Matrix metalloproteinase-2 and -14 in p16-Positive and -Negative HNSCC after Exposure To 5-FU and Docetaxel *In Vitro*

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Abstract. *Background: Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer in the world. While the incidence of HNSCC associated with tobacco and alcohol abuse is falling, the incidence of HNSCC associated with human papilloma virus (HPV) is rising. Proliferation, cell migration and formation of metastases are dependent on interactions between the tumor cells, tumor stromal cells and the extracellular matrix (ECM). Degradation of the ECM is a crucial step in the process of local tumor infiltration and formation of locoregional and distant metastases. Matrix metalloproteinases (MMPs) are a family of enzymes that are able to degrade the ECM. Locally advanced HNSCC with cervical node metastases are treated with docetaxel in induction chemotherapy (ICT) combined with platinum-based chemotherapy and 5-fluorouracil (5-FU) as standard clinical anti-neoplastic regimens. This study evaluated the expression of MMP-14 and MMP-2 in HPV-positive (CERV196) and HPV-negative squamous cell carcinoma (HNSCC 11A and 14C) and the alteration of expression levels after exposure to either docetaxel or 5-FU. Materials and Methods: Tumor cells were exposed to 5-FU or docetaxel in concentrations of 1.0 and 5.0 μmol/ml. MMP-protein expression was evaluated by enzyme-linked immunosorbent assay (ELISA) after 2, 3, 5, 8 and 10 days of incubation. Results: Docetaxel exposure significantly decreased MMP-14 expression in HNSCC 11A and especially 14C but not in CERV196 apart from an apoptotic process. 5-FU had no significant effect on MMP-14 expression independent of the HPV-status. Significant alterations of MMP-2 could be detected in HNSCC 11A only. Conclusion: Although neither of the applied drugs were selective inhibitors of MMP-expression, surprisingly docetaxel significantly decreased MMP-14 in HNSCC 14C and 11A in this study. Interestingly, HPV-positive CERV196 was not sensitive to decreased MMP-14 or -2 expression following incubation with 5-FU or docetaxel.*

Abbreviations: 5-FU: 5-Fluorouracil; BSS: balanced salt solution; CAFs: cancer-associated fibroblasts; CIS: carcinoma *in situ*; DMEM: dulbecco's modified essential medium; DNA: deoxyribonucleic acid; DPD: dihydropyrimidine dehydrogenase; dTMP: deoxythymidine monophosphate; dUMP: deoxyuridine monophosphate; ECM: extracellular matrix; ELISA: enzyme-linked immunosorbent assay; FCS: fetal calf serum; HNSCC: head and neck squamous cell carcinoma; HPV: human papilloma virus; ICT: induction chemotherapy; MMPs: matrix metalloproteinases; MT-MMPs: membrane-type matrix metalloproteinases; NCCN: National Comprehensive Cancer Network; PBS: phosphate buffered saline; RNA: ribonucleic acid; SCC: squamous cell carcinoma; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; siRNA: small interfering ribonucleic acid; TIMPs: tissue inhibitors of metalloproteinases.

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Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide with a global incidence of approximately 631,000 cases and 352,000 associated deaths in 2008 (1). Local tumor size and the presence of cervical lymph node metastases are the most important prognostic factors. Despite advances in surgical techniques, radiation and chemotherapy, the survival rate has remained almost unchanged (2). Identification of new predictive markers is mandatory to improve treatment and outcome for patients. The incidence of HNSCC has decreased in recent years, which is attributed to a falling prevalence of tobacco abuse (3). Still, smoking and alcohol abuse are important risk factors and have synergistic effects in cancerogenesis (4, 5). In contrast, the incidence of oropharyngeal cancer, especially of the tonsils and tongue base, has increased in patients 20-44 years old (6).

Human papilloma virus (HPV) and its cancerogenic potential is well known for carcinomas of the uterine cervix. Risk factors for HPV infection are promiscuity and unsafe

sex. Consequently, HPV is associated with anogenital and oropharyngeal cancer (7). The growing relevance of the viral etiology of HNSCC is illustrated by the rising incidence of HPV-associated oropharyngeal cancer (8, 9). HPV-negative and -positive HNSCC differ in molecular and clinical aspects (10-12). The viral oncogenes suppress inflammatory signaling as a mechanism of immune escape (13). It is assumed that HPV-positive HNSCCs have a higher sensitivity to radiation and chemotherapy and may be associated with a better prognosis (14, 15).

Endothelium, cancer-associated fibroblasts (CAFs), pericytes and the infiltrating inflammatory cells are part of the tumor stroma and are referred to as tumor stromal cells. They form the extracellular matrix (ECM) and are attributed with important functions during tumor progression (16). The ECM consists of various glycoproteins, hyaluronic acid, proteoglycans, collagen and elastic fibres. It is a physical barrier for cellular migration. The basement membrane is the most relevant structure concerning the depth of tumor infiltration. It connects the epithelium with the subepithelial connective tissue. The most important components of the basement membrane are type IV collagen and various glycoproteins, such as laminin, which interacts with integrins in the cell membrane of the epithelial cells. Tumor cell invasion of the basement membrane defines an invasive carcinoma and differentiates it from a carcinoma *in situ*. Without invasion of the basement membrane, the formation of lymphatic or hematogenous metastases is impossible because the epithelial layer is free of vessels (17).

Currently, matrix metalloproteinases (MMPs) are a group of over 20 zinc-dependent endopeptidases that exist as soluble and membrane-bound enzymes (18, 19). Tumor cells as well as the tumor stromal cells can synthesize MMPs (20, 21). A study by Bodnar *et al*. demonstrated that MMP-2 expression is higher in tumor stromal cells and MMP-14 expression is higher in HNSCC tumor cells (22). Jerome Gross and Charles Lapière first described MMPs in 1962 (23). MMPs are synthesized as inactive proenzymes and cleavage of the propeptide leads to activation of the enzyme. Classification of MMPs can be based on substrate specificity or cellular location. Known groups of MMPs are collagenases, gelatinases, stromelysins and membrane-type MMPs (MT-MMPs). The enzyme activity of MMPs is strictly regulated in healthy tissues since uncontrolled activity could result in tissue damage. Currently, four specific tissue inhibitors of metalloproteinases (TIMPs) are known (24). It has been shown that the expression of TIMP-2 in HNSCC, which is involved in the activation of MMP-2 by MMP-14, is associated with later lymph node and hematogenous metastases as well as poorer 5-year survival rate (25). Although the primary function of MMPs in the formation of metastases is the local degradation of the ECM including the basement membrane, they have a crucial role in the maintenance of the microenvironment of the tumor that supports tumor growth and angiogenesis (18).

MMP-14, also known as MT1-MMP, was the first membrane-type MMP to be discovered. Its potency to promote invasion and metastasis of cancer cells was demonstrated by various studies (26). Substrates of MMP-14 are collagen types I, II and III, fibrin, laminin and aggrecan. Additionally, MMP-14 activates other MMPs, such as MMP-2 and MMP-13, by cleavage of their propeptides. Thus, MMP-14 is said to initiate several enzymatic cascades. Besides promotion of cancer cell invasion and formation of metastasis, MMP-14 is involved in physiological processes, such as wound healing, skeletal development and angiogenesis (27-29). The latter emphasizes that MMPs not only support tumor growth and invasion by degradation of the ECM but also by supporting a tumor microenvironment. In the process of MMP-2 activation, MMP-14 builds a complex with TIMP-2 that on one hand abolishes the enzyme activity of MMP-14 and on the other hand works as a receptor for MMP-2 that brings MMP-2 into close contact with other unbound and active MMP-14 (30). An association of MMP-14 with TIMP-2 expression in particular was found in patients with lymph node metastasis of HNSCC (31). Zhang *et al*. demonstrated that MMP-14 expression increased significantly in advanced-stage HNSCC and lymph node metastasis compared to stage I or II HNSCC. Furthermore, elevated MMP-14 expression was associated with poorer prognosis (32).

Activation of MMP-2 is believed to be a crucial step during tumor invasion (33). MMP-2 is a secreted gelatinase that degrades gelatin, laminin and various types of collagen including collagen IV, a major component of the basement membrane (34). The active form of MMP-2 correlates with tumor invasiveness (35). Local degradation of the basement membrane is a mandatory step for tumor invasion and the formation of lymphatic or hematogenous metastases. Ruokolainen and colleagues showed that MMP-2 expression is strongly related to the formation of lymph node or distant metastases (25).

Paclitaxel, the first known taxane, was extracted from the rare tree *Taxus brevifolia*. Docetaxel is a semi-synthetic analog of paclitaxel and well established as a cytostatic chemotherapeutic drug. Taxanes bind tubulin reversibly and prevent depolymerization of the microtubules. This arrests mitosis between metaphase and anaphase. Docetaxel does not block the start of mitosis. Further properties of docetaxel are induction of apoptosis, anti-angiogenic effects and stimulation of the immune system (36). Over 90% of advanced-stage human tumor grafts displayed docetaxel sensitivity in mice (37). Combinations of docetaxel with 5-fluorouracil (5-FU), cyclophosphamide, etoposide or anti-

angiogenic drugs showed synergistic effects in vivo. Demonstrated side effects were hematological, gastrointestinal and neuromotoric toxicity (36, 37). At present, docetaxel is used for the treatment of breast, ovarian, prostate, and non-small cell lung cancer. For locally advanced HNSCC, docetaxel is recommended by the National Comprehensive Cancer Network (NCCN) for induction chemotherapy (ICT) in combination with platinum-based chemotherapeutic drugs and 5-FU (38, 39). Patients who received docetaxel in addition to ICT had significantly higher survival rates compared to patients who received cisplatin and 5-FU ICT plus radiotherapy (40, 41).

The pyrimidine analog 5-FU irreversibly binds to thymidylate synthase and inhibits the methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), leading to a deficit of dTMP. dTMP is a necessary nucleotide for DNA replication. Different cytotoxic metabolites of 5-FU can be incorporated into DNA as substitutes for dTMP. This causes abortion of the DNA strand replication, leading to cell cycle arrest and apoptosis. Disturbance of nuclear RNA maturation is possible by incorporation of 5-FU metabolites into RNA. However, inhibition of DNA synthesis is considered to be the main effect of 5-FU. Cellular resistance to 5-FU is gained by alterations of 5-FU metabolism, mutations of thymidylate synthase or altered kinetics in respect to nucleotides or folates. Biochemical modulation of 5-FU metabolism may allow to overpower resistance to 5-FU (42). 5-FU is applied mainly by intravenous infusion (43) and primarily eliminated by dihydropyrimidine dehydrogenase (DPD) in the liver. A partial DPD deficiency can be found in about 3% of patients, which may lead to increased 5-FU-related side-effects. Although multiple mechanisms may lead to 5-FU resistance, DPD activity in tumor cells is significantly related to 5-FU sensitivity. Lower DPD activity is attributed to increased 5-FU efficacy (44).

This study sought to evaluate the expression of MMP-14 and MMP-2 in HPV-positive and -negative squamous cell carcinoma cells *in vitro* and to assess alterations of expression under the influence of docetaxel or 5-FU.

Materials and Methods

Cell lines. The human cell lines HNSCC 11A and 14C were received from Dr T.E. Carey (University of Michigan, Ann Arbor, MI, USA) and originated from SCC (squamous cell carcinoma) of the oropharynx and larynx. They were kept in Dulbecco's modified essential medium (DMEM) (Fisher Scientific and Co., Pittsburgh, PA, USA) with 10% fetal calf serum (FCS) and antibiotics (Life Technologies Inc., Gaithersburg, MD, USA). A p16-positive SCC of the uterine cervix was the origin of the cell line CERV196 (CLS, Eppelheim, Germany) and was cultured in Eagle's minimum essential medium with 2 mM L-glutamine, 10% FCS and Earle's balanced salt solution (BSS) with 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids and 1.0 mM sodium pyruvate. Cell cultures were kept at 37˚C in a fully humidified atmosphere with 5% CO_2. Docetaxel and 5-FU were stored at 4˚C and solved in sterile water at the time of use. Cell lines were incubated with either docetaxel or 5-FU at concentrations of 1.0 or 5.0 μmol/ml for 48, 72, 120, 192 or 240 hours. The drug concentrations were selected after performing the alamarBlue® (AbD Serotec, Oxford, UK) cell proliferation assay, measuring proliferation of SCC cells quantitatively and establishing the relative cytotoxicity of the chemotherapeutic drugs examined. After incubation, cells were lysed and centrifuged, and the supernatants were collected in sterile tubes and stored at –20˚C until further analysis.

Enzyme-linked immunosorbent assay (ELISA) for total MMP-2 and MMP-14. After incubation with different concentrations of either docetaxel or 5-FU, the cells were washed with phosphate buffered saline (PBS). Afterwards, 350 μl of lysis buffer was added to each well. Lysed cells were stirred up with a vortex at 2-8˚C for 30 min and then microcentrifuged at 14,000 *g* for 5 min. The supernatant was pipetted into a clean tube.

MMP-2 levels were determined with a sandwich ELISA (R&D Systems, Wiesbaden, Germany). The system used a solid-phase monoclonal antibody and an enzyme-linked polyclonal antibody against MMP-2 (binding both the proenzyme and the active form). The specificity of antibodies to MMP-2 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 50 μl of supernatant. All analyses and calibrations were carried out twice. The calibrations on each microtiter plate included recombinant human MMP-2 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 were reported as pg/ml. The inter-assay coefficient of variation reported by the manufacturer varied from 5.4-9.8% and the range of detection was 289-50,000 pg/ml.

An ELISA kit for human MMP-14 (USCN Life Science Inc., Houston, TX, USA) provided a 96-well plate precoated with an antibody specific to human MMP-14. A serial dilution of an MMP-14 standard provided in the kit was prepared according to the manufacturer's instructions as well as the supernatant samples. Each ELISA was performed with 100 μl of supernatant. Optical density was measured using a microplate reader at 450 nm. The inter-assay coefficient of variation reported by the manufacturer was <10% and the range of detection for MMP-14 was 630-100,000 pg/ml. All analyses and calibrations were carried out twice.

Measuring total protein. Total protein levels were determined using DC Protein Assay (Bio-Rad, Hercules, CA, USA). Incubation, cell lysis and centrifugation were performed as previously described. A serial dilution of a protein standard was prepared according to the manufacturer's instructions. Measurement was carried out with 100 μl of protein standard or cell supernatant using a spectrophotometer set to 750 nm; concentrations were reported as μg/ml.

Statistical analysis. Statistical analysis was performed in cooperation with PD Dr. C. Weiss, Institute of Biomathematics, Faculty of Medicine, Mannheim, Germany. A p-value ≤0.05 was considered statistically significant. The two-coefficient variance test (SAS Statistics, Cary, NC, USA) and Dunnett's test were performed.

Results

Total protein assay. The total protein was measured in order to differentiate the decrease in protein expression of MMP-2 or MMP-14 from cytotoxicity (apoptosis) caused by the applied drugs. Total protein levels of the cell lysate were compared to levels of MMP expression. For the negative control and 5-FU, a minor fluctuation of the protein quotient (MMP expression level/total protein level) was determined but no increase of the protein quotient could be stated. Thus a measured alteration of MMP expression levels cannot be the result of apoptosis due to cytotoxicity of 5-FU. For docetaxel, an increase of the protein quotient could be detected in a time-dependent manner irrespective of the HPV-status. Consequently, a cytotoxic effect of docetaxel can be stated (data not shown).

ELISA for MMP-14 expression in HNSCC 11A, 14C and CERV196 cells. MMP-14 was expressed in all evaluated cell lines. Expression levels of MMP-14 in HNSCC 14C exceeded the expression levels in HNSCC 11A and CERV196 at least twofold. A trend towards decreased expression levels after incubation with chemotherapeutic drugs was observed, especially in HNSCC 14C but also in HNSCC 11A.

A statistically significant reduction of MMP-14 expression in HNSCC 14C cells was found with 5-FU after 3 and 5 days of incubation (p-value=0.002 and 0.014), which corresponds to an average reduction of 63.5%. 5-FU had no significant impact on MMP-14 expression in HNSCC 11A or CERV196.

Docetaxel significantly reduced MMP-14 levels (30%) in HNSCC 11A after 3 days of incubation with a p-value of 0.043. For HNSCC 14C cells, there was a significant reduction in MMP-14 expression after 3, 5, 8 and 10 days of incubation with docetaxel (p-values were 0.004, <0.001, 0.03 and 0.024, respectively). On average, docetaxel reduced MMP-14 levels by 31%. Taking into account the increased protein quotient after incubation with docetaxel (see section on total protein assay), this reduction could be due to cytotoxicity and increased apoptosis, not a specific effect on MMP-14 expression. However, although the increase of protein quotient was detected in all three cell lines, a similar decrease of MMP-14 could not be found in HNSCC 11A and CERV196. Docetaxel did not significantly alter MMP-14 expression in CERV196 independent of the incubation time. Different drug concentrations had no statistically significant effect on the expression of MMP-14. For simplification, only the data for 5-FU and docetaxel at a concentration of 5.0 μmol/ml are shown (Table I, Figure 1).

Comparing the impact of 5-FU and docetaxel on the reduction of MMP-14 expression, there was a significant difference in HNSCC 14C cells after 5, 8 and 10 days of incubation (p-value=0.004, 0.014 and 0.001 respectively). For HNSCC 11A and CERV196, no significant differences between the effect of 5-FU and docetaxel could be detected.

Table I. *ELISA of matrix metalloproteinase-14 (MMP-14) expression (pg/ml) in HNSCC 11A, 14C and CERV196 cells after incubation with 5-fluorouracil (5-FU) and docetaxel compared to the negative control (statistical significant results are shown in bold).*

Incubation time (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	703.333	997.333	0.116	952.333	0.629
72	1112.767	811.333	0.102	780.4	**0.043**
120	900.133	669	0.639	704	0.345
192	732.667	865.333	0.565	643.667	0.652
240	899.333	931	0.986	564.933	0.334
HNSCC 14C					
48	1989.1	1045.533	0.714	2127.133	0.996
72	2803.8	967.81	**0.002**	2118.333	**0.004**
120	2677.667	1031	**0.014**	1566.067	**<0.001**
192	2181	888.36	0.574	1457.667	**0.03**
240	1775.667	940.04	0.847	1329.333	**0.024**
CERV196					
48	910.567	1045.533	0.879	1061.767	0.153
72	1060	967.81	0.37	934.633	0.112
120	930	1031	0.748	850.817	1.0
192	1029.667	888.36	0.746	862.22	0.388
240	1031	940.04	0.349	765	0.532

ELISA for MMP-2 expression in HNSCC 11A, 14C and CERV196 cells. Expression of MMP-2 was detected in all studied cell lines. Expression levels in CERV196 were slightly lower compared to the HPV-negative SCC cell lines. With two exceptions, both 5-FU and docetaxel showed no significant alteration of MMP-2 expression. A statistically significant alteration of MMP-2 levels (47% reduction) could be observed only in HNSCC 11A after 8 days of incubation with 5-FU (p-value=0.005) and with docetaxel (49% reduction; p-value=0.003). No statistically significant alteration of MMP-2 expression could be observed in HNSCC 14C and CERV196 after incubation with 5-FU or docetaxel irrespective of the incubation time or drug concentration (Table II) (Figure 2).

Discussion

The aim of the present study was to evaluate the expression of MMP-14 and MMP-2 in HPV-positive and -negative SCC and to determine alterations of MMP expression after incubation of these tumor cells with 5-FU or docetaxel as standard chemotherapeutic drugs in clinical regimens.

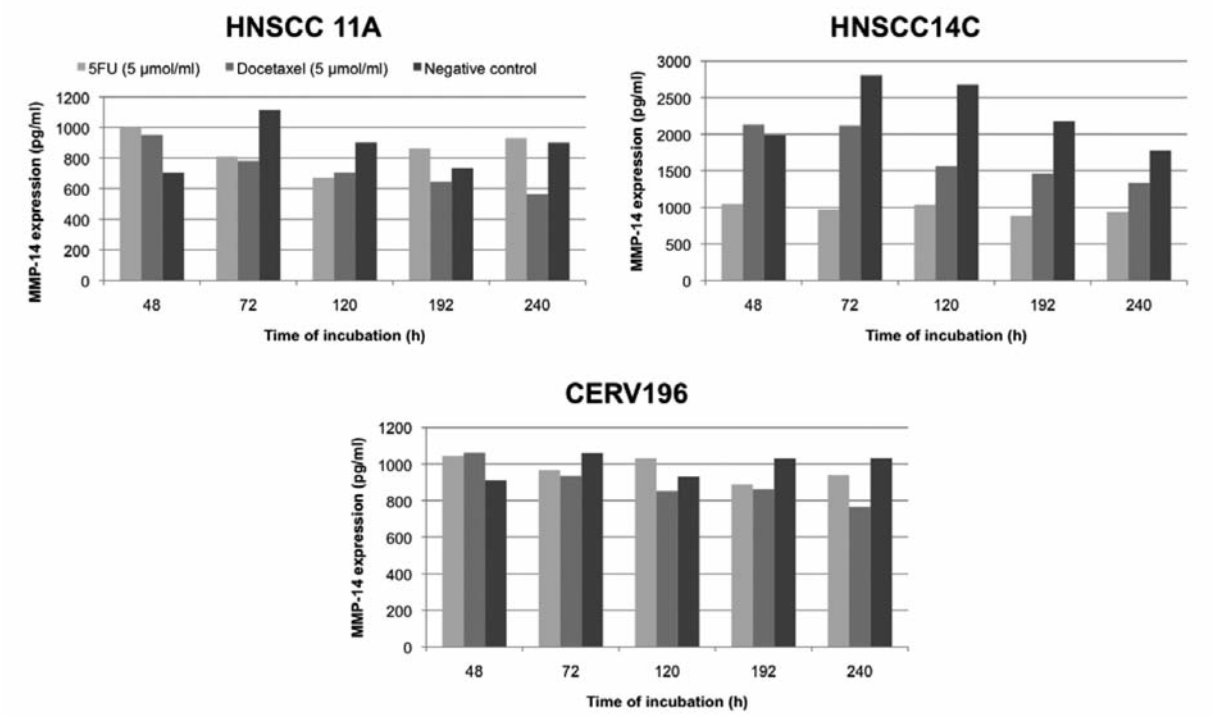

Figure 1. *Matrix metalloproteinase 14 (MMP-14) expression in HNSCC 11A, 14C and CERV196 cells after incubation with 5-FU and docetaxel compared to the negative control.*

Docetaxel and 5-FU are not direct inhibitors of MMPs. Docetaxel affects mitosis by inhibition of the spindle apparatus, while 5-FU disturbs DNA replication. Only a small number of studies have investigated the alteration of MMPs after therapy with 5-FU or docetaxel. Yamamoto *et al*. demonstrated suppression of lung micrometastasis progression after therapy with docetaxel in combination with an MMP-inhibitor (45). Further studies showed possible interactions of MMP-inhibition and chemotherapeutic drugs. For instance, a knockdown of MMP-7 expression by siRNA (small interfering ribonucleic acid) increased sensitivity to 5-FU and radiation in colon carcinoma (46).

Our study showed that MMP-2 and MMP-14 were expressed in all observed cell lines. MMP-2 expression levels were slightly lower in HPV-positive CERV196 compared to HPV-negative SCC cells and they were also lower compared to the expression levels of MMP-14 in all cell lines. As previously mentioned, Bodnar *et al*. demonstrated that MMP-2 expression is higher in tumor stromal cells and MMP-14 expression is higher in HNSCC tumor cells, which might explain our results and might indicate that epithelial cells are less sensitive to an alteration of MMP-2 expression following chemotherapy (22). Interestingly, MMP-14 levels in HNSCC 14C exceeded the expression levels in HNSCC 11A and CERV196 by at least two-fold. Higher levels of

Table II. *ELISA of matrix metalloproteinase 2 (MMP-2) expression (pg/ml) in HNSCC 11A, 14C and CERV196 cells after incubation with 5-fluorouracil (5-FU) and docetaxel compared to the negative control (statistical significant results are shown in bold).*

Incubation time (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	601.333	535.333	0.588	566.667	0.254
72	662.667	669.333	0.996	545	0.288
120	726.667	483.667	0.12	481.333	0.113
192	955.667	503.667	**0.005**	485.667	**0.003**
240	793.667	691.333	0.466	564	0.503
HNSCC 14C					
48	591	573	0.692	568	0.888
72	746.333	604.667	0.26	556.667	0.256
120	714.333	572.667	0.261	510	0.092
192	601.333	418	0.382	475.333	0.61
240	622	544.667	0.674	561.333	0.992
CERV196					
48	335.333	318.667	0.993	352.333	1.0
72	411.667	331.333	0.735	325	0.515
120	412.333	405.667	0.952	291.667	0.276
192	570.667	522	0.987	363.333	0.478
240	570	755	0.775	331	0.163

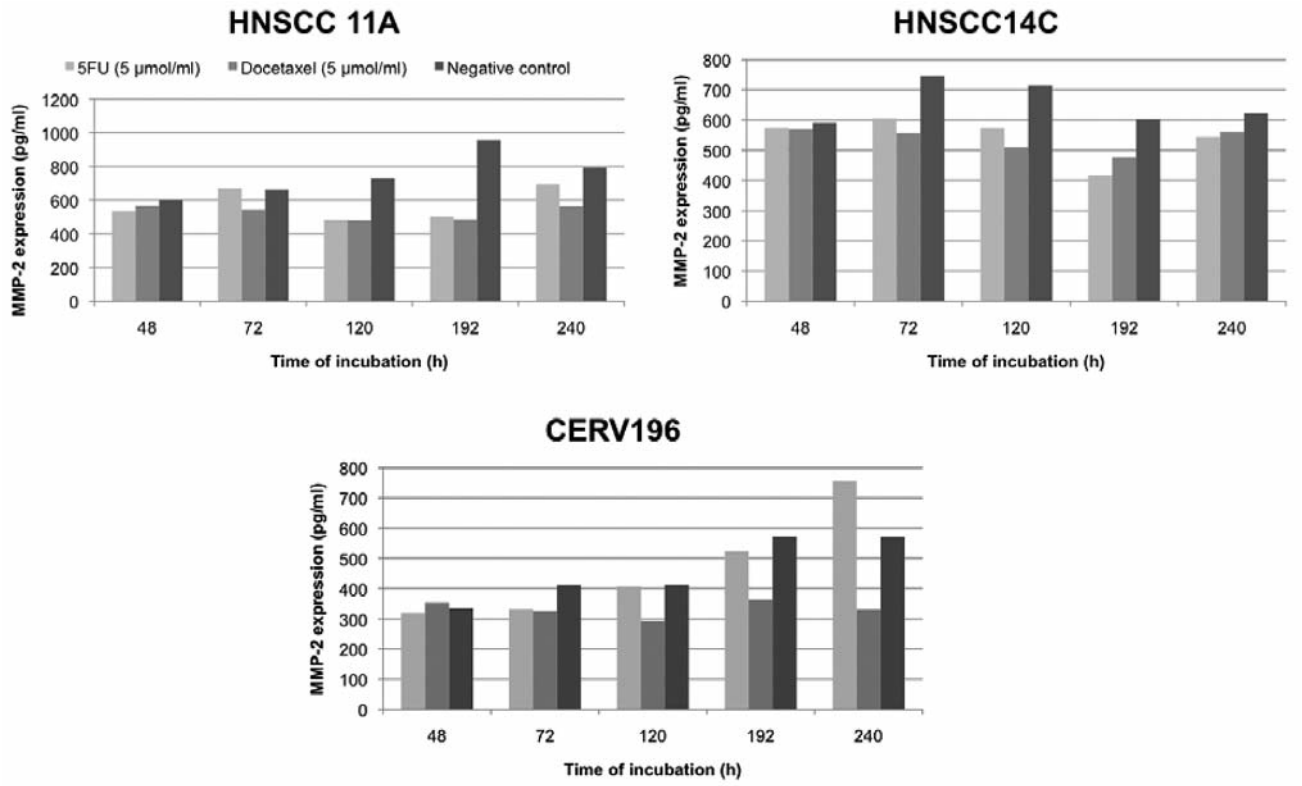

Figure 2. *Matrix metalloproteinase-2 (MMP-2) expression in HNSCC 11A, 14C and CERV196 cells after incubation with 5-FU and docetaxel compared to the negative control.*

MMP expression can often be found in more advanced tumors or metastases (18, 32, 47).

We were able to demonstrate that docetaxel and 5-FU reduce the protein expression of MMP-14 and MMP-2 in HPV-negative SCC. As previously described, 5-FU appeared to be less effective in altering protein expression compared to docetaxel (48). However, in the case of a significant reduction of MMP levels following incubation with 5-FU, the reduction was equal to the effect of docetaxel. In fact, 5-FU revealed the highest levels of suppression, with 63.5% for MMP-14 expression in HNSCC 14C. As previously mentioned, 5-FU disturbs RNA synthesis as one possible mechanism of action. This inhibition of protein expression may include expression of MMPs, which may be a possible mechanism for MMP reduction after exposure to 5-FU.

Suppression of MMP-14 expression after incubation with docetaxel in HNSCC 11A was rather sparse compared to the broad and significant reduction of MMP-14 in HNSCC 14C, which also showed higher expression levels than HNSCC 11A in negative controls. Comparison of the total protein levels with MMP expression as the target variable in a protein quotient (MMP expression level/total protein level) revealed an increasing protein quotient after incubation with docetaxel in an incubation time-dependent manner due to a decrease of the denominator (total protein level). This can be interpreted as a sign of a cytotoxic effect of docetaxel. Cytotoxicity is known to be higher in docetaxel than 5-FU because docetaxel directly affects mitosis and the spindle fibers. However, the protein quotient increased under docetaxel incubation in all three cell lines. Still, a similar decrease of the target proteins MMP-14 and MMP-2 was not present in HNSCC 11A and CERV196. Therefore, the alterations of MMP expression under docetaxel cannot only be explained as a cytotoxic effect and/or increase of apoptosis.

A statistically significant alteration of MMP-2 expression was observed in HNSCC 11A after 8 days of incubation with docetaxel and with 5-FU, but no differences in HNSCC 14C could be detected. HPV-positive CERV196 cells showed no significant differences in MMP-14 or MMP-2 expression after exposure to docetaxel or 5-FU, which was shown in a previously published study (48). This contradicts the hypothesis that p16-positive HNSCCs have increased sensitivity to chemotherapy.

MMPs are said to support tumor cell promotion in invasive carcinoma, primary tumor growth, angiogenesis, invasion, intravasation, extravasation, local migration and sustained tumor growth (18). MMP-2 and MMP-14 are

important for the local degradation of the ECM, which is crucial for tumor cell invasion (especially for an early-stage tumor) and the formation of metastasis. Most clinical studies that investigated the effectiveness of MMP-Inhibitors included patients with advanced-stage tumors. Prolonged progression-free survival in combination with standard antitumor therapy was observed, but overall survival did not improve with MMP-Inhibitors. Furthermore, MMP-Inhibitors revealed several side effects, such as nausea, fatigue, fevers, abdominal pain, elevation of hepatic enzymes, thrombocytopenia, arthralgia, myalgia and maculopapular rash (49-51). In advanced-stage tumors, the processes of local degradation of the ECM, neoangiogenesis, generation of a tumor microenvironment and the formation of metastases have occurred already. Therefore, inhibition of this process cannot be expected to make a significant therapeutic impact apart from delayed local tumor progress. Advanced-stage HNSCCs also tend to express a wide set and higher levels of MMPs (47). It is not surprising that MMP-Inhibitors are insufficient for therapy of advanced-stage tumors (52-55). Local degradation of the ECM is most relevant in early-stage tumors. It is reasonable to suggest that MMP-Inhibition would be most effective before cancer cells infiltrate the basement membrane, but a carcinoma *in situ* (CIS) is either not detected or can be successfully treated by local excision.

Branca *et al*. showed that expression of MMP-2 increases with the grade of an intraepithelial neoplasia. Increased MMP-2 expression was not associated with high-risk HPV, which is consistent with our findings (56). In contrast, Shiau and colleagues detected that HPV-16 up-regulates the expression of MMP-2 and -9 via Interleukin 8 in adenocarcinomas of the lung (57). Our results did not reveal HPV-status dependent differences of MMP-2 and MMP-14 expression. This contradicts the findings of da Silva *et al*. who demonstrated an HPV-16-associated increase of MMP-2 and -14 expression (58). Furthermore, CERV196 appeared to be less sensitive to reduced MMP expression following incubation with either 5-FU or docetaxel. Our study evaluated the MMP expression in tumor cells only. As previously mentioned, MMP-2 is said to be expressed mainly by stromal cells. As tumor stromal cells are not present in cell cultures, the relevance of stromal expression of MMPs could not be evaluated in this study.

Further studies are planned to investigate the relevance of MMPs in the progression of HNSCC with eventual examination in an animal model.

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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, Howe HL, Ward E, Ries LA, Schrag D, Jamison PM, Jemal A, Wu XC, Friedman C, Harlan L, Warren J, Anderson RN and Pickle LW: 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 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.

4 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.

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

6 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.

7 Romanitan M, Nasman A, Ramqvist T, Dahlstrand H, Polykretis L, Vogiatzis P, Vamvakas P, Tasopoulos G, Valavanis C, Arapantoni-Dadioti P, Banis K and Dalianis T: Human papillomavirus frequency in oral and oropharyngeal cancer in Greece. Anticancer Res *28*: 2077-2080, 2008.

8 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.

9 Gillison ML, D'Souza G, Westra W, Sugar E, Xiao W, Begum S and Viscidi R: 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.

10 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.

11 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.

12 Stransky N, Egloff AM, Tward AD, Kostic AD, Cibulskis K, Sivachenko A, Kryukov GV, Lawrence MS, Sougnez C, McKenna A, Shefler E, Ramos AH, Stojanov P, Carter SL, Voet D, Cortes ML, Auclair D, Berger MF, Saksena G, Guiducci C, Onofrio RC, Parkin M, Romkes M, Weissfeld JL, Seethala RR, Wang L, Rangel-Escareno C, Fernandez-Lopez JC, Hidalgo-Miranda A, Melendez-Zajgla J, Winckler W, Ardlie K, Gabriel SB, Meyerson M, Lander ES, Getz G, Golub TR, Garraway LA and Grandis JR: The mutational landscape of head and neck squamous cell carcinoma. Science *333*: 1157-1160, 2011.

13 Le Buanec H, D'Anna R, Lachgar A, Zagury JF, Bernard J, Ittele D, d'Alessio P, Hallez S, Giannouli C, Burny A, Bizzini B, Gallo RC and Zagury D: HPV-16 E7 but not E6 oncogenic protein triggers both cellular immunosuppression and angiogenic processes. Biomed Pharmacother *53*: 424-431, 1999.

14 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.
15 Fakhry C, Westra WH, Li S, Cmelak A, Ridge JA, Pinto H, Forastiere A and Gillison ML: 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.
16 Park CC, Bissell MJ and Barcellos-Hoff MH: The influence of the microenvironment on the malignant phenotype. Mol Med Today *6*: 324-329, 2000.
17 Wilson DF, Jiang DJ, Pierce AM and Wiebkin OW: Oral cancer: role of the basement membrane in invasion. Aust Dent J *44*: 93-97, 1999.
18 Nelson AR, Fingleton B, Rothenberg ML and Matrisian LM: Matrix metalloproteinases: biologic activity and clinical implications. J Clin Oncol *18*: 1135-1149, 2000.
19 Ravanti L and Kahari VM: Matrix metalloproteinases in wound repair (review). Int J Mol Med *6*: 391-407, 2000.
20 Basset P, Bellocq JP, Wolf C, Stoll I, Hutin P, Limacher JM, Podhajcer OL, Chenard MP, Rio MC and Chambon P: A novel metalloproteinase gene specifically expressed in stromal cells of breast carcinomas. Nature *348*: 699-704, 1990.
21 Monsky WL, Kelly T, Lin CY, Yeh Y, Stetler-Stevenson WG, Mueller SC and Chen WT: Binding and localization of M(r) 72,000 matrix metalloproteinase at cell surface invadopodia. Cancer Res *53*: 3159-3164, 1993.
22 Bodnar M, Szylberg L, Kazmierczak W and Marszalek A: Differentiated expression of membrane type metalloproteinases (MMP-14, MMP-15) and pro-MMP2 in laryngeal squamous cell carcinoma. A novel mechanism. J Oral Pathol Med *42*: 267-274, 2013.
23 Gross J and Lapiere CM: Collagenolytic activity in amphibian tissues: a tissue culture assay. Proc Natl Acad Sci USA *48*: 1014-1022, 1962.
24 Gomez DE, Alonso DF, Yoshiji H and Thorgeirsson UP: Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. Eur J Cell Biol *74*: 111-122, 1997.
25 Ruokolainen H, Paakko P and Turpeenniemi-Hujanen T: Tissue and circulating immunoreactive protein for MMP-2 and TIMP-2 in head and neck squamous cell carcinoma – tissue immunoreactivity predicts aggressive clinical course. Mod Pathol *19*: 208-217, 2006.
26 Sato H, Takino T, Okada Y, Cao J, Shinagawa A, Yamamoto E and Seiki M: A matrix metalloproteinase expressed on the surface of invasive tumour cells. Nature *370*: 61-65, 1994.
27 Okada A, Tomasetto C, Lutz Y, Bellocq JP, Rio MC and Basset P: Expression of matrix metalloproteinases during rat skin wound healing: evidence that membrane type-1 matrix metalloproteinase is a stromal activator of pro-gelatinase A. J Cell Biol *137*: 67-77, 1997.
28 Holmbeck K, Bianco P, Caterina J, Yamada S, Kromer M, Kuznetsov SA, Mankani M, Robey PG, Poole AR, Pidoux I, Ward JM and Birkedal-Hansen H: MT1-MMP-deficient mice develop dwarfism, osteopenia, arthritis, and connective tissue disease due to inadequate collagen turnover. Cell *99*: 81-92, 1999.
29 Hiraoka N, Allen E, Apel IJ, Gyetko MR and Weiss SJ: Matrix metalloproteinases regulate neovascularization by acting as pericellular fibrinolysins. Cell *95*: 365-377, 1998.
30 Kinoshita T, Sato H, Okada A, Ohuchi E, Imai K, Okada Y and Seiki M: TIMP-2 promotes activation of progelatinase A by membrane-type 1 matrix metalloproteinase immobilized on agarose beads. J Biol Chem *273*: 16098-16103, 1998.
31 de Vicente JC, Lequerica-Fernandez P, Santamaria J and Fresno MF: Expression of MMP-7 and MT1-MMP in oral squamous cell carcinoma as predictive indicator for tumor invasion and prognosis. J Oral Pathol Med *36*: 415-424, 2007.
32 Zhang H, Liu M, Sun Y and Lu J: MMP-14 can serve as a prognostic marker in patients with supraglottic cancer. Eur Arch Otorhinolaryngol *266*: 1427-1434, 2009.
33 Zeng ZS, Cohen AM and Guillem JG: Loss of basement membrane type IV collagen is associated with increased expression of metalloproteinases 2 and 9 (MMP-2 and MMP-9) during human colorectal tumorigenesis. Carcinogenesis *20*: 749-755, 1999.
34 Stetler-Stevenson WG, Aznavoorian S and Liotta LA: Tumor cell interactions with the extracellular matrix during invasion and metastasis. Annu Rev Cell Biol *9*: 541-573, 1993.
35 Tokuraku M, Sato H, Murakami S, Okada Y, Watanabe Y and Seiki M: Activation of the precursor of gelatinase A/72 kDa type IV collagenase/MMP-2 in lung carcinomas correlates with the expression of membrane-type matrix metalloproteinase (MT-MMP) and with lymph node metastasis. Int J Cancer *64*: 355-359, 1995.
36 Armand JP: Focus on cellular pharmacology of docetaxel. Bull Cancer *90*: 1067-1070, 2003.
37 Bissery MC: Preclinical pharmacology of docetaxel. Eur J Cancer *31A(Suppl 4)*: S1-6, 1995.
38 Schultz JD, Bran G, Anders C, Sadick H, Faber A, Hormann K and Sauter A: 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.
39 Pointreau Y, Garaud P, Chapet S, Sire C, Tuchais C, Tortochaux J, Faivre S, Guerrif S, Alfonsi M and Calais G: Randomized trial of induction chemotherapy with cisplatin and 5-fluorouracil with or without docetaxel for larynx preservation. J Natl Cancer Inst *101*: 498-506, 2009.
40 Posner MR, Hershock DM, Blajman CR, Mickiewicz E, Winquist E, Gorbounova V, Tjulandin S, Shin DM, Cullen K, Ervin TJ, Murphy BA, Raez LE, Cohen RB, Spaulding M, Tishler RB, Roth B, Viroglio Rdel C, Venkatesan V, Romanov I, Agarwala S, Harter KW, Dugan M, Cmelak A, Markoe AM, Read PW, Steinbrenner L, Colevas AD, Norris CM Jr. and Haddad RI: Cisplatin and fluorouracil alone or with docetaxel in head and neck cancer. N Engl J Med *357*: 1705-1715, 2007.
41 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.
42 Pinedo HM and Peters GF: Fluorouracil: biochemistry and pharmacology. J Clin Oncol *6*: 1653-1664, 1988.
43 Diasio RB and Harris BE: Clinical pharmacology of 5-fluorouracil. Clin Pharmacokinet *16*: 215-237, 1989.
44 Milano G and Etienne MC: Dihydropyrimidine dehydrogenase (DPD) and clinical pharmacology of 5-fluorouracil (review). Anticancer Res *14*: 2295-2297, 1994.
45 Yamamoto A, Yano S, Shiraga M, Ogawa H, Goto H, Miki T, Zhang H and Sone S: A third-generation matrix metallo-proteinase (MMP) inhibitor (ONO-4817) combined with docetaxel suppresses progression of lung micrometastasis of MMP-expressing tumor cells in nude mice. Int J Cancer *103*: 822-828, 2003.

46 Zhang W, Li Y, Yang L, Zhou B, Chen KL, Meng WJ, Liu Y, Hu JK, Sun XF and Zhou ZG: Knockdown of MMP-7 inhibits cell proliferation and enhances sensitivity to 5-Fluorouracil and X-ray irradiation in colon cancer cells. Clin Exp Med *14*: 99-106, 2014.

47 Kurahara S, Shinohara M, Ikebe T, Nakamura S, Beppu M, Hiraki A, Takeuchi H and Shirasuna K: Expression of MMPS, MT-MMP, and TIMPs in squamous cell carcinoma of the oral cavity: correlations with tumor invasion and metastasis. Head Neck *21*: 627-638, 1999.

48 Aderhold C, Umbreit C, Faber A, Sauter A, Sommer JU, Birk R, Erben P, Hofheinz RD, Stern-Straeter J, Hormann K and Schultz JD: Chemotherapeutic Alteration of VEGF, PDGF and PDGFRalpha/beta Expression Under 5-FU *vs*. Docetaxel in HPV-transformed Squamous Cell Carcinoma Compared To HPV-negative HNSCC In Vitro. Anticancer Res *33*: 1951-1961, 2013.

49 Hirte H, Goel R, Major P, Seymour L, Huan S, Stewart D, Yau J, Arnold A, Holohan S, Waterfield B, Bates S, Bennett K, Walsh W and Elias I: A phase I dose escalation study of the matrix metalloproteinase inhibitor BAY 12-9566 administered orally in patients with advanced solid tumours. Ann Oncol *11*: 1579-1584, 2000.

50 Heath EI, O'Reilly S, Humphrey R, Sundaresan P, Donehower RC, Sartorius S, Kennedy MJ, Armstrong DK, Carducci MA, Sorensen JM, Kumor K, Kennedy S and Grochow LB: Phase I trial of the matrix metalloproteinase inhibitor BAY12-9566 in patients with advanced solid tumors. Cancer Chemother Pharmacol *48*: 269-274, 2001.

51 Erlichman C, Adjei AA, Alberts SR, Sloan JA, Goldberg RM, Pitot HC, Rubin J, Atherton PJ, Klee GG and Humphrey R: Phase I study of the matrix metalloproteinase inhibitor, BAY 12-9566. Ann Oncol *12*: 389-395, 2001.

52 Primrose JN, Bleiberg H, Daniel F, Van Belle S, Mansi JL, Seymour M, Johnson PW, Neoptolemos JP, Baillet M, Barker K, Berrington A, Brown PD, Millar AW and Lynch KP: Marimastat in recurrent colorectal cancer: exploratory evaluation of biological activity by measurement of carcinoembryonic antigen. Br J Cancer *79*: 509-514, 1999.

53 Nemunaitis J, Poole C, Primrose J, Rosemurgy A, Malfetano J, Brown P, Berrington A, Cornish A, Lynch K, Rasmussen H, Kerr D, Cox D and Millar A: Combined analysis of studies of the effects of the matrix metalloproteinase inhibitor marimastat on serum tumor markers in advanced cancer: selection of a biologically active and tolerable dose for longer-term studies. Clin Cancer Res *4*: 1101-1109, 1998.

54 Rosemurgy A, Harris J, Langleben A, Casper E, Goode S and Rasmussen H: Marimastat in patients with advanced pancreatic cancer: a dose-finding study. Am J Clin Oncol *22*: 247-252, 1999.

55 Evans JD, Stark A, Johnson CD, Daniel F, Carmichael J, Buckels J, Imrie CW, Brown P and Neoptolemos JP: A phase II trial of marimastat in advanced pancreatic cancer. Br J Cancer *85*: 1865-1870, 2001.

56 Branca M, Ciotti M, Giorgi C, Santini D, Di Bonito L, Costa S, Benedetto A, Bonifacio D, Di Bonito P, Paba P, Accardi L, Syrjanen S, Favalli C, Syrjanen K and Group HP-PS: Matrix metalloproteinase-2 (MMP-2) and its tissue inhibitor (TIMP-2) are prognostic factors in cervical cancer, related to invasive disease but not to high-risk human papillomavirus (HPV) or virus persistence after treatment of CIN. Anticancer Res *26*: 1543-1556, 2006.

57 Shiau MY, Fan LC, Yang SC, Tsao CH, Lee H, Cheng YW, Lai LC and Chang YH: Human papillomavirus up-regulates MMP-2 and MMP-9 expression and activity by inducing interleukin-8 in lung adenocarcinomas. PLoS One *8*: e54423, 2013.

58 da Silva Cardeal LB, Brohem CA, Correa TC, Winnischofer SM, Nakano F, Boccardo E, Villa LL, Sogayar MC and Maria-Engler SS: Higher expression and activity of metalloproteinases in human cervical carcinoma cell lines is associated with HPV presence. Biochem Cell Biol *84*: 713-719, 2006.

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