Effect of selective small molecule inhibitors on MMP-9 and VEGFR-1 expression in p16-positive and -negative squamous cell carcinoma

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Abstract. The identification of molecular targets in the therapy of human papilloma virus (HPV)-associated head and neck squamous cell carcinoma (HNSCC) is a primary aim of cancer research. Matrix metalloproteinase 9 (MMP-9) and vascular endothelial growth factor receptor (VEGFR) have important roles in the development of HNSCC. The tyrosine kinase inhibitors, nilotinib, dasatinib, erlotinib and gefitinib are well established in the targeted therapy of tumors other than HNSCC. The present study aimed to investigate the alteration of MMP-9 and VEGFR-1 expression patterns following treatment with these tyrosine kinase inhibitors in p16-positive and -negative squamous carcinoma cells. MMP-9 and VEGFR-1 expression was evaluated using an ELISA in HNSCC 11A, HNSCC 14C and p16-positive CERV196 tumor cell lines, following treatment with nilotinib, dasatinib, erlotinib and gefitinib. A statistically significant reduction in MMP-9 and VEGFR-1 expression was observed in the p16-negative HNSCC 11A cells following treatment with all inhibitors (P<0.05). VEGFR-1 expression was significantly increased in p16-positive SCC cells following treatment with nilotinib, dasatinib, erlotinib and gefitinib (P<0.05). The expression of MMP-9 and VEGFR-1 was significantly altered by treatment with nilotinib, dasatinib, erlotinib and gefitinib *in vitro*. The results of the present study are attributed to the efficacy of the tested drugs and present potential compensatory strategies of cancer cells to avoid the antiangiogenic properties of the tested tyrosine kinase inhibitors *in vitro*.

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Abbreviations: AKT, protein kinase B; BCR-ABL, BCR-ABL fusion protein; BM, basement membrane; c-KIT, mast/stem cell growth factor receptor Kit; DDR, epithelial discoidin domain-containing receptor; ECM, extracellular matrix; EGFR, epidermal growth factor receptor; HNSCC, head and neck squamous cell carcinoma; HPV, human papilloma virus; HRP, horseradish peroxidase; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; NSCLC, non-small cell lung cancer; PDGFR, platelet-derived growth factor receptor; Src, proto-oncogene tyrosine-protein kinase Src; VEGFR, vascular endothelial growth factor receptor

Introduction

Pharmaceutical approaches are important in the treatment of head and neck squamous cell carcinoma (HNSCC). Current therapeutic options include surgery, radiotherapy, chemotherapy and immunotherapy; however, the 5-year survival rate has not improved significantly in previous decades (1,2). Tobacco and alcohol abuse remain the primary risk factors of HNSCC incidence (3,4). In contrast to a decreasing incidence of laryngeal cancer, the incidence of oropharyngeal cancer is increasing (5). This increase may be due to an overall human papilloma virus (HPV) infection prevalence of >20% (6,7). HPV subtypes 16 and 18 are key regulators in the formation of several tumor entities, including carcinoma of the uterine cervix and oropharyngeal squamous cell carcinoma (8). Within this group HPV 16 may be detected in >90% of HPV-associated tumors (9). The mechanism of HPV-induced cancerous lesions involves an increased risk of viral DNA integration into the host genome (10). This genomic alteration leads to the overexpression of viral oncogenes E6 and E7, and subsequently results in a disruptive viral infection with an abrogation of cell cycle checkpoints (11). However, HPV-associated HNSCC is associated with an improved outcome following current treatment options (12,13).

The extracellular matrix (ECM) is important to nutritive cellular support, and functions as a physical barrier to cellular migration and a regulator of intercellular communication (14). The ECM consists of proteoglycans, non-proteoglycan polysaccharides, including hyaluronic acid, fibers and other components, including fibronectin and laminin (14,15). During the process of tumor formation, the basement membrane (BM) is essential as it connects the epithelium to the subepithelial connective tissue and therefore must be penetrated for invasive tumor growth. Two major components of the basement membrane, type IV collagen and fibronectin, have been demonstrated to be disregulated in HNSCC (16). ECM degradation occurs through the secretion of several proteases, which leads to local tumor invasion following pentration of the BM and ultimately, the occurrence of lymphonodal and distant metastasis (17). Under normal conditions matrix metalloproteinases (MMP) are important components in tissue remodeling of the ECM, and participate in the regulation of angiogenesis, tissue repair and morphogenesis (18). Currently, the MMP family consists of >20 distinct zinc-dependent endopeptidases (19,20). MMPs occur as either membrane-bound or soluble as collagenases, gelatinases and stromelysins, and are synthesized as inactive proenzymes by tumor cells and surrounding tumor stromal cells (21). Among MMPs, the catalytic gelatinase MMP-9 has been previously investigated due to its ability to degrade type IV and V collagen in the BM (22). The degradation of type IV collagen is associated with increased levels of MMP-9 in HNSCC (23). In HNSCC increased levels of MMP-9 are also associated with increased lymphonodal metastasis (24).

Angiogenesis and neovascularization are essential in tumor cell formation (25). Vascular endothelial growth factor (VEGF), and the VEGF receptors (VEGFR)-1, -2 and -3, serve an important role in the proliferation and differentiation of endothelial cells (25). Increased expression of VEGF and VEGFR has been reported in various tumor entities, including HNSCC (26,27). Tumor growth and supporting processes, including angiogenesis, are directly associated with VEGF in HNSCC (28). The importance of molecular indicators, including VEGF, in the microenvironment of tumor cells is increasing as previous studies have revealed that plasma levels of VEGF can be used as prognostic markers in HNSCC (27,29). Increased expression of VEGFR has also been identified in HPV-positive SCC cell lines (30,31). The role of VEGFR-2 is well understood and it is known to be overexpressed by tumor endothelial cells, and promotes cell proliferation and migration (25). By contrast, the role of VEGFR-1 in tumor formation is poorly understood; it may serve a role in the process of VEGF sequestration or stimulation of hematopoietic stem cell migration (32). Furthermore, the expression of VEGFR-1 appears to be associated with cell survival and radiosensitivity (33). In HPV-associated tumor disease, several HPV-dependent oncoproteins have been reported to alter VEGFR-1 expression *in vitro* (34,35). Intracellular VEGF signaling is mediated by the activation and transphosphorylation of its tyrosine kinase receptors, VEGFR-1, -2 and -3 (25). A major principle of targeted therapy in tumor disease involves the selective inhibition of tyrosine kinase receptors, to inhibit the process of subsequent intracellular signaling cascades. Small molecule targeted therapies have been established in multiple types of cancer (36-45). Erlotinib and gefitinib are orally available selective tyrosine kinase inhibitors of epidermal growth factor receptor (EGFR) and are approved for the therapy of non-small cell lung cancer (NSCLC) (36-38). Gefitinib functions through the competitive inhibition of ATP binding to EGFR and consecutive inhibition of receptor autophosphorylation, leading to a subsequent decrease in proangiogenic proteins, including VEGF (39,40). It has also been reported that gefitinib affects the synthesis of MMPs and other extracellular matrix proteins in tumor tissues (41). BCR-ABL fusion protein (BCR-ABL) inhibitors were designed for the treatment of chronic myeloid leukemia (42). A reciprocal translocation between chromosomes 9 and 22, known as the Philadelphia chromosome, forms the BCR-ABL oncogene (42). Furthermore, the BCR-ABL inhibitors nilotinib and dasatinib also function by inhibiting the platelet-derived growth factor receptor (PDGFR) and mast/stem cell growth factor receptor Kit (c-KIT) (43,44). The inhibitory effects of dasatinib are mediated through the inhibition of proto-oncogene tyrosine-protein kinase Src (Src), a process associated with tumor proliferation and angiogenesis (45). To the best of our knowledge, the present study is the first to investigate the alteration of VEGFR-1 and MMP-9 expression in HPV-associated SCC cells *in vitro*, following treatment with the small molecule inhibitors erlotinib, gefitinib, nilotinib and dasatinib.

Materials and methods

Cell lines. A total of two distinct HPV-negative cell lines originating from oropharyngeal and laryngeal SCC (HNSCC 11A and HNSCC 14C) were donated by Dr T. E. Carey (University of Michigan, Ann Arbor, MI, USA). The p16 positive CERV196 cell line was obtained from poorly differentiated SCC cells of the uterine cervix (Cell Line Service GmbH, Eppelheim, Germany). The CERV196 tumor cells were cultured in Eagle's minimum essential medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 2 mM L-glutamine and Earle's balanced salt solution (Thermo Fisher Scientific, Inc.), adjusted to contain 1.0 g/l sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate and 10% fetal bovine serum (Thermo Fisher Scientific, Inc.). The HNSCC 11A and 14C tumor cells were cultured in Dulbecco's Eagle's minimum essential medium (Thermo Fisher Scientific, Inc.), supplement with 10% fetal calf serum (Thermo Fisher Scientific, Inc.), 2 mM L-glutamine and an antibiotic/antimycotic solution (penicillin-streptomycin, 10,000 U/ml; working dilution, 1/100; Thermo Fisher Scientific, Inc.). Cell cultures were incubated at 37°C and 5% CO_2 for 24, 48, 72 or 96 h. Orally available nilotinib, dasatinib, gefitinib and erlotinib were donated by Dr Hofheinz (Department of Oncology, Medical Faculty Mannheim, University of Heidelberg, Mannheim, Germany). The drugs were stored at room temperature and dissolved in dimethyl sulfoxide. The tumor cells were incubated at 37°C with 20 μmol/l of each of the four substances for 24, 48, 72 and 96 h and compared with the negative control (untreated cells). The alamarBlue (AbD Serotec, Raleigh, NC, USA) cell proliferation assay was used

to quantify proliferating HNSCC tumor cells and establish the relative cytotoxicity of the tyrosine kinase inhibitors according to the manufacturer's protocol.

VEGFR-1 and MMP-9 ELISA. Determination of protein concentrations was performed using the ELISA technique. Subcultures of the cells were generated by diluting and dissolving the cells from the culture. A PBS solution supplemented with a combination of 0.05% trypsin and 0.02% EDTA (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added at 37˚C for 5 min to passage the cells. Subcultures were transferred to microplates for further analysis of proliferation at a 70% confluence. Protein expression was analyzed following centrifugation at 8,050 x *g* for 10 min at room temperature and collection of the medium supernatant. The DuoSet IC Human Total VEGFR-1 (catalog no. DY321B; R&D Systems, Inc., Minneapolis, MN, USA) and DuoSet IC Human Total MMP-9 (catalog no. DY911; R&D Systems, Inc.) kits were used. For the sandwich ELISA, a solid-phase capture antibody specific for VEGFR-1 or MMP-9 was used, as well as a specific detection antibody [standard streptavidin-horseradish peroxidase (HRP) format]. The capture antibody was diluted to the working concentration in PBS (VEGFR-1, 2.0 μg/ml; MMP-9, 1.0 μg/ml) and incubated overnight at room temperature according to the manufacturer's protocol. Three washing steps with the washing buffer containing Tween 20 were performed. The ELISA plates were blocked by adding 300 μl of the Reagent Diluent (Reagent Diluent, DY995; R&D Systems, Inc.) to each well and were incubated for 1 h at room temperature and were washed again with the washing buffer for three times. The detection antibody was diluted to its working concentration (VEGFR-1, 0.5 μg/ml; MMP-9, 0.1 μg/ml) and incubated with the ELISA plate for 2 h at room temperature. The ELISA plate was washed three times with Tween 20 and incubated with streptavidin-HRP (diluted according to the manufacturer's protocol) for 20 min at room temperature. The wells were subsequently washed with Tween 20. The visualization reaction was initiated by adding the substrate solution for 20 min followed by 50 μl stop solution at room temperature according to the manufacturer's protocol. Each ELISA was performed according to the manufacturer's protocol. Each experiment was performed for three times. The calibrations on the microtiter plates included recombinant human VEGFR-1 and MMP-9 standards that were provided in the kits. Optical density was measured using a microplate reader (MRX ELISA Reader; Dynatech, El Paso, TX, USA) and a wavelength of 450 nm. Wavelength correction was set to 540 nm and concentrations were reported in pg/ml. The range of detection was between 28.4 and 281.5 pg/ml for VEGFR-1, and between 0.02 and 1021.9 pg/ml for MMP-9. Inter-assay coefficient of variation reported by the manufacturer was <10%.

Statistical analysis. Statistical analysis was performed using the mean values from each experiment. Each experiment was performed for at least three times (n=3). Comparisons were made with the negative control to evaluate statistical significance. Values are presented as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference. The two-coefficient variance test (SAS Statistics software, version 9.3; SAS Institute, Inc., Cary, NC, USA) and Dunnett's test were used.

Results

VEGFR-1 expression levels in HNSCC 11A, HNSCC 14C and CERV196 cells. VEGFR-1 expression was detected in all three cell lines. Treatment with 20 μmol/l dasatinib, gefitinib and erlotinib between 48 and 96 h significantly reduced VEGFR-1 expression in HNSCC 11A cells compared with the negative control (all P<0.001; Fig. 1A). Treatment with 20 μmol/l nilotinib for 48 h significantly reduced VEGFR-1 expression in HNSCC 11A cells compared with the negative control (P=0.002; Fig. 1A). Treatment with nilotinib for 96 h significantly reduced VEGFR-1 expression in HNSCC 11A cells compared with the negative control (P=0.003; Fig. 1A). There was no significant decrease in VEGFR-1 protein expression after 24 h treatment with any of the drugs (Fig. 1A). Treatment with nilotinib exhibited a significant decrease in VEGFR-1 expression after 24 h in the HNSCC 14C cells compared with the negative control cells (P=0.040; Fig. 1B). A significant increase in VEGRF-1 expression in HNSCC 14C cells was observed following treatment with nilotinib for 96 h compared with the negative control cells (P=0.038; Fig. 1B). Following treatment with nilotinib for 48 and 72 h VEGFR-1 expression appeared to increase. Treatment with gefitinib also markedly increased VEGFR-1 expression in HNSCC 14C cells after between 48 and 96 h compared with the negative control cells. In the HNSCC 14C cells, treatment with dasatinib markedly decreased VEGFR-1 expression after between 24 and 72 h compared with the negative control cells. No significant alteration was observed following treatment with erlotinib (Fig. 1B). In the CERV196 cells increased VEGFR-1 protein expression was typically observed following treatment compared with the negative control cells (Fig. 1C). Treatment with nilotinib for 24 and 72 h significantly increased VEGFR-1 expression compared with the negative control cells (P=0.008 and P=0.023, respectively; Fig. 1C). Treatment with dasatinib exhibited a significant increase in VEGFR-1 expression in the CERV196 cells after 24 and 72 h compared with the negative controls (P=0.037 and P=0.040, respectively; Fig. 1C). Treatment with erlotinib significantly increased VEGFR-1 expression after 48 h compared with the negative control cells (P=0.001; Fig. 1C). Treatment with gefitinib significantly increased protein levels of VEGFR-1 after 48 and 72 h compared with the negative control cells (P=0.018 and P=0.041, respectively; Fig. 1C). Decreased VEGFR-1 expression was observed following treatment with gefitinib for 24 h and nilotinib for 96 h (Fig. 1C). The quantified VEGFR-1 expression levels are presented in Table I.

MMP-9 expression levels in HNSCC 11A, HNSCC 14C and CERV196 cells. MMP-9 expression was evaluated in all three cell lines. Treatment with 20 μmol/l dasatinib, gefitinib and erlotinib between 24 and 96 h significantly decreased MMP-9 expression in the HNSCC 11A cells compared with the negative control cells (all P<0.001; Fig. 2A). Treatment with 20 μmol/l nilotinib for 24 and 48 h also significantly decreased MMP-9 expression compared with the negative control cells (P<0.001 and P=0.014, respectively; Fig. 2A). In addition, a marked decrease in MMP-9 expression in HNSCCC 11A cells was observed following treatment nilotinib for 72 and 96 h. In
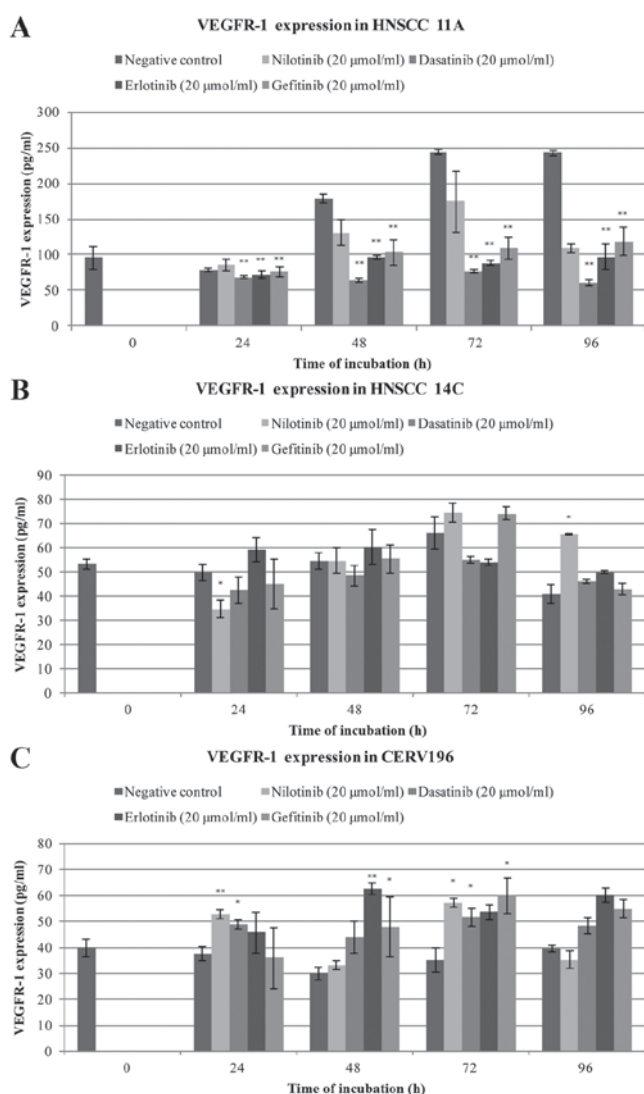
Figure 1. Alterations in VEGFR-1 expression in (A) HNSCC 11A, (B) HNSCC 14C and (C) CERV196 cells following incubation with nilotinib, dasatinib, erlotinib and gefitinib at a concentration of 20 μmol/l. Values are presented as the mean ± standard deviation (*P<0.05, **P<0.01 compared with negative control cells). VEGFR-1, vascular endothelial growth factor receptor 1.
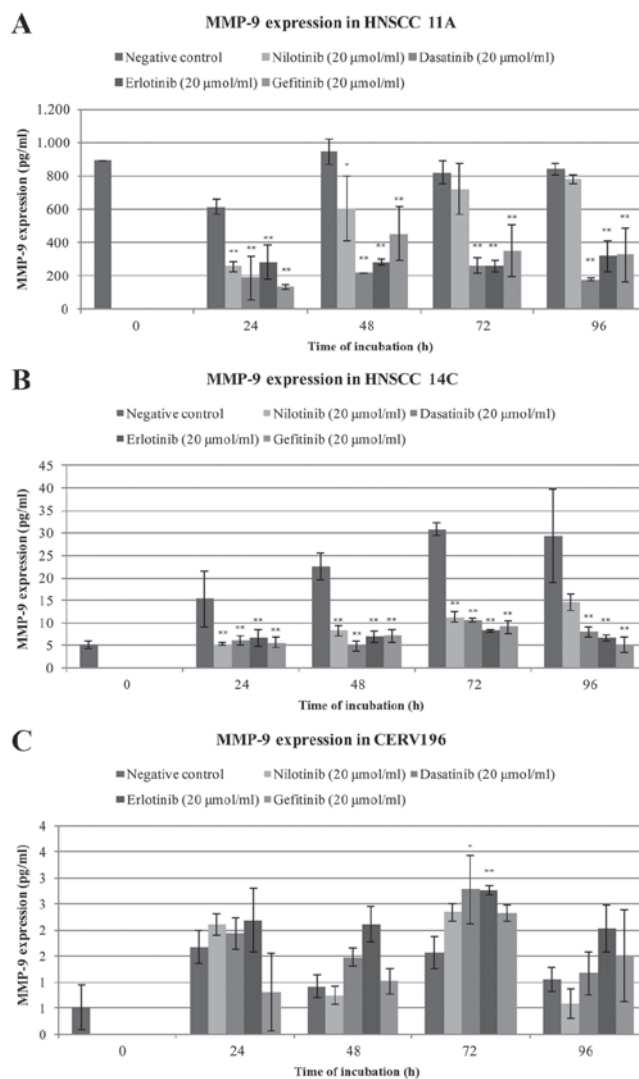

Figure 2. Alterations in MMP-9 expression in (A) HNSCC 11A, (B) HNSCC 14C and (C) CERV196 cells following incubation with nilotinib, dasatinib, erlotinib or gefitinib at a concentration of 20 μmol/l. Values are presented as the mean ± standard deviation (*P<0.05, **P<0.01 compared to negative control cells). MMP-9, matrix metalloproteinase 9.

the HNSCC 14C cells a significant decrease in MMP-9 protein expression was observed following treatment with dasatinib, gefitinib and erlotinib across all time points compared with the negative control cells (all P<0.001; Fig. 2B). Treatment with nilotinib for between 24 and 72 h also significantly decreased MMP-9 expression compared with the negative control cells (all P<0.001; Fig. 2B). Treatment with dasatinib and erlotinib for 72 h led to a significant increase in MMP-9 expression in the CERV196 cells compared with the negative control cells (P=0.014 and P=0.007, respectively; Fig. 2C). The majority of treatment types and durations induced no significant alteration in MMP-9 expression in the CERV196 cells compared with the negative control cells.

Discussion

The present study was performed in order to evaluate the expression of VEGFR-1 and MMP-9 in HPV-positive and

-negative SCC cells and to measure the altered expression patterns of these biomarkers following treatment with the well-established tyrosine kinase inhibitors nilotinib, dasatinib, erlotinib and gefitinib.

The tumor microenvironment is essential for tumor progression, survival and the formation of metastases (25). In the context of tumor growth, penetration of the BM is important as the BM functions as a barrier and differentiates between non-invasive and invasive tumor growth. MMPs are known to degrade ECM substrates and promote the invasion of tumorous vasculature (46). The role of MMP-9 is decisive as it dissolves the BM by degrading type IV and V collagen (22). In the present study, a statistically significant decrease in MMP-9 expression was observed in HPV-negative HNSCC tumor cells following treatment with tyrosine kinase inhibitors in a time-dependent manner, despite none of the drugs functioning as a direct inhibitor of MMP-9. MMP-9 expression may be regulated by multiple factors including cytokines and growth factors including EGFR and transforming growth factors (47).

Table I. Quantification of vascular endothelial growth factor receptor 1 expression levels in HNSCC 11A, 14C and CERV196 cells following incubation with nilotinib, dasatinib, erlotinib and gefitinib at a concentration of 20 μmol/l.

| | | | Treatment | | | | | | | |
| | | NC | Nilotinib | | Dasatinib | | Erlotinib | | Gefitinib | |
Cell line	Incubation time, h	Mean	Mean	P-value	Mean	P-value	Mean	P-value	Mean	P-value
HNSCC 11A										
	24	78.6	86.3	0.358	68.2	0.340	72.2	1.00	76.3	0.818
	48	179.3	131.1	0.003[a]	64.4	<0.001[a]	96.5	<0.001[a]	102.7	<0.001[a]
	72	243.3	174.2	<0.001[a]	77.1	<0.001[a]	88.2	<0.001[a]	109.7	<0.001[a]
	96	242.2	109.4	0.002[a]	60.6	<0.001[a]	97.1	<0.001[a]	118.6	<0.001[a]
HNSCC 14C										
	24	49.8	34.8	0.040[a]	42.5	0.999	59.2	0.637	45.2	0.999
	48	54.6	54.7	0.995	48.5	0.513	60.2	0.974	55.6	0.570
	72	66.3	74.4	0.997	55.1	0.172	54.0	0.071	74.2	0.962
	96	40.9	65.8	0.038[a]	46.1	0.949	50.0	0.461	43.1	0.995
CERV196										
	24	37.5	52.8	0.008[a]	48.9	0.037[a]	45.8	0.218	36.0	0.753
	48	30.1	33.0	0.783	44.1	0.076	62.4	0.001[a]	47.9	0.018[a]
	72	35.2	57.1	0.023[a]	51.8	0.040[a]	53.6	0.141	60.1	0.041[a]
	96	39.5	35.4	0.760	48.3	0.966	60.1	0.147	54.8	0.279

[a]P<0.05 vs. the NC cells. Mean values, pg/ml. NC, negative control.

Table II. Quantification of matrix metalloproteinase 9 expression levels in HNSCC 11A, 14C and CERV196 cells following incubation with nilotinib, dasatinib, erlotinib and gefitinib at a concentration of 20 μmol/l.

| | | | Treatment | | | | | | | |
| | | NC | Nilotinib | | Dasatinib | | Erlotinib | | Gefitinib | |
Cell line	Incubation time, h	Mean	Mean	P-value	Mean	P-value	Mean	P-value	Mean	P-value
HNSCC 11A										
	24	613.3	256.7	<0.001[a]	188.0	<0.001[a]	281.5	<0.001[a]	133.1	<0.001[a]
	48	944.1	603.0	0.014[a]	217.2	<0.001[a]	280.6	<0.001[a]	451.9	<0.001[a]
	72	820.4	719.4	0.188	260.1	<0.001[a]	257.0	<0.001[a]	351.4	<0.001[a]
	96	839.7	778.8	0.987	177.7	<0.001[a]	319.1	<0.001[a]	325.8	<0.001[a]
HNSCC 14C										
	24	15.3	5.4	<0.001[a]	6.2	<0.001[a]	6.7	<0.001[a]	5.6	<0.001[a]
	48	22.4	8.3	<0.001[a]	4.9	<0.001[a]	7.0	<0.001[a]	7.1	<0.001[a]
	72	30.8	11.3	<0.001[a]	10.6	<0.001[a]	8.3	<0.001[a]	9.1	<0.001[a]
	96	29.4	14.6	0.170	8.0	<0.001[a]	6.7	<0.001[a]	5.1	<0.001[a]
CERV196										
	24	1.7	2.1	0.170	1.9	0.951	2.2	0.206	0.8	0.691
	48	0.9	0.8	0.998	1.5	0.866	2.1	0.438	1.0	0.746
	72	1.6	2.3	0.632	2.8	0.014[a]	2.8	0.007[a]	2.3	0.254
	96	1.1	0.6	0.978	1.2	0.999	2.0	0.451	1.5	0.988

[a]P<0.05 vs. the NC cells. Mean values in pg/ml. NC, negative control.

Activated EGFR may also promote cell migration through the regulation of MMP-9 expression in an epithelial-mesenchymal transition (EMT)-like process, which leads to the degradation of E-cadherin. This effect has also been reported in SCC

cells (48). Additionally, multiple signaling pathways including the mitogen-activated protein kinase (MAPK) and protein kinase B (AKT) pathways regulate the expression levels of MMPs including MMP-9 (49). The process of EMT may be activated through the MAPK signaling pathway which may lead to the migration and spread of tumor cells (50). Furthermore, the activation of MMPs including MMP-9 may result in long-term EMT (51).

Liu and Klominek (52) reported a decrease in MMP-9 production in malignant mesothelioma following treatment with erlotinib. Furthermore, a gefitinib-induced reduction in MMP-9 and MMP-2 expression was observed several tumor entities including oral HNSCC (41,53,54). Erlotinib and gefitinib function as inhibitors of EGFR (37), therefore, MMP-9 regulation in HNSCC 11A and HNSCC 14C cells may be EGFR-mediated. Selective EGFR-inhibiting proteins may therefore be used for the direct inhibition of EGFR and the indirect inhibition of MMP-9 expression in HPV-negative HNSCC. In the present study, treatment with dasatinib and nilotinib also significantly reduced the expression levels of MMP-9 in p16-negative HNSCC tumor cells. However, the mechanism of action remains unclear as nilotinib and dasatinib function through the inhibition of BCR-ABL, PDGFR and c-KIT, and Src in the case of dasatinib. The mechanism of decreased MMP-9 expression following treatment with nilotinib and dasatinib remain to be elucidated. A recent study demonstrated that MMP-9 expression may be decreased in pituitary adenomas, suggesting that nilotinib exerts its inhibitory effects through the inhibition of the tyrosine kinase receptor, epithelial discoidin domain-containing receptor 1 (DDR1) (55). The present study hypothesizes that DDR1 may be associated with the regulation of MMPs, including MMP-2 and MMP-9, and therefore, may be associated with the mechanism of decreased MMP-9 levels in HNSCC, although DDR1 expression patterns in HNSCC remain to be elucidated.

Src kinases are a subcategory of non-receptor protein tyrosine kinases and contribute to tumor growth in tumor stromal cells (56). The results of the present study support the study by Liang *et al* (57), which associated the dasatinib-induced reduction in MMP-9 expression in tumor cells with the inactivation of Src-dependent signaling pathways. Therefore, the targeting of the Src kinase family with dasatinib may be a promising objective for further investigation into selective therapeutic approaches in solid malignant tumors, including HNSCC. In the present study, a similar effect was not observed in the p16-positive CERV196 cells. By contrast, an increase in MMP-9 expression was detected following treatment with dasatinib and erlotinib for 72 h in CERV196 cells. These results indicated HPV-dependent mechanisms in SCC cells to evade decreased MMP-9 levels. The level of MMP-9 expression was decreased in p16-positive squamous cancer cells. p16-associated oncoproteins E6 and E7 promote the activity of MMPs, including MMP-9 in cervical SCC cells (58). Therefore, a potential explanation for the intransigence or increase in MMP-9 expression is the counter regulation of the drug-induced decrease in MMP-9 through the activation of viral oncoproteins. Hu *et al* (59) demonstrated that activation of β-catenin, a functional protein coordinating cell-cell adhesion and promoting the expression of ECM components, including fibronectin, may be induced by viral oncoproteins. It was hypothesized

that other metastasis-associated proteins may be facilitated by p16-induced oncoproteins, which is consistent with the results of the present study of increased MMP-9 expression by nilotinib, dasatinib, erlotinib and gefitinib as it is not affected by this type of selective tyrosine kinase inhibition. The role of MMP-9 is complex and requires elucidation in further studies to investigate the therapeutic potential of targeting MMPs in HNSCC.

VEGFR-induced angiogenesis is important in local tumor progression and the formation of distant metastases. VEGFR-1 is expressed on the surface of endothelial cells and its expression was evaluated in all three cell lines. The expression and function of VEGFR-1 in tumor cells as a vascular and non-vascular modulator is less well-understood compared with VEGFR-2. The role of VEGFR-1 as a target for selective inhibition is in the early stages (60). Currently, there are no published data investigating the effect of the indirect inhibitors nilotinib, dasatinib, erlotinib and gefitinib on VEGFR-1 expression in HNSCC with respect to HPV-status. In the present study, a decrease in VEGFR-1 expression was observed in the HNSCC 11A cells following treatment with all the tested drugs for between 48 and 96 h. There was also a tendency towards a decrease in VEGFR-1 expression in the HPV-negative HNSCC 14C cells following treatment with nilotinib and dasatinib. The cellular mechanism for this effect remains unclear as nilotinib and dasatinib are not direct inhibitors of VEGFR.

It has been reported that the activation of Src serves an essential role in the signal transduction downstream of various growth factor receptors including VEGFR (61). Also, Src kinase activity in tumor cells is elevated (61,62). As previously described, Liang *et al* (57) demonstrated that dasatinib inhibits the angiogenic potential of endothelial cells including the expression of VEGF in tumor-associated endothelial cells, suggesting Src to be a key downstream effector of angiogenic signaling pathways. A Src-induced stop signal in the sense of a negative feedback mechanism for further VEGFR-expression may therefore be a potential explanation of the nilotinib- and dasatinib-induced effects on VEGFR-1 in HPV-negative tumor cells. The selective tyrosine kinase inhibitors erlotinib and gefitinib function through EGFR-inhibition. The results of the present study support several previous studies, which suggested that EGFR activation regulates VEGF expression (63-65). This mechanism may provide an explanation for the decrease in VEGFR-1 expression though selective EGFR-inhibition in HNSCC 11A. In the present study, an alteration in VEGFR-1 expression following treatment with erlotinib and gefitinib in HNSCC 14C was not detected. The HPV-positive CERV196 cells exhibited markedly decreased levels of VEGFR-1 expression in comparison with the HPV-negative tumor cells. In addition, an increase in VEGFR-1 expression in the HPV-positive CERV196 tumor cells was observed following treatment with all of the tested drugs. It is known that viral oncogenes may induce the expression of angiogenic factors, including VEGF (30,34). Therefore, a potential mechanism for the increase in VEGFR-1 expression is the drug-induced activation or stimulation of viral oncogenic proteins, including E6 and E7. As a result, unknown cellular autocrine mechanisms may increase the production of angiogenic proteins. This may be a potential evasive strategy of malignant cells following

drug-induced dysregulation. Dias *et al* (66) discussed a similar mechanism in virally transformed cancer cells of the oropharynx as a result of treatment with cetuximab. However, this hypothesis remains to be completely elucidated.

In conclusion, the present study is one of the first to investigate the altered expression patterns of the viable molecular target proteins MMP-9 and VEGFR-1, in p16-positive and -negative SCC cells, following treatment with the non-direct selective tyrosine kinases nilotinib, dasatinib, erlotinib and gefitinib *in vitro*. The results of the present study provide an improved understanding of MMP-9 and VEGFR-1, and their interaction with selective small molecule inhibitors. These results may be used in further investigation into novel strategies of targeted therapy in p16-positive and -negative HNSCC.

Acknowledgements

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