Cilengitide and Cetuximab Reduce Cytokine Production and Colony Formation of Head and Neck Squamous Cell Carcinoma Cells *Ex Vivo*

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Abstract. *Background/Aim:* To analyze *ex vivo* effects of combined targeting of the epidermal growth factor-receptor (EGFR) by cetuximab (E) plus $\alpha V\beta 3$ and $\alpha V\beta 5$ integrins by cilengitide (Cil) on colony formation of epithelial cells (CF_{ec}) and release of pro-angiogenic and pro-inflammatory cytokines in head and neck squamous cell carcinoma (HNSCC) cells. *Materials and Methods:* Collagenase-IV digests of 43 histopathological confirmed HNSCC cases were seeded into laminin-coated 96-well plates containing E, Cil, or Cil+E in final concentrations of 66.7 μ g/ml, 10 μ M, and 10 μ M+66.7 μ g/ml, respectively. Following the FLAVINO-assay protocol, supernatants were harvested after 3 days and adherent cells fixed in ethanol. Counting of CF_{ec} was facilitated by FITC-labeled pan-cytokeratin antibodies. Out of 43 HNSCC cases, 39 had adherent growth (mean $CF_{ec} \geq 4$ /well in triplicate controls). Cytokines in supernatants were measured using ELISA were interleukin 6 (IL-6), monocyte chemoattractant protein 1 (MCP-1) and vascular endothelial growth factor A (VEGFA). *Results:* CF_{ec} on laminin was significantly reduced by Cil, E, and Cil+E. Cytokine measurements also revealed significant suppression of MCP-1, IL-6 and VEGFA. The strongest suppression of CF_{ec} , MCP-1 and VEGFA release was exerted by Cil and E

combined. Efficacy of Cil+E exceeded those of the solely applied pharmaceuticals but failed regarding significant synergism of both treatments as E was unable to significantly boost the effects of Cil. In contrast, IL-6 release was significantly suppressed by E but not by Cil, while their combination strongly reduced it. *Conclusion:* Combined targeting of EGFR and integrins with E and Cil heightens their suppressive effects regarding CF_{ec} as well as release of pro-angiogenic and pro-inflammatory cytokines.

Tumors of the head and neck region, mostly head and neck squamous cell carcinomas (HNSCC), especially locally advanced and recurrent and/or metastatic HNSCC, are still related with poor outcome regarding 5-year survival time despite advancements in multimodal therapy strategies such as combined surgery with radio chemotherapy (1). Individual personalized therapy approaches need further investigation to overcome tumor heterogeneity and incalculable therapy response rates. Cytokines can potentially serve as biomarkers or even as new targets for therapy. This study focuses on monocyte chemoattractant protein (MCP-1), vascular endothelial growth factor A (VEGFA) and interleukin 6 (IL-6) which are potentially important in tumor proliferation, vascularization and metastasis (2). Recent results showed that MCP-1 is of special interest since inhibition of MCP-1 may improve the power of standard cytotoxic substances against HNSCC and on the other hand, MCP-1 may act as agent in tumor defense (3). In 2005 it was shown that IL-6 levels in cancer patients' serum correlates with tumor stage and lymph node status suggesting an active role of IL-6 in tumor progression (4). High IL-6 expression is also associated with poor prognosis and cisplatin resistance in HNSCC and other cancers (5). Since HNSCC are highly vascularized, it makes sense to investigate therapeutic options to interfere with tumor angiogenesis. Proangiogenic VEGFA is overexpressed in HNSCC and associates with elevated aggressiveness of the

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tumor (6-9). Targeted therapy such as inhibiting epidermal growth factor receptor (EGFR) with monoclonal antibody E is already used in clinical routine treatments (10, 11). As the first approved EGFR inhibitor for treatment of HNSCC in 2006, E improves survival in curative treatment of advanced HNSCC (10, 12) but also in first-line treatment of recurrent and/or metastatic HNSCC when teamed up with cisplatin and 5-fluorouracil (5-FU) (13). Since EGFR upregulates VEGFA, a potential readout of the response of HNSCC to E may be VEGFA as reported in a number of studies (14-17). Other attractive targets are integrins as they are key players in tumor growth by binding to particular extracellular matrix (ECM) being crucial in cell communication, cell-to-cell and cell-to-ECM interaction, modulating signaling pathways important for cell growth, differentiation and survival (18). The RGD-peptide Cil is a selective inhibitor for $\alpha V\beta 3$ and $\alpha V\beta 5$ integrins (18, 19). As both integrins are excessively expressed during vascularization, and $\alpha V\beta 5$ is overexpressed in various tumor entities including HNSCC, targeting these integrins by Cil is therefore promising (20). In phase I and II trial of Cil combined with E, cisplatin and 5-FU, no dose-limiting toxicities were found (13, 21) which makes treatment with Cil combinations even more attractive for further investigations concerning heterogeneity in *ex vivo* response of HNSCC. This work demonstrates the effects of Cil and E, alone and in combination on CF_{ec} and release of MCP-1, IL-6 and VEGFA by HNSCC.

Patients and Methods

Patient characteristics. HNSCC samples of 43 patients were included in this study. With patients' informed consent, biopsies of tumor tissue were obtained during surgery or panendoscopy. 39 histopathologically-confirmed HNSCC patients of 34 males and 5 females (mean age at 60.3 years; Table I) could be analyzed regarding tumor cytokine production and colony formation after treating the specimens with or without Cil or E alone or in combination.

The primaries' localizations were mostly oropharynx (24/39; 61.5%), larynx and hypopharynx (12/39; 30.8%); three HNSCC were localized in the nasopharynx (1/39; 2.6%) and oral cavity (2/39; 5.1%).

Only 8/39 (21.5%) tumors were early stage (UICC I and II), while 31/39 (79.5%) cancers were locally advanced (7/39, 18.0%, UICC III; 24/39, 61.5%, UICC IV). 16/39 (41%) had no lymph node metastasis, while 23/39 (59.0%) had lymph node metastasis at first diagnosis (N1: 3/39, 7.7%; N2a: 1/39, 2.5%; N2b: 6/39, 15.4%; N2c: 12/39, 30.8%; N3: 1/39, 2.5%); two patients had distant metastasis (M1: 2/39, 5.1%). 8/39 (21.5%) patients were nonsmokers, 4/39 (10.3%) avoided alcohol. Only 2 patients had never consumed tobacco and alcohol; 1 patient denied information about these aspects (Table I).

FLAVINO-assay. The same procedures according the protocol of the FLAVINO assay were used as previously described (3). Briefly, freshly obtained tumor specimens were put into phenol red- and riboflavin-free medium supplemented with 10% fetal calf serum and antibiotics (TM). After mechanical disintegration and digestion by

230 mU/ml collagenase IV (Sigma-Aldrich, Deisenhofen, Germany) 10,000 viable HNSCC cells were added to wells in triplicates coated with human laminin (Roche, Germany) containing either 66.7 μ g/ml E, 10 μ M Cil, Cil+E, or (for reference) medium alone, adjusting the total volume to 300 μ L. According to the FLAVINO-assay protocol, supernatants were harvested after 3 days and adherent cells were ethanol-fixed. Counting of colonies of epithelial cells was facilitated by FITC-labeled pan-cytokeratin antibodies. 39 HNSCC had adherent growth (mean $CF_{ec} \geq 4$ /well in triplicate controls).

ELISA assay. The influence of Cil and E on the cytokine secretion by HNSCC was analyzed using ELISA. The levels of cytokines IL-6, MCP-1 and VEGFA in supernatants were measured using indirect sandwich ELISAs (OptEIA Kits; BD Biosciences, Heidelberg, Germany) following the manufacturers' instructions and using tetramethyl benzidine as substrate. The optical density of each well was determined measuring the optical density at $\lambda_1=450$ nm and $\lambda_2=620$ nm on the Synergy2™ multi-mode microplate reader (BioTek Instruments, Inc., Winooski, VT, USA); calibration curves were calculated using the Gen5 software (BioTek). The lower limit of detection for all cytokines was ≤ 4 pg/ml.

Statistical analysis. All data shown herein are based on triplicate measurements. Differences were compared by Student's *t*-test for paired samples using SPSS Statistics for Windows, version 20.0.0 (SPSS Inc., Chicago, IL, USA); *p*-values ≤ 0.05 were regarded as significant.

Results

Colony formation by HNSCC and release of IL-6, MCP-1 and VEGFA demonstrated a huge amount of heterogeneity. In untreated controls no significant association were found with localization of the primary tumor, T category, N category, UICC stage, risk factors (pack years of tobacco smoking, daily alcohol consumption), sex and other patient characteristics regarding un-normalized readouts.

Treatments however, showed different effects on all analyzed parameters when using *t*-tests for paired samples compared to heteroscedastic *t*-tests for comparisons of measures normalized to individual controls. The effects of E, Cil, and Cil+E are shown in Figure 1 for CF_{ec} , IL-6, MCP-1, and VEGFA normalized to controls. Targeting EGFR and integrins is suppressing CF_{ec} and cytokine release but shows variable strength of effects on individual readouts and among HNSCC patients. This is demonstrated by the scatter plots of Table II showing the correlation of normalized data for CF_{ec} , IL-6, MCP-1 and VEGFA regarding the three treatments.

Cilengitide and cetuximab affect colony formation and cytokine production. In contrast to an earlier study with smaller sample size and slightly different experimental conditions regarding coating of cell-culture microtiter-plates (3), Cil suppressed significantly CF_{ec} on laminin ($p=0.0003$; Table II). However, also E ($p=0.004$) and Cil and E combined ($p=0.0003$) reduced CF_{ec} (Table II). IL-6 production was not significantly affected by Cil ($p=0.07$) and only slightly by E ($p=0.03$) alone but combining Cil and

Table I. Characteristics of HNSCC patients and treatment for 39 patients with cytokine measurements among the cohort of 43 patients with sufficient colony formation ($CF_{ec} \geq 4$ per well of untreated control in laminin-coated microtiter-plates) allowing for reliable response assessment.

Patient ID	Gender	Localization of primary	Age at diagnosis (years)	UICC stage	T	N	M	Therapy	Therapy monomodal vs. multimodal	Tobacco smoking (Pack Years)	Alcohol	Grading	Pn	V	L	R
1	M	oroph	57.9	III	2	1	0	Op+PORT	multi	45	≥ 60	3	0	0	1	0
2	M	larynx	59.5	III	3	0	0	Op+PORT	multi	0	0	2	0	0	0	0
3	M	hypo	57.9	IVA	3	2c	0	ICCh+Op+RT	multi	40	< 30	2	1	0	1	0
5	F	oroph	78.3	II	2	0	0	Op	mono	50	≥ 60	3	0	0	1	0
6	M	oroph	58.2	IVA	4a	2c	0	pRChT	multi	40	< 60	3	--	--	--	--
7	M	larynx	69.7	III	3	0	0	Op	mono	--	--	2	0	0	0	0
8	M	oroph	48.9	II	2	0	0	Op+PORT	multi	40	< 30	2	1	0	0	0
9	M	hypo	43.8	IVA	1	2a	0	Op+PORT	multi	30	≥ 60	2	0	0	0	0
10	M	oroph	63.2	IVA	1	2b	0	ICCh+Op+RT	multi	0	< 60	3	0	0	1	0
11	F	larynx	64.1	IVA	4a	2c	0	Op	mono	8	< 30	2	0	0	1	1
12	M	hypo	73.2	IVA	4a	1	0	Op	mono	20	< 30	2	--	--	--	--
13	M	oroph	66.3	II	2	0	0	Op	mono	40	< 30	2	0	0	0	0
14	M	oroph	49.7	III	3	0	0	ICCh+Op+RT	multi	45	≥ 60	2	1	0	1	--
15	M	hypo	56.6	IVC	4a	2c	1	pRChT	multi	--	< 30	2	--	--	--	--
16	F	nasoph	50.8	IVA	4a	0	0	Op+PORChT	multi	0	< 30	3	0	1	0	0
17	M	oral	77.3	II	2	0	0	Op+PORT	multi	80	< 60	3	1	0	0	0
19	M	oroph	53.7	IVB	3	3	0	Op+PORT	multi	30	≥ 60	2	1	0	1	0
20	M	larynx	66.3	I	1	0	0	Op	mono	30	≥ 60	2	0	0	1	0
21	M	oroph	49.0	III	3	0	0	Op	mono	30	< 30	3	0	0	1	0
22	M	hypo	49.3	IVC	4a	2c	1	pRChT	multi	45	≥ 60	3	--	--	--	--
24	M	oroph	59.2	III	3	1	0	Op	mono	0	< 30	2	0	0	1	0
25	M	oroph	56.8	IVA	4a	2b	0	Op+PORChT	multi	40	< 60	3	1	1	1	0
26	F	oroph	74.2	I	1	0	0	Op	mono	0	0	2	1	0	0	0
27	M	oroph	61.1	IVA	1	2c	0	Op+PORChT	multi	90	< 60	3	0	0	1	0
28	F	larynx	50.4	IVA	3	2c	0	Op+PORChT	multi	40	--	2	0	1	1	0
29	M	oroph	58.7	IVA	3	2c	0	Op+PORT	multi	60	≥ 60	2	--	--	--	--
30	M	oroph	74.0	IVA	3	2b	0	Op+PORChT	multi	0	< 30	2	1	1	1	0
32	M	oroph	56.0	IVA	4a	2c	0	Op	mono	40	< 30	2	1	1	1	0
33	M	oroph	60.0	IVA	3	2b	0	Op	mono	0	< 30	3	1	0	1	0
34	M	oroph	64.4	IVA	4a	0	0	Op+PORT	multi	0	< 30	3	1	0	0	0
35	M	larynx	57.2	IVA	4a	2c	0	ICCh+Op+RT	multi	80	≥ 60	3	0	0	1	0
36	M	oroph	70.3	IVA	4a	2c	0	Op+PORT	multi	50	0	2	0	0	1	0
37	M	oroph	51.1	IVA	3	2b	0	ICCh+Op+RT	multi	30	< 30	3	0	0	0	0
38	M	oroph	41.7	IVA	4a	2c	0	Op	mono	20	< 60	3	0	1	1	0
39	M	oral	55.9	II	2	0	0	Op+PORT	multi	30	≥ 60	3	1	0	1	0
40	M	hypo	71.7	II	2	0	0	Op	mono	45	< 60	2	0	0	0	0
41	M	oroph	83.1	III	3	0	0	Op	mono	60	0	--	--	--	--	1
42	M	oroph	65.5	IVA	4a	0	0	Op	mono	125	< 60	2	1	0	1	0
43	M	oroph	48.2	IVA	3	2b	0	ICCh+Op+RT	multi	35	≥ 60	2	0	1	1	0

F, Female; M, male; oroph, oropharynx; hypo, hypopharynx; oral, oral cavity; nasoph, nasopharynx; Op, surgery; RT, radiotherapy; ICCh, induction-chemotherapy; PORT, post-operative radiotherapy; PORChT, post-operative radiochemotherapy; pRChT, primary concomitant radiochemotherapy.

E led to considerable suppression of IL-6 ($p=0.00024$). All treatments altered MCP-1 production significantly. Cil showed the strongest impact on MCP-1 production ($p=0.00008$) followed by Cil+E ($p=0.00038$) and E alone ($p=0.01$). While E alone was not able to achieve significant effects on VEGFA ($p=0.14$), Cil ($p=0.037$) or Cil+E ($p=0.03$) substantially suppressed VEGFA release.

As shown in Table II, this study proves that for chemoresponse assessment measuring cytokine production is

superior to colony formation. Readouts of CF_{ec} were more inconsistent than cytokine release in triplicate measurements although effects of Cil and E were considerable.

Discussion

By analyzing 39 HNSCC *ex vivo* we were able to demonstrate a significant impact of EGFR and integrin inhibition on CF_{ec} and production of IL-6, MCP-1 and

VEGFA in the majority of HNSCC samples (Figure 1, Table II). However, the inter-individual heterogeneity calls for a more detailed analysis. As far as we are aware, there are no other studies regarding this specific topic. Our findings showed that 10 μ M Cil in HNSCC tumor cells *ex vivo*, may suppress production of MCP-1 and VEGFA as well as CF_{ec} significantly. We demonstrated that activity of Cil increased in binary combination with E. Reynolds et al. demonstrated in 2009 that low dose integrin inhibition can lead to altered expression of α V β 3 and VEGFR-2 resulting in tumor angiogenesis and proliferation (22). In our study, some individual tumor samples reacted paradoxically with an increase in VEGFA production and CF_{ec} suggesting dose dependency of Cil effects.

In the ADVANTAGE study, Cil was given as fourth component in addition to cisplatin, 5-FU and E to patients with recurrent and/or metastatic HNSCC (13). In this setting no benefit in overall survival could be demonstrated for Cil. This may be related to limited response in this heavily pre-treated population that might be exploited already by the three other drugs to its maximum extend. This is also strengthened by the CeFCiD study that was unable to demonstrate superiority of the fourth component in HNSCC using the otherwise highly active taxol docetaxel (23, 24). Therefore, ADVANTAGE which is the only clinical trial of Cil in HNSCC (13) appears to be inadequate to elucidate the full potential of Cil in HNSCC and may not stand against potential efficacy of Cil or Cil+E.

To our knowledge, there are so far no clinical trials in HNSCC analyzing solely Cil or Cil in binary combination together with one additional drug, *e.g.* E. As most newly emerging drugs are tested, the sole investigation of Cil combined with cisplatin, 5-FU and E may have limited the chance to demonstrate any benefit of the fourth component Cil. No dose limiting toxicities and only mild side effects of Cil were observed (13) and potential benefit from Cil is highlighted by our data. This argues for new clinical trials as there might still be potential for including this pentapeptide in targeted therapy of eligible patients. These patients, as suggested by our data (Figure 1, Table II), could eventually be identified by *ex vivo* testing.

EGFR targeting by E improves overall survival in HNSCC patients (10, 11). E treatment decreased considerably the release of IL-6, MCP-1, VEGFA and CF_{ec} in our *ex vivo* experiments. As IL-6, MCP-1 and VEGFA could serve as prognostic biomarkers and increased levels of IL-6 (4), MCP-1 (3, 25) and VEGFA (6-8, 26, 27) are associated with inflammation and poor outcome, we interpret their reduction towards normal levels as indicators for response. The high degree of correlation of reduced colony formation and reduced cytokine release of our samples supports this interpretation. Pathways of EGFR and VEGFA are closely linked since they share common downstream pathways effecting tumor cells

Figure 1. Effect of cilengitide (Cil), cetuximab (E) and Cil+E on production of IL-6, MCP-1, VEGFA and colony formation of epithelial cells (CF_{ec}) normalized to individual control. (A) Mean and 95% confidence interval; (B) median and interquartile range of 39 HNSCC. Asterisks indicate significance (p-value ≤ 0.05).

directly and indirectly (28). Overexpression of EGFR leads to increased VEGFA expression (14, 29). This study, showing that VEGFA release was reduced after EGFR targeting, highlights VEGFA as a suitable biomarker for response to E (14, 29, 30). Our measurements highlight the suppressing effect of EGFR blockage on VEGFA and IL-6 as well as on MCP-1 (Figure 1, Table II) solidifying the value of E in anticancer therapy regimes.

In this study, actual tumor samples – primary HNSCC without prolonged propagation by cultivation under artificial conditions leading to loss of normal features and chemoresponse – were exposed to anticancer agents *ex vivo*. We did not use cell lines in order to provide most biologically appropriate conditions bypassing common problems with cell lines as they are highly selected and sometimes artificially immortalized hindering transfer of such data to treatment of individual tumors. We saw real-

Table II. Correlation obtained in 39 evaluable patients by analyzing formation of epithelial colonies (CF_{ec}; A), IL-6 (B), MCP-1 (C), and VEGFA (D) with their response to cilengitide (Cil), cetuximab (E), and Cil+E. The probability R², Pearson's correlation coefficient r and p-values for the number of pair-wise comparisons are shown accompanied by scatter plots showing the distribution of source data. Correlations with p-value ≤0.05 were considered significant and highlighted in bold.

A				B			
CF _{ec} ctrl (n)	CF _{ec} Cil+E (n)	CF _{ec} E (n)	CF _{ec} Cil (n)	CF _{ec} ctrl (n)	IL-6 ctrl (pg/ml)	IL-6 Cil (pg/ml)	IL-6 E (pg/ml)
R ²	0.224	0.599	0.300	IL-6 ctrl (pg/ml)	R ²	0.531	0.675
r	0.473	0.774	0.548		r	0.729	0.822
p	0.0003	0.0042	0.0003		p	0.0002	0.0301
CF _{ec} Cil (n)	0.300	0.260		IL-6 Cil (pg/ml)	R ²	0.609	0.593
r	0.548	0.510			r	0.780	0.770
p	0.209	0.0013			p	0.0148	0.8623
CF _{ec} E (n)	0.298			IL-6 E (pg/ml)	R ²	0.780	
r	0.546				r	0.883	
p	0.0018				p	0.0034	
CF _{ec} Cil+E (n)				IL-6 Cil+E (pg/ml)	R ²		
R ²					r		
p					p		
C				D			
MCP-1 ctrl (pg/ml)	MCP-1 Cil+E (pg/ml)	MCP-1 E (pg/ml)	MCP-1 Cil (pg/ml)	VEGFA ctrl (pg/ml)	VEGFA Cil+E (pg/ml)	VEGFA E (pg/ml)	VEGFA Cil (pg/ml)
R ²	0.611	0.811	0.575	VEGFA ctrl (pg/ml)	R ²	0.646	0.802
r	0.782	0.901	0.758		r	0.804	0.896
p	0.0004	0.0112	0.0001		p	0.0329	0.1416
MCP-1 Cil (pg/ml)	0.410	0.572		VEGFA Cil (pg/ml)	R ²	0.605	0.718
r	0.640	0.756			r	0.778	0.847
p	0.6194	0.0023			p	0.5492	0.1017
MCP-1 E (pg/ml)	0.652			VEGFA E (pg/ml)	R ²	0.723	
r	0.807				r	0.850	
p	0.0479				p	0.0783	
MCP-1 Cil+E (pg/ml)				VEGFA Cil+E (pg/ml)	R ²		
R ²					r		
p					p		

world heterogeneity in response to treatment as individual tumors responded differently to the same treatment. The impact of Cil and E varied enormously depending on distinct tumor features. This aspect suggests the presence of subgroups within HNSCC patients of which some react with suppressed cytokine production to treatment and some which react with a boost of cytokine release.

Targeting $\alpha V\beta 3$ and $\alpha V\beta 5$ integrins by Cil and EGFR by E leads to reduced CF_{ec} and cytokine release proving that Cil does have a potential in cancer treatment. Analyzing cytokine release is superior to counting epithelial cell colonies concerning chemoresponse testing in order to select suitable patients which could benefit from targeted therapy.

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