA *et al*: Annual report to the nation on the status of cancer, 1975-2002, featuring population-based trends in cancer treatment. J Natl Cancer Inst *97*: 1407-1427, 2005.

3 Shiboski CH, Schmidt BL and Jordan RC: Tongue and tonsil carcinoma: increasing trends in the U.S. population ages 20-44 years. Cancer *103*: 1843-1849, 2005.

4 Pindborg JJ, Zheng KH, Kong CR and Lin FX: Pilot survey of oral mucosa in areca (betel) nut chewers on Hainan Island of the People's Republic of China. Community Dent Oral Epidemiol *12*: 195-196, 1984.

5 Licitra L, Bernier J, Grandi C, Merlano M, Bruzzi P and Lefebvre JL: Cancer of the oropharynx. Crit Rev Oncol Hematol *41*: 107-122, 2002.

6 Sturgis EM and Cinciripini PM: Trends in head and neck cancer incidence in relation to smoking prevalence: an emerging epidemic of human papillomavirus-associated cancers? Cancer *110*: 1429-1435, 2007.

7 Brugere J, Guenel P, Leclerc A and Rodriguez J: Differential effects of tobacco and alcohol in cancer of the larynx, pharynx, and mouth. Cancer *57*: 391-395, 1986.

8 Slaughter DP, Southwick HW and Smejkal W: Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin. Cancer *6*: 963-968, 1953.

9 Romanitan M, Nasman A, Ramqvist T *et al*: Human papillomavirus frequency in oral and oropharyngeal cancer in Greece. Anticancer Res *28*: 2077-2080, 2008.

10 Chaturvedi AK, Engels EA, Anderson WF and Gillison ML: Incidence trends for human papillomavirus-related and -unrelated oral squamous cell carcinomas in the United States. J Clin Oncol *26*: 612-619, 2008.

11 Gillison ML, D'Souza G, Westra W *et al*: Distinct risk factor profiles for human papillomavirus type 16-positive and human papillomavirus type 16-negative head and neck cancers. J Natl Cancer Inst *100*: 407-420, 2008.

12 Nasman A, Attner P, Hammarstedt L *et al*: Incidence of human papillomavirus (HPV) positive tonsillar carcinoma in Stockholm, Sweden: an epidemic of viral-induced carcinoma? Int J Cancer *125*: 362-366, 2009.

13 Hansson BG, Rosenquist K, Antonsson A *et al*: Strong association between infection with human papillomavirus and oral and oropharyngeal squamous cell carcinoma: a population-based case-control study in southern Sweden. Acta Otolaryngol *125*: 1337-1344, 2005.

14 Brandsma JL and Abramson AL: Association of papillomavirus with cancers of the head and neck. Arch Otolaryngol Head Neck Surg *115*: 621-625, 1989.

15 Kreimer AR, Clifford GM, Boyle P and Franceschi S: Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review. Cancer Epidemiol Biomarkers Prev *14*: 467-475, 2005.

16 Applebaum KM, Furniss CS, Zeka A *et al*: Lack of association of alcohol and tobacco with HPV16-associated head and neck cancer. J Natl Cancer Inst *99*: 1801-1810, 2007.

17 Pintos J, Black MJ, Sadeghi N *et al*: Human papillomavirus infection and oral cancer: a case-control study in Montreal, Canada. Oral Oncol *44*: 242-250, 2008.

18 Smith EM, Ritchie JM, Summersgill KF *et al*: Human papillomavirus in oral exfoliated cells and risk of head and neck cancer. J Natl Cancer Inst *96*: 449-455, 2004.

19 Termine N, Panzarella V, Falaschini S *et al*: HPV in oral squamous cell carcinoma vs head and neck squamous cell carcinoma biopsies: a meta-analysis (1988-2007). Ann Oncol *19*: 1681-1690, 2008.

20 Smith EM, Hoffman HT, Summersgill KS, Kirchner HL, Turek LP and Haugen TH: Human papillomavirus and risk of oral cancer. Laryngoscope *108*: 1098-1103, 1998.

21 Koch WM, Lango M, Sewell D, Zahurak M and Sidransky D: Head and neck cancer in nonsmokers: a distinct clinical and molecular entity. Laryngoscope *109*: 1544-1551, 1999.

22 Tachezy R, Klozar J, Rubenstein L *et al*: Demographic and risk factors in patients with head and neck tumors. J Med Virol *81*: 878-887, 2009.

23 Gillison ML, Koch WM, Capone RB *et al*: Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. J Natl Cancer Inst *92*: 709-720, 2000.

24 D'Souza G, Kreimer AR, Viscidi R *et al*: Case-control study of human papillomavirus and oropharyngeal cancer. N Engl J Med *356*: 1944-1956, 2007.

25 Le Buanec H, D'Anna R, Lachgar A *et al*: HPV-16 E7 but not E6 oncogenic protein triggers both cellular immunosuppression and angiogenic processes. Biomed Pharmacother *53*: 424-431, 1999.
26 Le Buanec H, Lachgar A, D'Anna R *et al*: Induction of cellular immunosuppression by the human papillomavirus type 16 E7 oncogenic protein. Biomed Pharmacother *53*: 323-328, 1999.
27 Califano J, van der Riet P, Westra W *et al*: Genetic progression model for head and neck cancer: implications for field cancerization. Cancer Res *56*: 2488-2492, 1996.
28 Goodwin EC and DiMaio D: Repression of human papillomavirus oncogenes in HeLa cervical carcinoma cells causes the orderly reactivation of dormant tumor suppressor pathways. Proc Natl Acad Sci USA *97*: 12513-12518, 2000.
29 Wiest T, Schwarz E, Enders C, Flechtenmacher C and Bosch FX: Involvement of intact HPV16 E6/E7 gene expression in head and neck cancers with unaltered p53 status and perturbed pRb cell cycle control. Oncogene *21*: 1510-1517, 2002.
30 Wells SI, Francis DA, Karpova AY, Dowhanick JJ, Benson JD and Howley PM: Papillomavirus E2 induces senescence in HPV-positive cells *via* pRB- and p21(CIP)-dependent pathways. EMBO J *19*: 5762-5771, 2000.
31 Butz K, Geisen C, Ullmann A, Spitkovsky D and Hoppe-Seyler F: Cellular responses of HPV-positive cancer cells to genotoxic anti-cancer agents: repression of E6/E7-oncogene expression and induction of apoptosis. Int J Cancer *68*: 506-513, 1996.
32 Bristow RG, Benchimol S and Hill RP: The p53 gene as a modifier of intrinsic radiosensitivity: implications for radiotherapy. Radiother Oncol *40*: 197-223, 1996.
33 Lindquist D, Romanitan M, Hammarstedt L *et al*: Human papillomavirus is a favourable prognostic factor in tonsillar cancer and its oncogenic role is supported by the expression of E6 and E7. Mol Oncol *1*: 350-355, 2007.
34 Lopez-Ocejo O, Viloria-Petit A, Bequet-Romero M, Mukhopadhyay D, Rak J and Kerbel RS: Oncogenes and tumor angiogenesis: the HPV-16 E6 oncoprotein activates the vascular endothelial growth factor (VEGF) gene promoter in a p53 independent manner. Oncogene *19*: 4611-4620, 2000.
35 Toussaint-Smith E, Donner DB and Roman A: Expression of human papillomavirus type 16 E6 and E7 oncoproteins in primary foreskin keratinocytes is sufficient to alter the expression of angiogenic factors. Oncogene *23*: 2988-2995, 2004.
36 Tang X, Zhang Q, Nishitani J, Brown J, Shi S and Le AD: Overexpression of human papillomavirus type 16 oncoproteins enhances hypoxia-inducible factor 1 alpha protein accumulation and vascular endothelial growth factor expression in human cervical carcinoma cells. Clin Cancer Res *13*: 2568-2576, 2007.
37 Dahm-Daphi J: p53: biology and role for cellular radiosensitivity. Strahlenther Onkol *176*: 278-285, 2000.
38 Lindel K, Beer KT, Laissue J, Greiner RH and Aebersold DM: Human papillomavirus positive squamous cell carcinoma of the oropharynx: a radiosensitive subgroup of head and neck carcinoma. Cancer *92*: 805-813, 2001.
39 Fakhry C, Westra WH, Li S *et al*: Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a prospective clinical trial. J Natl Cancer Inst *100*: 261-269, 2008.
40 Park CC, Bissell MJ and Barcellos-Hoff MH: The influence of the microenvironment on the malignant phenotype. Mol Med Today *6*: 324-329, 2000.
41 De Wever O and Mareel M: Role of tissue stroma in cancer cell invasion. J Pathol *200*: 429-447, 2003.
42 Tlsty TD: Stromal cells can contribute oncogenic signals. Semin Cancer Biol *11*: 97-104, 2001.
43 Bhowmick NA, Neilson EG and Moses HL: Stromal fibroblasts in cancer initiation and progression. Nature *432*: 332-337, 2004.
44 Bhowmick NA, Chytil A, Plieth D *et al*: TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. Science *303*: 848-851, 2004.
45 Micke P and Ostman A: Exploring the tumour environment: cancer-associated fibroblasts as targets in cancer therapy. Expert Opin Ther Targets *9*: 1217-1233, 2005.
46 Joyce JA: Therapeutic targeting of the tumor microenvironment. Cancer Cell *7*: 513-520, 2005.
47 Hanahan D and Weinberg RA: Hallmarks of cancer: the next generation. Cell *144*: 646-674, 2011.
48 Hanahan D and Weinberg RA: The hallmarks of cancer. Cell *100*: 57-70, 2000.
49 Ferrara N: The role of vascular endothelial growth factor in pathological angiogenesis. Breast Cancer Res Treat *36*: 127-137, 1995.
50 Zhang HT, Craft P, Scott PA *et al*: Enhancement of tumor growth and vascular density by transfection of vascular endothelial cell growth factor into MCF-7 human breast carcinoma cells. J Natl Cancer Inst *87*: 213-219, 1995.
51 Eisma RJ, Spiro JD and Kreutzer DL: Vascular endothelial growth factor expression in head and neck squamous cell carcinoma. Am J Surg *174*: 513-517, 1997.
52 Mineta H, Miura K, Ogino T *et al*: Prognostic value of vascular endothelial growth factor (VEGF) in head and neck squamous cell carcinomas. Br J Cancer *83*: 775-781, 2000.
53 Salven P, Heikkila P, Anttonen A, Kajanti M and Joensuu H: Vascular endothelial growth factor in squamous cell head and neck carcinoma: expression and prognostic significance. Mod Pathol *10*: 1128-1133, 1997.
54 Riedel F, Gotte K, Schwalb J, Wirtz H, Bergler W and Hormann K: Serum levels of vascular endothelial growth factor in patients with head and neck cancer. Eur Arch Otorhinolaryngol *257*: 332-336, 2000.
55 Riedel F, Gotte K, Schwalb J, Schafer C and Hormann K: Vascular endothelial growth factor expression correlates with p53 mutation and angiogenesis in squamous cell carcinoma of the head and neck. Acta Otolaryngol *120*: 105-111, 2000.
56 Heldin CH and Westermark B: Mechanism of action and *in vivo* role of platelet-derived growth factor. Physiol Rev *79*: 1283-1316, 1999.
57 Plate KH, Breier G, Farrell CL and Risau W: Platelet-derived growth factor receptor-beta is induced during tumor development and upregulated during tumor progression in endothelial cells in human gliomas. Lab Invest *67*: 529-534, 1992.
58 Risau W, Drexler H, Mironov V *et al*: Platelet-derived growth factor is angiogenic *in vivo*. Growth Factors *7*: 261-266, 1992.
59 Pietras K, Ostman A, Sjoquist M *et al*: Inhibition of platelet-derived growth factor receptors reduces interstitial hypertension and increases transcapillary transport in tumors. Cancer Res *61*: 2929-2934, 2001.

60 Forsberg K, Valyi-Nagy I, Heldin CH, Herlyn M and Westermark B: Platelet-derived growth factor (PDGF) in oncogenesis: development of a vascular connective tissue stroma in xenotransplanted human melanoma producing PDGF-BB. Proc Natl Acad Sci USA *90*: 393-397, 1993.
61 Bran B, Bran G, Hormann K and Riedel F: The platelet-derived growth factor receptor as a target for vascular endothelial growth factor-mediated anti-angiogenetic therapy in head and neck cancer. Int J Oncol *34*: 255-261, 2009.
62 Aebersold DM, Froehlich SC, Jonczy M *et al*: Expression of transforming growth factor-alpha, epidermal growth factor receptor and platelet-derived growth factors A and B in oropharyngeal cancers treated by curative radiation therapy. Radiother Oncol *63*: 275-283, 2002.
63 Skobe M and Fusenig NE: Tumorigenic conversion of immortal human keratinocytes through stromal cell activation. Proc Natl Acad Sci USA *95*: 1050-1055, 1998.
64 Shao ZM, Nguyen M and Barsky SH: Human breast carcinoma desmoplasia is PDGF initiated. Oncogene *19*: 4337-4345, 2000.
65 Dong J, Grunstein J, Tejada M *et al*: VEGF-null cells require PDGFR alpha signaling-mediated stromal fibroblast recruitment for tumorigenesis. EMBO J *23*: 2800-2810, 2004.
66 Board R and Jayson GC: Platelet-derived growth factor receptor (PDGFR): a target for anticancer therapeutics. Drug Resist Updat *8*: 75-83, 2005.
67 Trafalis DT, Alifieris C, Dalezis P, Geromichalos G and Sitaras NM: Indications for an alternative effective treatment of head and neck squamous cell carcinoma with temsirolimus plus bevacizumab: from bench to bedside? Anticancer Drugs *23*: 874-882, 2012.
68 Armand JP: Focus on cellular pharmacology of docetaxel. Bull Cancer *90*: 1067-1070, 2003.
69 Bissery MC: Preclinical pharmacology of docetaxel. Eur J Cancer *31A(Suppl 4)*: S1-6, 1995.
70 Schultz JD, Bran G, Anders C *et al*: Induction chemotherapy with TPF (Docetaxel, Carboplatin and Fluorouracil) in the treatment of locally advanced squamous cell carcinoma of the head and neck. Oncol Rep *24*: 1213-1216, 2010.
71 Ahn JS, Cho SH, Kim OK *et al*: The efficacy of an induction chemotherapy combination with docetaxel, cisplatin, and 5-FU followed by concurrent chemoradiotherapy in advanced head and neck cancer. Cancer Res Treat *39*: 93-98, 2007.
72 Posner MR, Hershock DM, Blajman CR *et al*: Cisplatin and fluorouracil alone or with docetaxel in head and neck cancer. N Engl J Med *357*: 1705-1715, 2007.
73 Huang GC, Liu SY, Lin MH, Kuo YY and Liu YC: The synergistic cytotoxicity of cisplatin and taxol in killing oral squamous cell carcinoma. Jpn J Clin Oncol *34*: 499-504, 2004.
74 Pinedo HM and Peters GF: Fluorouracil: biochemistry and pharmacology. J Clin Oncol *6*: 1653-1664, 1988.
75 Diasio RB and Harris BE: Clinical pharmacology of 5-fluorouracil. Clin Pharmacokinet 16: 215-237, 1989.
76 Milano G and Etienne MC: Dihydropyrimidine dehydrogenase (DPD) and clinical pharmacology of 5-fluorouracil (review). Anticancer Res *14*: 2295-2297, 1994.
77 Schultz JD, Muhlheim K, Erben P *et al*: Chemotherapeutic alteration of VEGF-/PDGF- and PDGF-Ralpha/beta expression by imatinib in HPV-transformed squamous cell carcinoma compared to HPV-negative HNSCC *in vitro*. Oncol Rep *26*: 1099-1109, 2011.
78 Schultz JD, Rotunno S, Riedel F *et al*: Synergistic effects of imatinib and carboplatin on VEGF, PDGF and PDGF-Ralpha/ss expression in squamous cell carcinoma of the head and neck *in vitro*. Int J Oncol *38*: 1001-1012, 2011.
79 Fujita K, Sano D, Kimura M *et al*: Anti-tumor effects of bevacizumab in combination with paclitaxel on head and neck squamous cell carcinoma. Oncol Rep *18*: 47-51, 2007.

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