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SDF-1/CXCR4 expression in head and neck cancer and outcome after postoperative radiochemotherapy

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ABSTRACT

Introduction: Outcome after postoperative radiochemotherapy (RT-CT) for patients with advanced head and neck squamous cell carcinomas (HNSCC) remains unsatisfactory, especially among those with HPV negative tumours. Therefore, new biomarkers are needed to further define subgroups for individualised therapeutic approaches. Preclinical and first clinical observations showed that the chemokine receptor CXCR4 and its ligand SDF-1 (CXCL12) play an important role in tumour cell proliferation, survival, cancer progression, metastasis and treatment resistance. However, the data on the prognostic value of SDF-1/CXCR4 expression for HNSCC are conflicting. The aim of our hypothesis-generating study was to retrospectively explore the prognostic potential of SDF-1/CXCR4 in a well-defined cohort of HNSCC patients collected within the multicenter biomarker study of the German Cancer Consortium Radiation Oncology Group (DKTK-ROG).

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Prognostic
Biomarker
Postoperative radiochemotherapy

Material and methods: Patients with stage III and IVA HNSCC of the oral cavity, oropharynx and hypopharynx were treated with resection and adjuvant radiotherapy (RT) with ≥60 Gy and concurrent cisplatin-based chemotherapy (CT). Tissue micro-arrays (TMAs) from a total of 221 patients were generated from surgical specimens, 201 evaluated for the SDF-1 and CXCR4 expression by immunofluorescence and correlated with clinico-pathological and outcome data.

Results: In univariate and multivariate analyses intracellular SDF-1 expression was associated with lower loco-regional control (LRC) in the entire patient group as well as in the HPV16 DNA negative subgroup. CXCR4 expression showed a trend for lower LRC in the univariate analysis which was not confirmed in the multivariate analysis. Neither for SDF-1 nor CXCR4 expression associations with distant metastasis free or overall survival were found.

Conclusions: Our exploratory data support the hypothesis that overexpression of intracellular SDF-1 is an independent negative prognostic biomarker for LRC after postoperative RT-CT in high-risk HNSCC. Prospective validation is warranted and further exploration of SDF-1/CXCR4 as a potential therapeutic target to overcome treatment resistance in HNSCC appears promising.

Introduction

Head and neck squamous cell carcinomas (HNSCC) represent about 5% of the newly world-wide diagnosed tumours [1,2]. Radiotherapy (RT) is a corner stone of the HNSCC treatment and often performed in combination with chemotherapy (CT) either in a primary setting or as adjuvant therapy following surgery. Based on randomized studies that showed a benefit by adding CT to RT, high risk resected locally advanced HNSCC are currently commonly treated with adjuvant RT-CT, with a 5-years overall survival (OS) of about 50% [3–5]. In a previous publication [6] the Radiation Oncology Group of the German Cancer Consortium (DKTK-ROG) demonstrated that HPV16 DNA status is a strong prognostic factor for loco-regional control (LRC) for locally advanced HNSCC treated with surgery and adjuvant RT-CT. The LRC in patients with HPV16 DNA positive tumours was close to 100%, while the rate of loco-regional relapse among those with HPV negative tumours reached 20%. Therefore, biomarkers are needed, which help to identify patients with HPV negative tumours who are on high risk of relapse in order to subject them for treatment intensification, e.g., by molecular targeting, radiation dose escalation or hypoxia modification. Chemokines are emerging as potential biomarkers and molecular targets to overcome treatment resistance in several types of cancer including HNSCC [7,8].

Chemokines are small cytokines that bind to trans-membrane domain of G-protein-coupled receptors. They are responsible for leucocyte trafficking and homing and were shown to be involved in tumour development and progress. Particularly, CXCR4 together with its ligand SDF-1 (stromal derived factor 1, also called CXCL12) were reported to influence cell survival, proliferation and migration. They can interfere directly with intracellular signalling pathways such as MAPK and PI3K/AKT or promote angiogenesis, stimulate interleukins or recruit myeloid bone marrow-derived tumour-promoting cells to the tumour site [8–11]. Moreover, a higher CXCR4 expression was observed in the CD44 positive tumours cell fraction from tumour cell lines and patient-derived tumour tissue, indicating a key role of SDF-1/CXCR4 pathway in tumour aggressiveness and resistance to treatment strategies [12–17]. Studies conducted on HNSCC cell lines or in HNSCC nude mice models showed that SDF-1/CXCR4 expression enhances cell motility, proliferation and metastases via up-regulation of ERK1/2, AKT/PKB, PI3K-AKT, NF-kB and matrix metalloproteinases (MMP) [18–29]. Some clinical data suggest higher metastatic potential and worse outcomes in patients with SDF-1/CXCR4 positive HNSCC tumours [18–21,30–34]. However, the existing clinical evidence is partly conflicting, limited due to the small number of patients and the large heterogeneity in SDF-1/CXCR4 detection methods, patient and tumour characteristics, applied treatments and reported outcome parameters. In the present retrospective study, we aimed to explore the prognostic potential of SDF-1/CXCR4 expression in a well-defined large cohort of patients with locally advanced high-risk HNSCC treated with surgery and adjuvant CT-RT as part of a multicentre biomarker trial conducted by the DKTK-ROG.

Material and methods

Patients and treatment

The trial was approved by the ethical committees of all the DKTK-ROG centres. The eligibility criteria, along with the clinical characteristics and treatments details were previously described [6]. Briefly, 221 patients with locally advanced squamous cell carcinoma of the oral cavity, oropharynx or hypopharynx treated between 2004 and 2012 with surgery and adjuvant RT-CT were included in the study. All patients had received platinum-based CT (median dose 200 mg/m^2) and RT consisting of a median dose of 50.4 Gy elective nodal irradiation with a boost up to a median dose of 64 Gy to the former tumour region. All patients had one or more of the following high-risk factors: stage pT4, >3 positive lymph nodes, positive microscopic resection margins, extracapsular extension. Clinical information, RT treatment plans and follow up data were collected and evaluated centrally at the DKTK partner site Dresden. In addition, a central radiological review of the imaging of relapses was performed.

Tissue samples

Formalin-fixed paraffin-embedded (FFPE) materials of primary tumour specimens retrieved after surgery were collected and centrally processed at the DKTK partner site Dresden. All the samples were stained with haematoxylin and eosin for histology verification. Tissue microarrays (TMAs, 1-mm diameter each core) were generated, and the tumour content in each TMA core was reviewed by expert pathologists.

HPV16 DNA and p16 evaluation

Methods used for the determination of HPV16 DNA and p16 were previously described [6]. Briefly, HPV DNA was extracted from FFPE-sections, amplified by PCR and detected by hybridisation using QIAamp DNA FFPE tissue kit (Qiagen GmbH, Hilden, Germany), HotStarTaq Plus Master Mix (Qiagen GmbH) and LCD-Array

HPV 3.5 kit (CHIPRON GmbH, Berlin, Germany), respectively, according to the manufacturer's instruction. Samples from 6 patients were excluded because of insufficient DNA. Immunohistochemical staining of p16 was performed using the CINtec Histology Kit (Roche mtm laboratories AG, Basel, CH), according to the manufacturer's instruction. Tumour samples expressing p16 in ≥70% were considered positive (overexpression). 7 patients had to be excluded because of insufficient tumour material (<10%) in the FFPE samples.

Staining, imaging and scoring system

TMAs were stained for SDF-1 and CXCR4 using immunofluorescence. Under supervision of an experienced pathologist (BS), SDF-1 and CXCR4 staining was established using positive (tonsil tissue) and negative controls (no primary antibody and anti-IgG from the same specie). After deparaffinization, rehydration and epitope-retrieval technique, sections were stained with TSATM Kit T20912 (containing goat anti-mouse IgG and tyramide labelled with Alexa 488, Life Technologies GmbH, Molecular probes, Invitrogen, Darmstadt, Germany) for SDF-1 detection and with TSATM Kit T20922 (containing goat anti-rabbit IgG and tyramide labelled with Alexa 488, Life Technologies GmbH, Molecular probes, Invitrogen, Darmstadt, Germany) for CXCR4 detection, according to the manufactures instructions. An antibody dilution of 1:100 was used for SDF-1 (mouse monoclonal, Clone 79018, R&D Systems, Minneapolis, USA; dilution 1:100) and of 1:200 for CXCR4 (rabbit monoclonal [UMB2], Clone ab124824, Abcam, Cambridge Science Park Milton Rd, Milton, Cambridge, United Kingdom; dilution 1:200). Imaging of the TMAs was performed using a Zeiss Axio Imager MI fluorescence microscope controlled by AxioVision 4.8 software (Carl Zeiss, Jena, Germany). Whole TMA scans were performed using a motorised scanning stage and a monochrome digital camera (AxioCam MRm, Carl Zeiss, Jena, Germany; Maerzhaeuser, Wetzlar, Germany, 400× (EC Plan Neofluar)). Different specific staining patterns, i.e., membrane and intracellular (including cytoplasmic and nuclear), could be observed and were confirmed by an expert pathologist (BS). The staining extent and intensity were evaluated only in the tumour areas. The staining intensity was scored semi-quantitatively with arbitrary thresholds per each core from 0 to 3 as follow: negative (0), low (1), intermediate (2) and high (3). The score per each pattern type was calculated as the mean score of all the cores of the tumour specimen derived from each patient. At least one core having a score ≥2 (meaning a mean patient score >1) was considered as positive. This semi-quantitative analysis of the tissue staining was performed blinded to the clinical characteristics and oncological outcome of the patients.

Statistics

Loco-regional tumour control (LRC), distant metastasis free survival (DMFS) and overall survival (OS) defined as event from the date of RT-CT start were calculated and Kaplan–Meier curves generated. Comparisons between staining results and clinicopathological data were performed using the Fisher's exact test. Prognostic parameters were evaluated using univariate and multivariate analysis (Cox proportional hazard model). Hazard ratios and 95% confidence intervals were calculated. p-values < 0.05 were considered statistically significant and p-values between 0.05 and 0.1 were considered as a trend. No correction for multiple testing was performed. Analyses were performed using the open-source software R (version 3.2.3 www.r-project.org). Graphical representation was performed using GraphPad Prism version 7 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

Results

A total of 221 patients from 8 different DKTK partner sites with a median follow-up of 47.3 months (range, 2.5–100 months) were included in this retrospective biomarker study. The details of the entire study population and treatment have been previously published [6]. For the present study, TMA including one up to five cores from tumour specimens of 201 of these patients were available for SDF-1 and 190 for CXCR4 staining. The characteristics of this patient subgroup are summarised in Table 1. SDF-1 and CXCR4 showed a heterogeneous staining pattern between the tumours of different patients and within the cancer cells, i.e., a membranous and intracellular staining (Fig. 1). Based on the semi-quantitative criteria membranous expression of SDF-1 (mSDF-1) and CXCR4 (mCXCR4) was detected in tumours of 24 (11.9%) and 14 (7%) patients, respectively. The intracellular expression of SDF-1 (icSDF-1) and CXCR4 (icCXCR4) was found to be positive in tumours of 53 (26.4%) and 55 patients (27.4%), respectively (Table 1). The distribution of icSDF-1 and icCXCR4 expression in relation to clinical and pathological characteristics are summarised in Table 2, indicating significantly higher proportion of icSDF-1 expression among histological grade 2 (G2), HPV16 DNA and p16 positive tumours. icCXCR4 staining was associated with histological G2 and lower pT-stages.

Neither mSDF-1 nor mCXCR4 expression was associated with LRC, DMFS or OS (Table 3). LRC rates were significantly lower in icSDF-1 positive tumours in the entire cohort and in the HPV16 DNA positive subgroup (Fig. 2). As a trend, higher icCXCR4 expression was associated with lower LRC (Fig. 2). In the univariate analysis of the entire cohort and the subgroup of patients with HPV16 DNA negative tumours icSDF-1 expression was associated with significantly lower LRC (HR 2.67, 95% CI 1.29–5.54 and HR 2.54, 1.19–5.4, respectively) but without association with DMFS or OS (Table 3). IcCXCR4 expression showed a trend towards lower LRC. The combined expression, i.e., tumours positive for icSDF-1 and icCXCR4 versus all other, was significantly associated with lower LRC. No significant heterogeneity of the effect was observed between the different centres (data not shown). The multivariate analysis revealed an independent negative prognostic value of icSDF-1 for LRC in the entire patient cohort and in the subgroup of patients with HPV16 DNA negative tumours (Table 4). CXCR4 was not significantly associated with LRC. The combined parameter of SDF-1/CXCR4 expression did not outperform SDF-1 expression alone.

Discussion

The present exploratory study suggests that SDF-1 expression in tumours is an independent negative prognostic marker for LRC after postoperative RT-CT that might stratify patients with high-risk HPV negative HNSCC for treatment modification. CXCR4 showed a trend in univariate analysis but was not prognostic in multivariate analysis. To our knowledge, this is the largest biomarker study in a patient population with locally advanced HNSCC who received postoperative RT-CT. Our data suggest a role of SDF-1 for resistance to postoperative RT-CT.

Signalling via the SDF-1/CXCR4 axis is well established as a chemokine-receptor pathway playing an important role in cancer progression and malignancy. Tumour infiltrating leukocytes but also fibroblasts, endothelial and especially tumour epithelial cells are able to produce chemokines and express chemokine receptors, determining a chemokine gradient and network that influence tumour cell growth, survival and migration [9]. The autocrine and paracrine actions of SDF-1 on CXCR4-expressing cells have been well described on ovarian cancer cells, where modulation of

Table 1
Patients characteristics, SDF-1/CXCR4 expression (A) and treatment details (B) for the 201 patients included in the present study.

Age (years)		57 (median)			24 (min)		76 (max)
(A)							
Gender		Male			161		80,1%
		Female			40		19,9%
Site		Oral cavity			56		27,9%
		Oropharynx			116		57,7%
		Hypopharynx			29		14,4%
pT stage		T1			34		16,9%
		T2			92		45,8%
		T3			45		22,4%
		T4			30		14,9%
R status		Negative			113		56,2%
		Positive			87		43,3%
		Unknown			1		0,5%
ECE		Negative			95		47,3%
		Positive			106		52,7%
p16		Negative			121		60,2%
		Positive			75		37,3%
		Unknown			5		2,5%
HPV16 DNA		Negative			134		66,7%
		Positive			67		33,3%
icSDF1		Negative			148		73,6%
		Positive			53		26,4%
icCXCR4		Negative			135		67,2%
		Positive			55		27,4%
		Missing			11		5,5%
mSDF1		Negative			177		88,1%
		Positive			24		11,9%
mCXCR4		Negative			176		87,6%
		Positive			14		7,0%
		Missing			11		5,5%

		Median	Percentiles				Range	
			10%	25%	75%	90%	Min	Max
(B)								
Cisplatin dose (mg/m^2)		200	100	200	200	240	100	300
RT dose (Gy)	Boost volume	64,0	60,0	63,8	66,0	66,0	56,0	68,4
	Per fraction	2,0	1,8	2,0	2,0	2,1	1,8	2,2
	Adjuvant volume	50,4	50,0	50,0	55,8	60,0	46,8	66
	Per fraction	2,0	1,8	1,8	2,0	2,0	1,8	2,1
Time between surgery and RCT (weeks)		6,0	4,6	5,1	7,6	9,6	0,6	22,9
Overall treatment time of RCT (days)		44,0	41,0	43,0	47,0	51,0	31,0	57,0
Follow-up time (months)		46,2	9,7	30,1	60,5	70,4	2,5	100,1

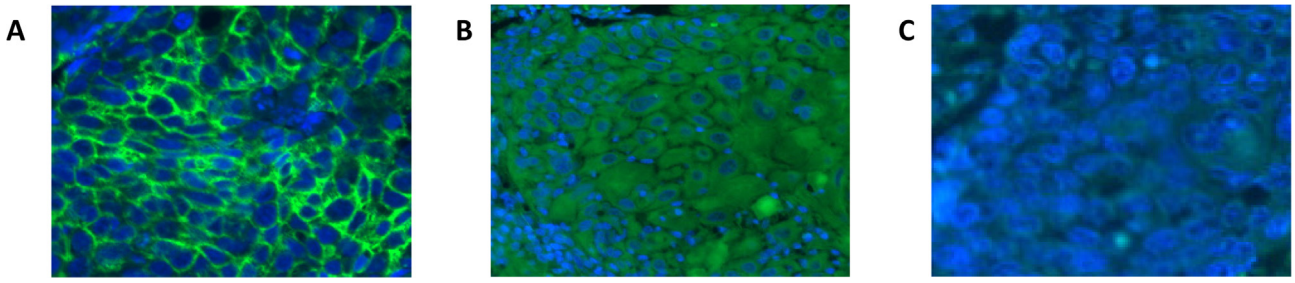

Fig. 1. Representative images of SDF-1 immunofluorescent stained tumour sections. (A) Membrane staining (score 3). (B) Intracellular staining (score 2). (C) Negative staining (score 0). SDF-1 is shown in green, DAPI in blue. Similar staining patterns and intensities were observed for CXCR4. Original image magnification: 400×.

dendritic cells, increasing integrin expression, enhanced matrix metalloproteinase activity, induced TNF-α production and angiogenesis have been observed [35]. SDF-1 and CXCR4 expression might be mechanistically linked to other biomarkers of treatment resistance in HNSCC such as p53 mutation and up-regulation of focal adhesion kinase (FAK) as well as immune markers like PD-L1. Importantly in the context of radiation, SDF-1/CXCR4 signalling is involved in radiation-induced infiltration of the tumour by bone-marrow derived immune cells such CD11b positive myelomonocytes which in turn contribute to radiation resistance [36,37]. $p53^{null}$ mice showed a faster tumour growth associated with increased presence of CD11b positive myeloid-derived suppressor cells and production of SDF-1 [38]. In addition, reduced p53 activity was shown to enhance the migration of multipotent fibroblast-like cells from the bone marrow to the tumour through up-regulation of SDF-1 [39]. In squamous cell carcinomas up-regulation of FAK inhibits the anti-tumoral CD8 positive T cell activity through the regulation of the chemokine network [40]. An increased SDF-1 expression in breast cancer cells was shown to stimulate cell adhesion and migration through FAK [41]. In three-dimensional grown human HNSCC FAK overexpression was responsible for an increased radiation resistance through

Table 2
Clinico-pathological characteristics and icSDF-1/icCXCR4 expression. *P*-values for comparisons using the Fischer's exact test, significant *p*-values in bold.

	icSDF1 pos.		icSDF1 neg.		*p*	icCXCR4 pos.		icCXCR4 neg.		*p*
Age										
<Median (57 y)	23	43%	75	51%	0,42	30	55%	65	48%	0,52
≥Median	30	57%	73	49%		25	45%	70	52%	
Gender										
M	43	81%	118	80%	1,00	42	76%	110	81%	0,43
F	10	19%	30	20%		13	24%	25	19%	
Site										
Oral cavity	18	34%	38	26%	0,31	19	35%	34	25%	0,41
Oropharynx	26	49%	90	61%		29	53%	82	61%	
Hypopharynx	9	17%	20	14%		7	13%	19	14%	
pT stage										
pT1-2	33	62%	93	63%	1,00	27	49%	89	66%	**0,03**
pT3-4	20	38%	55	37%		28	51%	46	34%	
pN stage										
pN0-1	12	23%	38	26%	0,71	12	22%	36	27%	0,58
pN2-3	41	77%	110	74%		43	78%	99	73%	
Grading (3 missing)										
G1	0	0%	5	3%	**0,01**	2	4%	3	2%	0,08
G2	38	73%	75	51%		37	69%	71	53%	
G3	14	27%	66	45%		15	28%	59	44%	
Resection margin (1 missing)										
R0	32	62%	81	55%	0,42	32	59%	73	54%	0,63
R1	20	38%	67	45%		22	41%	62	46%	
ECE										
No	25	47%	70	47%	1,00	22	40%	66	49%	0,34
Yes	28	53%	78	53%		33	60%	69	51%	
HPV16 DNA										
Negative	41	77%	93	63%	0,06	40	73%	88	65%	0,39
Positive	12	23%	55	37%		15	27%	47	35%	
p16 (5/4 missing)										
Negative	39	76%	82	57%	**0,01**	37	69%	78	59%	0,25
Positive	12	24%	63	43%		17	31%	54	41%	
Smoking (69/67 missing)										
Yes	27	84%	90	90%	0,36	29	85%	80	90%	0,53
No (never)	5	16%	10	10%		5	15%	9	10%	

phosphorylation of AKT and ERK [42]. AKT, together with other intracellular proteins like IP3, mitogen activated protein kinases (MAPKs) and factors as VEGF mediate the SDF-1 activity [8]. Synergistic effects of anti-PD-L1 immunotherapy and SDF-1 targeting support the functional cross-talk between chemokines and CD8 lymphocytes with tumour hypoxia as an important microenvironmental modulator [43,44]. SDF-1 is the only known ligand for CXCR4. CXCR4 expression has been demonstrated in many tumour entities, such as breast, ovarian, prostate, melanoma, oesophageal, non-small cell lung cancer, head and neck, bladder, colo-rectal, pancreatic, stomach, sarcoma, leukaemia and glioma [10]. In a meta-analysis of more than five thousand patients affected by different tumour types, Zhao and colleagues demonstrated a shorter progression free survival and overall survival in patients with CXCR4-overexpressing tumours [45]. Data suggest, that CXCR4 expression could be indicative of cancer stem cell (CSC) characteristics [16]. In gliomas, SDF-1/CXCR4 have been found to be overexpressed in perihypoxic tumour areas and, particularly CXCR4, in glioma stem cells, conferring characteristics of aggressiveness and resistance to RT-CT induced apoptosis [46]. Analysing HNSCC cell lines, Faber and colleagues observed an increased formation of podia (key structures for cell adhesion) in CD44, a putative CSC marker, and CXCR4 positive cells under the influence of SDF-1, suggesting that the SDF-1-CXCR4 pathway may be important for the interaction between CSCs and their supportive cells in the CSC niche [14]. In tumour specimen of patients with HNSCC, the same authors showed that CD44 is mainly located on the membrane of cells at the invasive tumour front and that CXCR4 and SDF-1 are located in the membrane and in the cytoplasm of the tumour cells [13]. Similarly, we observed a membrane and an intracellular (mainly cytoplasmic but also nuclear) type of staining for SDF-1 and CXCR4. Interestingly, we did not find any correlation between the membrane expression and the outcome, that might suggest a possible role of SDF-1/CXCR4 after its internalization in the cell cytoplasm and translocation to the nucleus. However, the significance of the SDF-1/CXCR4 subcellular location remains a matter of debate. Some studies conducted on patients with colorectal cancer indicated a negative correlation between the nuclear expression and the clinical outcome [47–49], whereas others studies showed a positive correlation for the nuclear staining but a negative correlation for the cytomembrane staining and the clinical outcome in patients with lung cancer [50,51]. The importance of SDF-1/CXCR4 signalling is supported by in vitro and in vivo experiments including HNSCC models using CXCR4 targeting approaches. These experiments showed a decreased cell proliferation, motility, invasion and a reduced metastatic potential under treatment with AMD3100, a pharmacological antagonist of CXCR4 listed under pubchem CID 65015 [25,52,53]. Importantly, in preclinical experiments CXCR4 blockade also enhanced radiation response in xenografted breast, lung and glioblastoma tumours indicating that SDF-1/CXCR4 influences tumour radiation sensitivity [36,54,55]. In addition, in glioblastoma SDF-1 is involved in the radiation-induced migratory phenotype [56]. Collectively, the preclinical and clinical data support the concept that the SDF-1/CXCR4

Table 3
Univariate analysis in A: all patients and B: patients with HPV16 DNA negative tumours only. Significant results in bold.

	LRC				DM				OS			
	HR	CI lower	CI upper	*p*	HR	CI lower	CI upper	*p*	HR	CI lower	CI upper	*p*
(A) N = 201 (all patients)												
Age (<Median 57 y vs. ≥Median)	2,68	1,21	5,91	**0,015**	1,39	0,73	2,64	0,310	1,43	0,87	2,35	0,158
Gender (M vs. F)	0,62	0,27	1,39	0,242	1,66	0,65	4,26	0,289	0,80	0,45	1,43	0,446
Tumour Site (OP vs. OC+HP)	0,37	0,17	0,77	**0,009**	0,34	0,17	0,66	**0,001**	0,55	0,34	0,90	**0,017**
pT stage (pT3-4 vs. pT1-2)	2,75	1,32	5,75	**0,007**	2,24	1,18	4,24	**0,013**	2,48	1,51	4,05	**0,000**
pN stage (pN 2-3 vs. pN 0-1)	1,33	0,54	3,28	0,532	3,02	1,07	8,52	**0,036**	1,10	0,62	1,93	0,752
Grading (G1 vs. G2 vs. G3)	0,57	0,28	1,16	0,121	0,92	0,51	1,69	0,793	0,75	0,47	1,21	0,240
Resection margin (R1 vs. R0)	1,07	0,51	2,22	0,867	1,09	0,57	2,06	0,797	1,12	0,68	1,83	0,655
ECE (yes vs. no)	1,63	0,77	3,46	0,203	2,49	1,19	4,86	**0,014**	1,70	1,03	2,83	**0,040**
p16 (pos. vs. neg.)	0,22	0,08	0,62	**0,005**	0,21	0,08	0,53	**0,001**	0,34	0,19	0,63	**0,001**
HPV16 DNA (pos. vs. neg.)	0,13	0,03	0,54	**0,005**	0,38	0,17	0,87	**0,024**	0,31	0,16	0,61	**0,001**
Smoking (yes vs. never)	1,53	0,36	6,62	0,566	4,13	0,56	30,47	0,164	2,57	0,79	8,34	0,115
mSDF1 (pos. vs. neg.)	0,81	0,25	2,68	0,729	1,28	0,54	3,07	0,578	0,82	0,37	1,80	0,623
mCXCR4 (pos. vs. neg.)	2,13	0,74	6,14	0,162	1,10	0,34	3,59	0,871	1,72	0,78	3,78	0,180
icSDF1 (pos. vs. neg.)	2,67	1,29	5,54	**0,008**	1,02	0,50	2,10	0,955	1,17	0,69	2,01	0,561
icCXCR4 (pos. vs. neg.)	2,02	0,97	4,21	0,059	1,07	0,53	2,16	0,856	1,26	0,75	2,13	0,384
icSDF1+icCXCR4 (pos. vs. neg.)	2,87	1,30	6,29	**0,009**	0,77	0,27	2,15	0,606	1,15	0,59	2,27	0,681
(B) N = 134 (HPV16 neg. patients)												
Age (<Median 57 y vs. ≥Median)	2,38	1,06	5,34	**0,035**	1,31	0,65	2,66	0,453	1,44	0,84	2,49	0,187
Gender (M vs. F)	0,63	0,28	1,45	0,277	1,98	0,69	5,65	0,204	0,90	0,48	1,69	0,754
Tumour Site (OP vs. OC+HP)	0,68	0,31	1,48	0,327	0,53	0,25	1,13	0,102	0,86	0,50	1,47	0,572
pT stage (pT3-4 vs. pT1-2)	2,69	1,24	5,81	**0,012**	1,95	0,96	3,97	0,063	2,05	1,20	3,51	**0,009**
pN stage (pN 2-3 vs. pN 0-1)	1,46	0,59	3,62	0,417	3,94	1,20	12,96	**0,024**	1,38	0,74	2,58	0,313
Grading (G1 vs. G2 vs. G3)	0,68	0,32	1,45	0,321	0,93	0,47	1,86	0,837	0,62	0,36	1,06	0,081
Resection margin (R1 vs. R0)	1,16	0,54	2,48	0,702	1,07	0,53	2,17	0,858	1,03	0,60	1,75	0,925
ECE (yes vs. no)	1,62	0,75	3,50	0,223	2,63	1,21	5,71	**0,015**	1,94	1,11	3,37	**0,019**
Smoking (yes vs. never)	0,89	0,20	3,93	0,874	Cox PH model did not converge				2,15	0,50	9,29	0,303
mSDF1 (pos. vs. neg.)	0,96	0,23	4,04	0,950	1,69	0,59	4,83	0,329	0,93	0,34	2,59	0,897
mCXCR4 (pos. vs. neg.)	1,24	0,37	4,14	0,722	1,03	0,31	3,39	0,963	1,24	0,53	2,90	0,622
icSDF1 (pos. vs. neg.)	2,54	1,19	5,40	**0,016**	0,94	0,43	2,05	0,883	1,11	0,63	1,95	0,729
icCXCR4 (pos. vs. neg.)	2,04	0,96	4,33	0,065	1,13	0,53	2,41	0,757	1,27	0,73	2,20	0,405
icSDF1+icCXCR4 (pos. vs. neg.)	2,52	1,13	5,60	**0,024**	0,76	0,26	2,17	0,605	1,04	0,52	2,07	0,910

axis contributes to radiation resistance through the mechanistic links to stemness, hypoxia, infiltration by immune cells, intracellular signalling and migration.

The prognostic value of SDF-1 and CRCXR4 in HNSCC patients has been reported before from different studies. High SDF-1/CXCR4 expression in lymphnode metastases from 30 patients is associated with poor OS [21]. In 47 tumour samples from patients who underwent surgery for HNSCC of the mobile tongue, high CXCR4 expression correlated with T and N stage and is associated with poor OS in the univariate but not in multivariate analysis [32]. In contrast, SDF-1 does not correlate with OS. Regarding the latter, the apparent difference to the study reported here is that in the study of Albert et al. [32] also earlier tumour stages were included which are likely managed with surgery alone. In a retrospective analysis of 56 patients with tumours of the oral cavity stage I to IV treated with either neoadjuvant RT-CT, surgery or RT alone, CXCR4 expression has been found to be associated with poor OS and disease specific survival in univariate and multivariate analyses [20]. The discrepancy with our results may be explained by differences in stage distribution and therapeutic management. It may be speculated that CXCR4 has a specific role in oral cavity tumours, which represent only 27.9% in our study. In a large study on 233 HNSCC patients with inoperable tumours undergoing primary RT/RT-CT CXCR4 expression was associated with increased risk of distant metastasis but not with LRC or OS [57]. High SDF-1 expression was associated with better OS. However, in multivariate analysis SDF-1 expression was not significantly correlated with LRC, DMFS or OS. Interestingly, discrepant from our findings p16 positive tumours showed on average a higher SDF-1/CXCR4 expression than p16 negative tumours. In our study SDF-1 was on average lower in p16 positive tumours and no association between CXCR4 expression and p16 could be found. A more complex prognostic pattern for SDF-1 and CXCR4 is suggested from a study on 111 HNSCC patients who were treated with surgery, surgery plus postoperative RT/RT-CT or with primary RT/RT-CT [58]. In addition, Clatot and colleagues [59] found that lower SDF-1 expression determined by PCR in tumour specimen of 71 HNSCC patients was correlating with worse disease-free survival and cancer specific survival. No associations were found for CXCR4. Taken together, the published data regarding the prognostic role of SDF-1 and CXCR4 in HNSCC is conflicting and may be explained by heterogeneity in patient and treatment characteristics as well as in methodological differences for determination of SDF-1/CXCR4 expression in the different studies. Our study contains a large, more homogenous and well-defined patient group. However, also taken into account the semi-quantitative scoring with arbitrary thresholds, our results are exploratory and need validation, which is planned in the currently recruiting prospective HNprädBio study of the DKTK-ROG as well as in a cohort of patients affected by locally advanced HNSCC treated with primary RT-CT (De-Colle et al., submitted). In addition, an overall biometry is going to be performed including all promising biomarkers that have been exploratively analysed in the retrospective DKTK-ROG cohorts of patients with locally advanced HNSCC such as hypoxia-associated gene expression, CSC marker expression [60,61], CD8-positive tumour-infiltrating lymphocytes [62], distinct mutation profiles [63] as well as SDF-1/CXCR4 in order to develop robust prognostic profiles for patient stratification for individualised therapy.

In summary, our exploratory data support further investigations of the SDF-1/CXCR4 axis to stratify HNSCC patients for

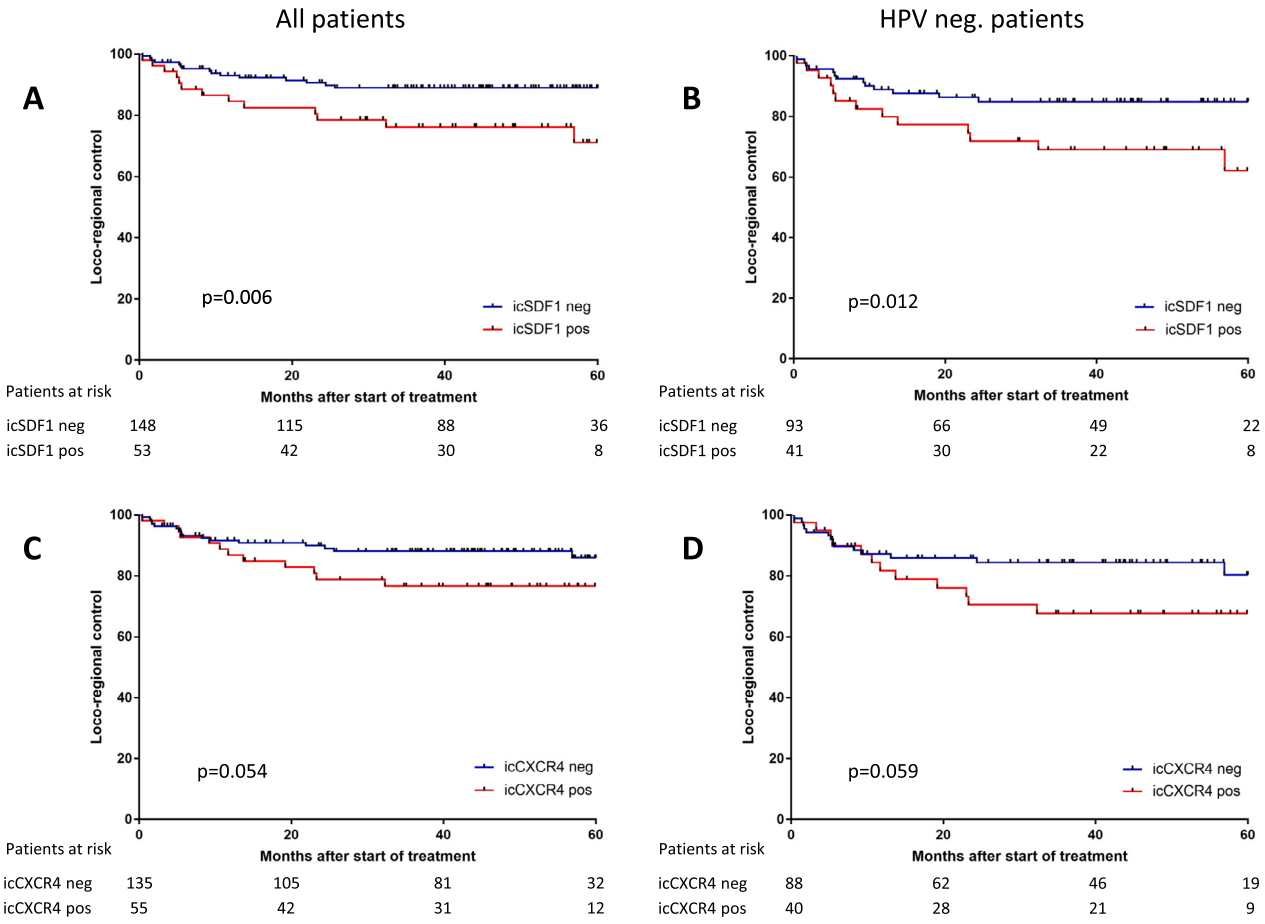

Fig. 2. A–D: Kaplan-Meier curves for locoregional tumour control in all patients (A and C) and patients with HPV16 DNA negative tumours only (B and D) according to the SDF-1 and CXCR4 intracellular expression.

Table 4
Multivariate analysis in A: all the patients and B: patients with HPV16 DNA negative tumours only. Significant results in bold.

	LRC			
	HR	CI lower	CI upper	*p*
(A) N = 201 (all patients)				
Age (<Median 57 y vs. ≥Median)	3,33	1,48	7,47	**0,004**
Tumour Site (OP vs. OC+HP)	0,63	0,29	1,38	0,246
pT stage (pT3-4 vs. pT1-2)	2,28	1,08	4,81	**0,031**
HPV16 DNA (pos. vs. neg.)	0,20	0,04	0,87	**0,032**
icSDF1 (pos. vs. neg.)	2,72	1,24	5,93	**0,012**
icCXCR4 (pos. vs. neg.)	1,13	0,52	2,46	0,764
Age (<Median 57 y vs. ≥Median)	3,33	1,48	7,50	**0,004**
Tumour Site (OP vs. OC+HP)	0,64	0,29	1,42	0,273
pT stage (pT3-4 vs. pT1-2)	2,31	1,09	4,88	**0,028**
HPV16 DNA (pos. vs. neg.)	0,19	0,04	0,86	**0,031**
icSDF1+icCXCR4 (pos. vs. neg.)	2,66	1,18	6,01	**0,018**
(B) N = 134 (HPV16 DNA neg. patients)				
Age (<Median 57 y vs. ≥Median)	3,12	1,37	7,13	**0,007**
Tumour Site (OP vs. OC+HP)	0,70	0,32	1,56	0,386
pT stage (pT3-4 vs. pT1-2)	2,45	1,12	5,35	**0,025**
icSDF1	2,99	1,33	6,75	**0,008**
icCXCR4	1,22	0,55	2,74	0,621
Age (<Median 57 y vs. ≥Median)	3,11	1,35	7,14	**0,007**
Tumour Site (OP vs. OC+HP)	0,72	0,32	1,59	0,410
pT stage (pT3-4 vs. pT1-2)	2,51	1,15	5,47	**0,021**
icSDF1+icCXCR4	2,87	1,25	6,59	**0,013**

biologically individualised RT-CT as well as a potential therapeutic target to overcome treatment resistance.

Conflict of interest disclosure

We certify that there is no actual or potential conflict of interest in relation to this article.

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