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Tumour-infiltrating lymphocytes predict response to definitive chemoradiotherapy in head and neck cancer

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Background: We aimed to investigate the prognostic value of tumour-infiltrating lymphocytes' (TILs) expression in pretreatment specimens from patients with head and neck squamous cell carcinoma (HNSCC) treated with definitive chemoradiotherapy (CRT).

Methods: The prevalence of CD3+, CD8+, CD4+ and FOXP3+ TILs was assessed using immunohistochemistry in tumour tissue obtained from 101 patients before CRT and was correlated with clinicopathological characteristics as well as local failure-free- (LFFS), distant metastases free- (DMFS), progression-free (PFS) and overall survival (OS). Survival curves were measured using the Kaplan–Meier method, and differences in survival between the groups were estimated using the log-rank test. Prognostic effects of TIL subset density were determined using the Cox regression analysis.

Results: With a mean follow-up of 25 months (range, 2.3–63 months), OS at 2 years was 57.4% for the entire cohort. Patients with high immunohistochemical CD3 and CD8 expression had significantly increased OS ($P=0.024$ and $P=0.028$), PFS ($P=0.044$ and $P=0.047$) and DMFS ($P=0.021$ and $P=0.026$) but not LFFS ($P=0.90$ and $P=0.104$) in multivariate analysis that included predictive clinicopathologic factors, such as age, sex, T-stage, N-stage, tumour grading and localisation. Neither CD4 nor FOXP3 expression showed significance for the clinical outcome. The lower N-stage was associated with improved OS in the multivariate analysis ($P=0.049$).

Conclusion: The positive correlation between a high number of infiltrating CD3+ and CD8+ cells and clinical outcome indicates that TILs may have a beneficial role in HNSCC patients and may serve as a biomarker to identify patients likely to benefit from definitive CRT.

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignancy in the world in developed countries (Argiris *et al*, 2008). Despite the improvement in local control and survival with the use of combined chemoradiotherapy (CRT), locoregional recurrence is encountered in ~30–40% of patients and distant metastases develop in 20–30% of HNSCC (Argiris *et al*, 2008). Locoregional and distal recurrence after definitive CRT constitute a major cause of morbidity and mortality in patients with HNSCC and has stimulated substantial efforts in identifying biological markers that predict patients at risk for disease recurrence (Begg, 2012).

The immune response to tumours is complex, involves the interaction of several cell types of the adaptive and the innate immune systems and has an important role in the progression of a variety of solid tumours (Bhardwaj, 2007; Lesterhuis *et al*, 2011). The immune system promotes the elimination of tumour cells and control of tumour growth; however, cancers are characterised by a highly suppressive tumour microenvironment that hinders T-cell

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function (Bhardwaj, 2007). The presence of tumour-infiltrating lymphocytes (TILs), such as CD3+ and CD8+ cells, has been associated with improved outcome in patients with cancer (Gooden *et al*, 2011). Indeed, studies have revealed that a pre-existing immunologic response might enhance the effects of chemotherapy and radiotherapy (Grabenbauer *et al*, 2006; Kawai *et al*, 2008; Denkert *et al*, 2010).

Human papillomaviruses (HPV), mainly type 16, leads to tumorigenesis and is encountered in ~20–25% of the patients with HNSCC (Chaturvedi *et al*, 2011). p16INK4 is a marker of HPV that expresses E7 mRNA. HPV-positive patients present distinct clinicopathological features and are more radio- and chemosensitive compared with HPV-negative patients, possibly because of the presence of wild-type TP53 (Fakhry *et al*, 2008; Lassen *et al*, 2009; Ang *et al*, 2010; Rischin *et al*, 2010). Recent studies have examined the role of the immune system in HPV-associated HNSCC. One study demonstrated an increased T-cell response against the E7 oncoprotein epitope in the blood of HPV-positive patients, whereas another work revealed an increased T-cell proliferation in response to HPV E6 and E7 oncoproteins (Bontkes *et al*, 2000). Some studies have shown that strong CD8 expression is associated with better prognosis in HPV-positive patients (Nasman *et al*, 2012; Nordfors *et al*, 2013) but others have failed to reproduce this finding (Kong *et al*, 2009; Wansom *et al*, 2012)

Although previous reports have examined the role of TILs in HNSCC, the majority of the studies were characterised by small sample size and/or use of heterogeneous treatment schedules (Ginos *et al*, 2004; Le *et al*, 2005; Badoual *et al*, 2006). In the present work, we aimed to investigate the potential biologic importance of TILs in patients with HNSCC treated with primary CRT. We examined pretherapeutic core biopsy samples from a total of 101 patients with HNSCC treated with definitive CRT and investigated the protein expression of CD3+, CD8+, CD4+ and FOXP3+ TILs in tumour specimens. The correlation with clinical parameters and patient outcome was calculated in order to determine the potential prognostic role of TIL. Finally, we investigated the prognostic significance of the HPV surrogate p16INK4 (further denoted as HPV16) and also of TILs according to the HPV16.

PATIENTS AND METHODS

Patients. Between 2007 and 2010, a total of 101 patients with histologically proven advanced HNSCC were treated with definitive CRT at the Department of Radiotherapy and Oncology, University Hospital Frankfurt am Main. Pretreatment evaluation consisted of physical examination, pretherapeutic biopsy, CT/MRI of head and neck and chest/abdomen, chest X-ray, serum chemistry and complete blood count in all patients. Patients were staged according to the system adopted by the Union International Contre le Cancer (UICC) and the American Joint Committee on Cancer. Patients were included in this study according to the following criteria: histopathological confirmation of HNSCC; age between 18 and 75 years; sufficient haematological, liver and renal functions; absence of distant metastases and second cancer; curative intent.

Treatment protocol and follow-up. All patients received a 3D-conformal RT planning and immobilisation using thermoplastic masks. In addition, CT and MRI scans were performed for target volume delineation. All patients prophylactically received a feeding tube before the initiation of CRT. Patients were treated with definitive CRT using ⩾6-MeV energy photon beams that were administered once daily with standard fractionation of 1.8–2 Gy to a total dose of 70–72 Gy. During the second half of the radiotherapy (after 30 Gy in 2 Gy fractions was reached), a hyperfractionated accelerated fractionation schedule (1.4–1.5 Gy twice daily) was applied. Four patients received a different RT fractionation schedule. Two cycles of chemotherapy were applied on days 1–5, and 29–33 consisting of 5-FU (600 $mg\,m^{-2}$ per day) as a continuous 120-h intravenous infusion and Cisplatin (20 $mg\,m^{-2}$ per day) as short intravenous infusion. In patients with poor kidney function ($<60\,ml\,h^{-1}$ creatinine clearance), carboplatin or mitomycin were administered instead of cisplatin. Chemotherapy dose was reduced during the second cycle in patients who developed leucopenia (leucocyte count $<3\,nl^{-1}$) and/or thrombocytopaenia (platelet count $<100\,000\,nl^{-1}$). All patient cases were presented at the Head and Neck Cancer interdisciplinary in-house tumour board. Definitive CRT was applied in the following cases: (1) patients with functionally inoperable tumours (⩾T3 stage); (2) patients considered inoperable because of poor general health status; and (3) patients who have electively opted for CRT instead of surgery. Follow-up examinations were first performed 6 weeks after the completion of CRT and thereafter at 3-month intervals using clinical examination, endoscopy and CT/MRI imaging. A panendoscopy with biopsy was performed in patients that presented suspicious and/or inconclusive radiologic and clinical findings in the follow-up examination. Patient characteristics are shown in Supplementary Table 1.

Immunohistochemical evaluation of CD3, CD8, CD4 and FOXP3. Formalin-fixed, paraffin-embedded (FFPE) blocks of all patients included in the study were obtained from the Pathology Department, University Hospital Frankfurt archives. Immunohistochemical staining of CD3, CD8, CD4 and FOXP3 was performed with a horseradish-peroxidase technique using a DAKO Autostainer Link 48 (DAKO, Hamburg, Germany) at the Pathology Department. Antigen retrieval was carried out by the pretreatment of microscope slides (Star Frost, Engelbrecht, Germany) with an Epitope Retrieval Solution (Trilog, Cell Marque, Rocklin, CA, USA) for 30 min. Following that, staining was conducted with standardised Dako EnVision FLEX Peroxidase Blocking reagent (K800, DAKO) and polyclonal antibodies for CD3, CD8, CD4 (dilution 1 : 50; Dako) and FOXP3 (dilution 1 : 100: Sigma, Munich, Germany) following incubation for 120 min at room temperature. Next, dextran polymer-conjugated horseradish-peroxidase and 3,3′-diaminobenzidine (DAB) chromogen were used for visualisation followed by counterstaining with hematoxylin solution (Gill 3, Sigma). Negative control slides in the absence of primary antibodies were included for each staining.

To determine HPV status, immunohistochemical staining of p16INK4a protein (HPV16) was performed on cancer paraffin-embedded tissue using a CINtec histology Kit (Roche, Heidelberg, Germany) according to the manufacturer's recommendations using a DAKO Autostainer Link 48 (DAKO).

Immunohistochemistry scoring. TIL scoring of tumours was performed semiquantitatively by measuring the densities of CD3+, CD8+, CD4+ and FOXP3+ cells as previously described (Dahlin *et al*, 2011): (1) no, or sporadic cells; (2) moderate numbers of cells; (3) abundant occurrence of cells; and (4) highly abundant occurrence of cells. TILs were evaluated in the following three different areas of the tumour: the intraepithelial compartment (cells within tumour cell nests); the stroma (cells within the intratumoural stroma) and the tumour periphery (cells localised in tumour periphery). Three random fields were examined, whereas necrotic areas were excluded from the measurements. Sections were examined by two observers (E Fokas, P Balermpas). In case of discrepancy, a final decision was made upon further examination of the slides in a microscope based on consensus by the investigators. Discrepancy occurred in seven cases. The total score for CD3, CD8, CD4 and FOXP3 was

measured as the sum of the individual scores from the three tumour areas (intra-epithelial compartment, stroma and tumour periphery), respectively. The total score ranged from 3 to 12, and the median value was used as a cutoff point to separate the patient cohort into two groups with either low or high CD3+, CD8+, CD4+ and FOXP3+ expressions. Furthermore, the ratios of CD3 and CD8 to both CD4 and FOXP3 (CD3:CD4; CD8:CD4; CD3:FOXP3; and CD8:FOXP3 ratio, respectively) were calculated for each individual tumour based on the cutoff value of each TIL marker, and their prognostic significance on clinical end points was examined. In addition, we assessed the prognostic role of the TIL score for each of the three different tumour areas (intra-epithelial compartment, stroma and tumour periphery). For that purpose, the median TIL score of each area was measured and the cutoff point was used to separate the cohort into two groups with either low or high TIL score. We also validated the semiquantitative scoring of TIL expression using a quantitative approach. For that purpose, the median number of cells, as assessed in three randomised ×10 magnification fields per sample, was used as a cutoff point to separate the patient cohort into two groups with either low or high TILs cell number.

Following that, we analysed the expression of HPV16 and assessed the clinical outcome according to HPV16 immunoreactivity (positive *vs* negative). We also analysed p16 immunoreactivity by considering both the percentage of positive cells and the intensity of staining. For that purpose, the staining intensity was scored as: 1+ (weak), 2+ (moderate) and 3+ (intense). The fraction of tumour cells with p16 positivity was assigned to the following: 1 (0–25%), 2 (26–50%), 3 (51–75%) and 4 (>75%). To minimise the interobserver variability, scoring was performed by two independent investigators without the knowledge of the clinicopathological data. The percentage of positive tumour cells and the staining intensity were then multiplied to produce an individual labelling score for each case, ranging from 0 (no positive tumour cells) to 12 (>75% of tumour cells with intense staining). Finally, we examined the prognostic value of the four TIL markers separately in HPV16-positive- and HPV16-negative patients. Images were acquired using ×10 and ×40 magnifications.

Statistical analysis. The differences between categorical variables were assessed by the Fisher's exact test. Overall survival (OS) and time to recurrence were calculated from the date of CRT-beginning to the day of death and recurrences, respectively. Patients without tumour recurrence were censored at the last follow-up contact. Progression free survival (PFS) was defined as the time between the start of therapy and tumour relapse (locoregional recurrence and/or distant metastases) or death because of non-tumour related causes. Local failure-free survival (LFFS) was defined as the time from the start of CRT to the first local tumour detection after CRT (that is, non-complete response), local tumour recurrence after complete response or death from any cause. Distant metastasis-free survival (DMFS) was assessed separately. A $P<0.05$ was considered statistically significant. Survival curves were plotted according to the Kaplan–Meier method using SPSS 19 for Windows (SPSS Inc., Chicago, IL, USA). Univariate and multivariate analyses were performed using the log-rank (Mantel–Cox) test and the Cox proportional hazard models, respectively.

RESULTS

TIL immunostaining on pretreatment biopsies. The immunohistochemistry characteristics and labelling scores are shown in Table 1. As a dichotomous variable, CD3 expression was defined as 'low' (weighted score ⩽6) in 60 patients (59.4%) and 'high' (weighted score >6) in 41 patients (40.6%) based on the median score. Similarly, CD8 expression was defined as 'low' (weighted score ⩽6) in 75 patients (74.2%) and 'high' (weighted score >6) in 26 patients (25.8%). The median score for both CD4 and FOXP3 was 4. Immunohistochemical analysis revealed 'low' CD4 score in 58 (56.9%) patients and 'high' in 43 (42.2%) patients, whereas FOXP3 expression was 'low' in 97 (95.1%) and 'high' in 4 (3.9%) patients. As shown in Table 2, patients with lower CD8 expression presented a significantly higher incidence of N2c-3 stage – that is, advanced lymphadenopathy (CD8 weighted score ⩽6 *vs* >6: 57 *vs* 31%; $P=0.035$), indicating a more aggressive disease. We did not find any other significant relationship between TILs' expression and clinicopathologic parameters (Table 2). Moreover, we validated the semiquantitative scoring of TILs expression in a representative patient subgroup ($n=30$) by using a quantitative method based on the median number of cells per tumour field (×10 magnification). CD3 and CD8 retained significance for all four clinical end points (OS, PFS, LFFS and DMFS), whereas neither FOXP3 nor CD4 was significantly correlated with improved outcome. The results are shown in Supplementary Table 2.

Table 1. Results of CD3, CD8, CD4 and FOXP3 immunohistochemistry

TIL markers	CD3	CD8	CD4	FOXP3
Dichotomized labelling score[a]	⩽6 *vs* >6	⩽6 *vs* >6	⩽4 *vs* >4	⩽4 *vs* >4
Low score	60 (59.4)	75 (74.2)	58 (56.9)	76 (74.5)
High score	41(40.6)	26 (25.8)	43 (42.2)	25 (24.5)

Abbreviation: AbbTILs = tumour-infiltrating lymphocytes.
[a]Dichotomised labelling (low *vs* high score) based on the median value of TILs' expression.

TIL immunostaining and treatment response. With a mean follow-up of 25 months (range, 2.3–63 months), OS at 2 years was 57.4% for the entire cohort. In univariate analysis, patients with high CD3 expression had a significantly superior OS (low *vs* high CD3: mean 30.3 *vs* 46.4 months; $P=0.002$), PFS (low *vs* high CD3: mean 26.1 *vs* 42.7 months; $P=0.002$), LFFS (low *vs* high CD3: mean 26.9 *vs* 42.7 months; $P=0.003$) and DMFS (low *vs* high CD3: mean 28.6 *vs* 46 months; $P=0.001$) (Figure 1A–D and Table 3). Similarly to CD3, high expression of CD8 correlated with better outcome. Indeed, patients with high CD8 expression had a significantly superior OS (low *vs* high CD8: mean 31.2 *vs* 50.1 months; $P=0.002$), PFS (low *vs* high CD8: mean 28.4 *vs* 45.3 months; $P=0.005$), LFFS (low *vs* high CD8: mean 29.2 *vs* 45.3 months; $P=0.008$) and DMFS (low *vs* high CD8: mean 30.0 *vs* 50.3 months; $P=0.002$) (Figure 1A–D and Table 3). Neither CD4 nor FOXP3 expression was significantly associated with any of the clinical end points (OS, PFS, LFFS and DMFS) in the univariate analysis (Table 3). Figure 2 illustrates the representative examples of low and high intra-epithelial CD3 and CD8 expressions.

Of note, the N-stage was the only clinicopathological parameter that reached statistical significance for a clinical end point (OS, $P=0.036$) (Table 3). Multivariate analysis was performed including the four TIL markers and all clinicopathological factors (Table 3). In the Cox model, high CD3 expression was confirmed as an independent prognostic parameter for OS ($P=0.024$), PFS ($P=0.044$) and DMFS ($P=0.021$), whereas no significance was found for LFFS ($P=0.90$). Similarly, high CD8 expression retained prognostic significance for OS ($P=0.028$), PFS ($P=0.047$) and DMFS ($P=0.026$) but lost significance for LFFS ($P=0.104$). We did not observe any significance for either CD4 or FOXP3 in the multivariate analysis. Early N-stage was associated with improved OS in the multivariate analysis ($P=0.046$). A trend towards improved DMFS was noticed for lower N-stage in the multivariate analysis ($P=0.060$) (Table 3).

Table 2. Tumour and treatment characteristics

	Low CD3 *n* (%)	High CD3 *n* (%)	*P*-value	Low CD8 *n* (%)	High CD8 *n* (%)	*P*-value	Low CD4 *n* (%)	High CD4 *n* (%)	*P*-value	Low FOXP3 *n* (%)	High FOXP3 *n* (%)	*P*-value
Age												
<61 years	27 (45%)	24 (59%)	0.225	37 (49%)	14 (54%)	0.821	32 (55.2%)	19 (44.2%)	0.318	42 (55.3%)	9 (36%)	0.111
⩾61 years	33 (55%)	17 (41%)		38 (51%)	12 (46%)		26 (44.8%)	24 (55.8%)		34 (44.7%)	16 (64%)	
Gender												
Male	47 (78%)	33 (80%)	0.499	61 (81%)	19 (73%)	0.406	46 (79.3%)	34 (79.1%)	0.584	61 (80.3%)	19 (76%)	0.777
Female	13 (22%)	8 (20%)		14 (19%)	7 (27%)		12 (20.7%)	9 (20.9%)		15 (19.7%)	6 (24%)	
Concomitant CT-dose												
100%	41 (68.3%)	24 (58.5%)	0.168	50 (66.7%)	15 (57.7%)	0.685	41 (70.7%)	24 (55.8%)	0.173	48 (63.1%)	17 (68%)	0.709
>50%	10 (16.9%)	9 (22%)		14 (18.9%)	5 (18.2%)		11 (19%)	8 (18.6%)		15 (19.7%)	4 (16%)	
⩽50%	8 (13.6%)	8 (19.5%)		10 (13.5%)	6 (23.1%)		6 (10.3%)	10 (23.3%)		13 (17.1%)	3 (12%)	
0%	1 (1.6%)	0 (0%)		1 (1.3%)	0 (0%)		0(0%)	1 (2.3%)		0 (0%)	1 (4%)	
Fractionation mode												
Normofractionated	41 (68.3%)	36 (87.8)	0.083	55 (73.3%)	22 (84.6%)	0.244	46 (79.3%)	31 (72.1%)	0.600	59 (77.6%)	18 (72%)	0.788
HART*	16 (26.7%)	4 (9.8%)		17 (22.7%)	3 (11.5%)		10 (17.2%)	10 (23.3%)		14 (18.4%)	6 (24%)	
Completely hyperfr.	1 (1.7%)	0 (0%)		1 (1.3%)	0 (0%)		1 (1.7%)	0 (0%)		1 (1.3%)	0 (0%)	
SIB#	0 (0%)	1 (2.4%)		0 (0%)	1 (3.8%)		0 (0%)	1 (2.3%)		1 (1.3%)	0 (0%)	
Other	2 (3.3%)	0 (0%)		2 (2.7%)	0 (0%)		1 (1.7%)	1 (2.3%)		1 (1.3%)	1 (4%)	
Tumour site												
Oral cavity	12 (20%)	12 (29%)	0.302	17 (22%)	7 (27%)	0.480	13 (22.4%)	11 (25.6%)	0.184	17 (22.4%)	7 (28%)	0.114
Oropharynx	25 (42%)	19 (46%)		33 (44%)	11 (42%)		21 (36.2%)	23 (53.2%)		30 (39.5%)	14 (56%)	
Hypopharynx	19 (31%)	7 (17%)		21 (28%)	5 (19%)		19 (32.8%)	7 (16.3%)		23 (30.3%)	3 (12%)	
Larynx	4 (7%)	1 (3%)		3 (4%)	2 (8%)		4 (6.9%)	1 (2.3%)		5 (6.6%)	0 (0%)	
Other	0 (0%)	2 (5%)		1 (1%)	1 (1%)		1 (1.7%0	1 (2.3%)		1 (1.3%)	1 (4%)	
T-staging												
cT1-2	5 (17%)	5 (12%)	0.522	7 (9%)	3 (11%)	0.715	7 (12.1%)	3 (7%)	0.397	6 (7.9%)	4 (16%)	0.239
cT3-4	55 (87%)	36 (88%)		68 (91%)	23 (89%)		51 (87.9%)	40 (93%)		70 (92.1%)	21 (84%)	
N-staging												
cN0-1	6 (10%)	9 (22%)	0.171	8 (11%)	7 (27%)	**0.035**	9 (15.5%)	6 (14%)	0.976	13 (17.1%)	2 (8%)	0.536
cN2a-2b	20 (33%)	15 (36%)		24 (32%)	11 (42%)		45 (77.6%)	34 (79.1%)		58 (76.3.%)	21 (84%)	
cN2c-3	34 (61%)	17 (52%)		43 (57%)	8 (31%)		4 (6.9%)	3 (7%)		5 (6.6%)	2 (8%)	
Grading												
G1	2 (4%)	3 (7%)	0.646	2 (3%)	5 (5%)	0.104	2 (3.7%)	3 (7.1%)	0.682	3 (4.2%)	2 (8%)	0.761
G2	42 (75%)	30 (75%)		56 (80%)	72 (75%)		42 (77.8%)	30 (71.4%)		54 (76.1%)	18 (72%)	
G3	12 (21)	7 (18%)		12 (17)	19 (20%)		10 (18.5%)	9 (21.4%)		14 (19.7%)	5 (20%)	
Smoking history												
Yes	37 (61.6%)	18 (43.9%)	0.149	45 (60%)	10 (38.4%)	0.076	28 (48.3%)	23 (53.5%)	0.431	31 (40.8%)	10 (40%)	0.284
No	23 (38.4%)	23 (56.1%)		30 (40%)	16 (61.6%)		30 (51.7%)	20 (46.5%)		45 (59.2%)	15 (60%)	

Score was based on the median value of TIL expression; significant results have been marked in bold. *Indicates hyperfractionated stereotactic radiotherapy. #Indicates simultaneous integrated boost.

In addition, we investigated the impact of TIL expression, according to the three separate tumour compartments (tumour periphery, tumour stroma and tumour cells; Supplementary Table 3, Supplementary Figures 1–4). The density of all four TIL markers was usually heterogenous within a tumour section and varied among individual tumours. Indeed, in some cases strong infiltration of all three compartments occurred, whereas in other cases there was strong infiltration of only the stroma and/or tumour cell/periphery compartment. We found differences in prognostic significance of TIL markers, depending on the separate areas. High expression of CD3 in the tumour cell area was significantly associated with better OS ($P=0.001$), PFS ($P=0.003$), LFFS ($P=0.004$) and DMFS ($P=0.001$). High CD8 expression in tumour periphery was significantly correlated with improved PFS ($P=0.035$) and LFFS ($P=0.036$), whereas high CD8 expression in tumour cell area was associated with better OS ($P=0.031$) and DMFS ($P=0.022$). High CD8 expression in tumour stroma was prognostic for all four clinical end points (OS: $P=0.008$; PFS: $P=0.027$; LFFS $P=0.018$; and DMFS: $P=0.012$). Analysis of the prognostic role for CD4 and FOXP3, based on the three tumour compartments, failed to identify significance for any of the clinical end points (Supplementary Table 3; Supplementary Figures 1–4). Similarly, we did not observe any significance for any of the TIL ratios with regard to the clinical end points (Supplementary Table 4).

Following that, we investigated the impact of HPV16 on clinical outcome and the prognostic value of the four TIL markers both in HPV16-positive- and HPV16-negative patients (Supplementary

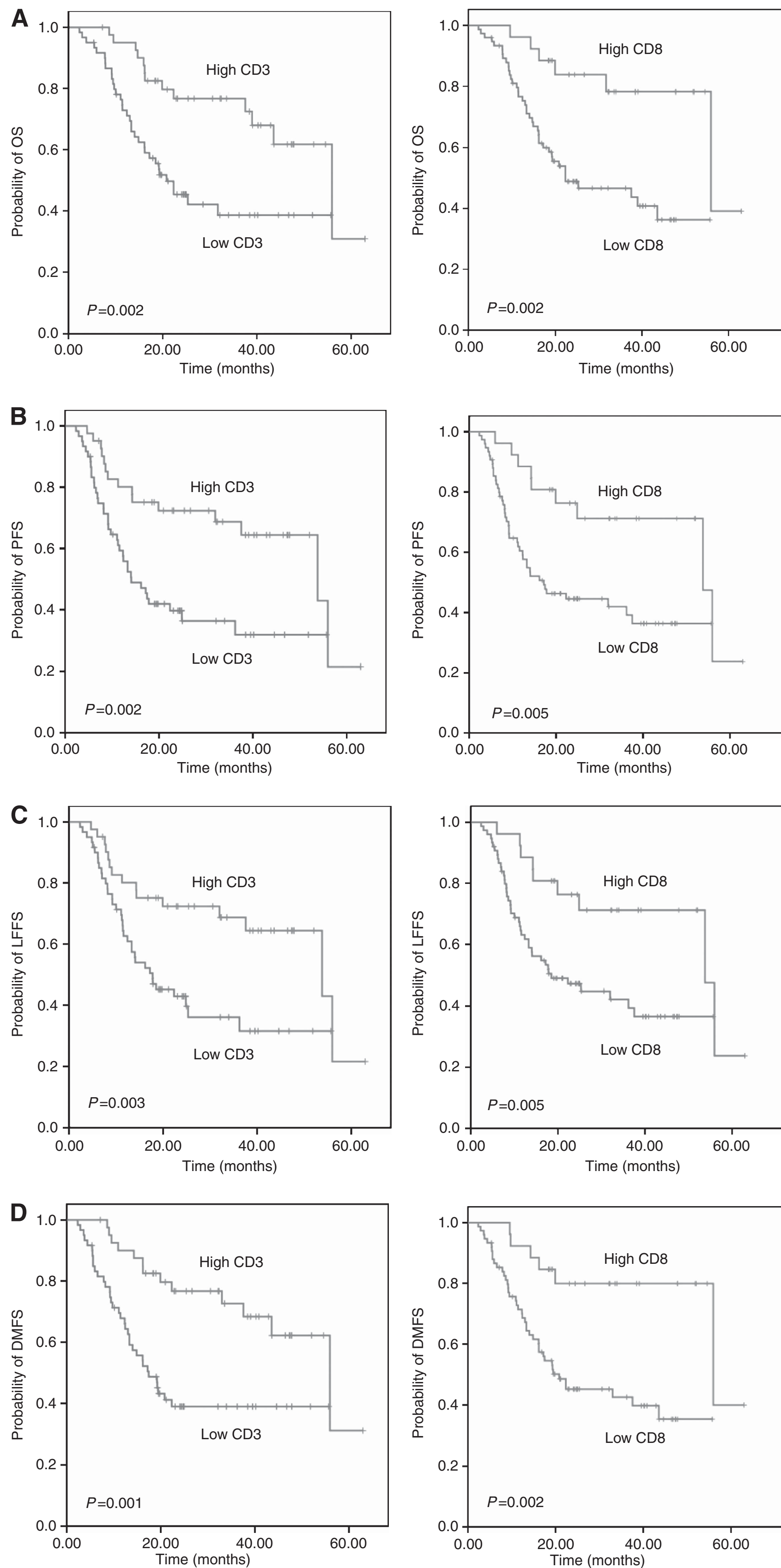

Figure 1. Prognostic role of CD3 and CD8 in the outcome of patients with head and neck squamous cell carcinoma after definitive chemoradiotherapy. (**A**) OS; (**B**) PFS; (**C**) LFFS; (**D**) and DMFS according to pretreatment CD3 and CD8 expression (low CD3 and CD8 expression: weighted score $\leqslant 6$; high CD3 and CD8 expression: weighted score >6; the cutoff score was based on the median value).

Table 3. Univariate and multivariate analyses of prognostic factors in patients with HNSCC

	Univariate *P*-value	Multivariate HR	95% CI Lower	95% CI Upper	*P*-value
OS					
CD3+ (low/high)	**0.002**	0.429	0.206	0.895	**0.024**
CD8+ (low/high)	**0.002**	0.359	0.130	0.990	**0.028**
CD4+ (low/high)	0.604	1.536	0.555	4.246	0.408
FOXP3+ (low/high)	0.878	1.040	0.227	4.765	0.960
Grade (G1/2/G3)	0.783	0.911	0.334	2.324	0.316
N-stage (N0-1/N2a-b/ N2c-N3)	**0.036**	1.324	1.004	1.746	**0.046**
T-stage (T1-2/T3-4)	0.178	0.396	0.162	0.970	0.143
Tumour localisation	0.658	0.915	0.623	1.344	0.649
Age (<61/⩾61)	0.102	1.414	0.762	2.624	0.272
Sex (male/female)	0.911	0.785	0.353	1.745	0.553
PFS					
CD3+ (low/high)	**0.002**	0.494	0.248	0.982	**0.044**
CD8+ (low/high)	**0.005**	0.464	0.198	1.087	**0.047**
CD4+ (low/high)	0.365	1.118	0.403	3.098	0.831
FOXP3+ (low/high)	0.810	0.719	0.154	3.368	0.675
Grade (G1/2/G3)	0.637	0.977	0.509	1.875	0.944
N-stage (N0-1/N2a-b/ N2c-N3)	0.160	1.176	0.926	1.493	0.184
T-stage (T1-2/T3-4)	0.282	0.462	0.181	1.179	0.106
Tumour localisation	0.587	0.896	0.628	1.277	0.542
Age (<61/⩾61)	0.260	1.006	0.541	1.870	0.986
Sex (male/female)	0.753	0.951	0.417	2.169	0.905
LFFS					
CD3+ (low/high)	**0.003**	0.547	0.272	1.100	0.090
CD8+ (low/high)	**0.005**	0.491	0.208	1.158	0.104
CD4+ (low/high)	0.400	1.211	0.437	3.359	0.713
FOXP3+ (low/high)	0.599	0.726	0.153	3.436	0.686
Grade (G1/2/G3)	0.646	0.768	0.386	1.528	0.452
N-stage (N0-1/N2a-b/ N2c-N3)	0.082	1.248	0.971	1.604	0.084
T-stage (T1-2/T3-4)	0.266	0.434	0.173	1.087	0.075
Tumour localisation	0.509	0.928	0.646	1.334	0.687
Age (<61/61)	0.121	1.344	0.734	2.458	0.338
Sex (male/female)	0.626	0.849	0.381	1.893	0.690
DMFS					
CD3+ (low/high)	**0.001**	0.431	0.211	.880	**0.021**
CD8+ (low/high)	**0.002**	0.314	0.113	.872	**0.026**
CD4+ (low/high)	0.440	1.444	0.526	3.962	0.475
FOXP3+ (low/high)	0.992	0.804	0.175	3.698	0.780
Grade (G1/2/G3)	0.822	0.839	0.411	1.712	0.629
N-stage (N0-1/N2a-b/ N2c-N3)	0.079	1.295	0.990	1.695	0.060
T-stage (T1-2/T3-4)	0.187	0.378	0.146	0.980	0.145
Tumour localisation	0.785	0.922	0.625	1.361	0.684
Age (<61/⩾61)	0.151	1.088	0.577	2.053	0.795
Sex (male/female)	0.949	1.144	0.494	2.649	0.753

Abbreviations: CI = confidence interval; DMFS = distant metastasis-free survival; HNSCC = head neck squamous cell carcinoma; HR = hazard ratio; LFFS = local failure-free survival; OS = overall survival; PFS = progression-free survival. Significant results have been marked in bold.

Tables 5 and 6; Supplementary Figures 5–8). HPV16 status did not affect outcome as no difference was detected in survival between HPV16-positive- and HPV16-negative patients (OS: $P = 0.541$; PFS: $P = 0.991$; LFFS $P = 0.814$; and DMFS: $P = 0.664$). Similarly, no significant difference in survival was obtained based on HPV16 labelling score (data not shown). Notably, we found no significance for any of the four TIL markers with regard to the outcome in HPV16-positive patients. In contrast, in HPV16-negative patients, high CD3 expression was a strong prognostic parameter for OS ($P = 0.007$), PFS ($P = 0.004$), LFFS ($P = 0.007$) and DMFS ($P = 0.003$). High CD8 expression also showed prognostic significance for OS ($P = 0.006$), PFS ($P = 0.015$), LFFS ($P = 0.020$) and DMFS ($P = 0.003$). Neither FOXP3 nor CD4 demonstrated a prognostic significance in either HPV16-positive- or HPV16-negative patients. Representative images of HPV16-positive- and -negative examples are shown in Supplementary Figure 9.

DISCUSSION

In the present study, HNSCC patients whose tumours were densely infiltrated by CD3+ and CD8+ T cells had a significantly longer OS, PFS and DMFS compared with patients whose tumours were poorly infiltrated. This finding was independent of clinicopathological parameters with a predictive role in this disease. Neither CD4 nor FOXP3 expression was significantly associated with any of the clinical end points.

The CD3 antigen is a protein complex comprising the T-cell receptor and has been commonly used as a T-cell marker (Gooden *et al*, 2011). The presence of higher numbers of CD3+ TILs has been associated with an improved outcome in different tumour types including colorectal cancer, breast, oesophageal, ovarian and anal cancer (Grabenbauer *et al*, 2006; Nedergaard *et al*, 2007; Denkert *et al*, 2010; Ducray *et al*, 2010; Dahlin *et al*, 2011; Gooden *et al*, 2011). Hence, our results are in accordance with previous observations.

High peritumoural lymphocytic infiltration has been associated with lower tumour stage and less invasive growth also in patients with HNSCC (Ginos *et al*, 2004; Rajjoub *et al*, 2007; Pretscher *et al*, 2009). However, these studies were characterised by low patient number and/or inhomogeneous treatment regimens (Ginos *et al*, 2004; Badoual *et al*, 2006; Rajjoub *et al*, 2007; Distel *et al*, 2009). Strengths of the present work rely on the fact that it includes a relatively large cohort of patients ($n = 101$) treated homogeneously with CRT in a single centre. Whiteshide and colleagues (Reichert *et al*, 2002) have shown that patients with HNSCC present defects in circulating T-cell signalling and tumour-directed cell killing, and TIL often undergoes apoptosis in tumours that are mediated by the Fas/FasL pathway. Le *et al* (2005) found a strong inverse correlation between the hypoxia-related marker galectin-1 and CD3 staining in patients with HNSCC, providing evidence on how hypoxia can help tumours in evading immune surveillance. In that work, patients with high infiltration of CD3+ cells presented better clinical outcome, similarly to our observations. However, treatment was heterogenous, as 80 patients received chemoradiation and 21 patients were treated with surgery ± radiation. In addition, no information with regard to the chemotherapy drugs in the chemoradiation treatment arm was provided (Le *et al*, 2005).

CD8 is a glycoprotein that constitutes a heterodimer of alpha and beta chains that are covalently linked by a disulphide bond (Gooden *et al*, 2011; Lieberman *et al*, 2001). A single immunoglobulin-like domain is contained to each dimer chain that is linked to the cell membrane via a polypeptide segment. CD8 has a key role in immune defense, as it serves as a coreceptor for the T-cell receptor (Gooden *et al*, 2011; Lieberman *et al*, 2001). Simultaneous

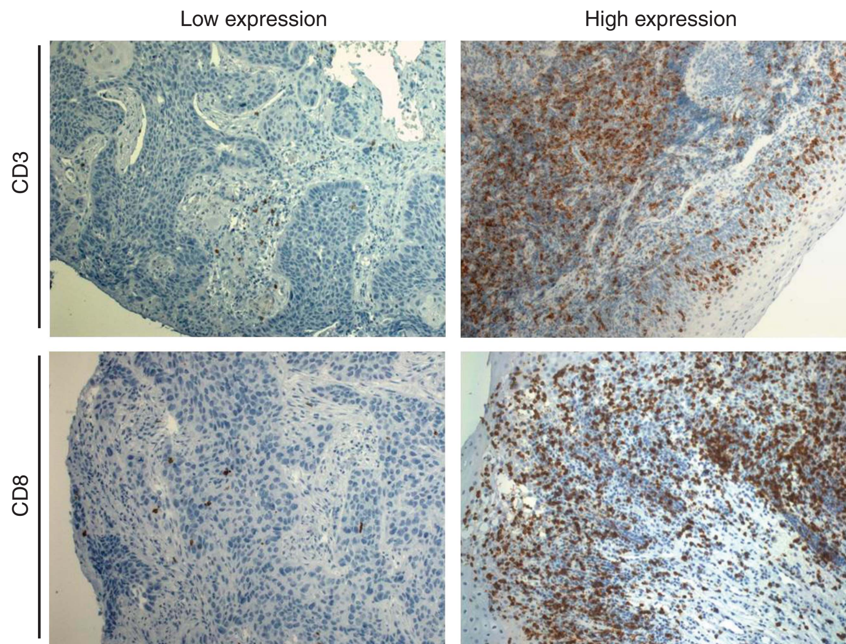

Figure 2. Representative examples of low and high CD3 and CD8 expression in head and neck squamous cell carcinoma samples, as indicated. Magnification, ×1.

binding of CD8 and T-cell receptor to the major histocompatibility complex class I molecule enhances profoundly the cytotoxic capability of T cells in killing tumour cells (Lieberman *et al*, 2001). Accordingly, a favourable outcome has been demonstrated for tumours with high tumour infiltration by CD8+ cells in patients with breast, colorectal, ovarian, renal and lung cancer, which is in agreement with our observations. With regard to HNSCC, Pretscher *et al* (2009) detected a reduced number of CD8+ cells in metastatic cervical lymph nodes. Distel *et al* (2009) reported better survival in 62 patients with low-risk HNSCC and high CD8 expression, after treatment with primary surgery followed by external radiotherapy. Ogino *et al* (2006) revealed that patients with laryngeal cancer and low CD8+ T cell infiltration had poor survival. Moreover, this constituted a heterogenous group of HNSCC patients, as 38 (60%) of the 63 patients were treated with surgery and only the remaining 25 (40%) received radiotherapy (Ogino *et al*, 2006), which makes interpretation of the results difficult.

Interestingly, we found differences in the prognostic value of CD3+ and CD8+ TILs that varied according to the tumour compartment. Indeed, high expression of CD3+ T cells in tumour cells correlated with better outcome, whereas high CD3 infiltration of tumour stroma or periphery did not affect outcome. High stromal CD8 infiltration was a positive prognostic factor for all four clinical end points, whereas high tumour cell CD8 expression correlated only with better OS and DMFS, and high tumour periphery CD8 expression correlated only with better PFS and LFFS. Several studies have observed differences in the prognostic value of TIL expression that varied according to the tumour compartment, similar to our work (Nedergaard *et al*, 2007; Distel *et al*, 2009; Pretscher *et al*, 2009; Denkert *et al*, 2010; Dahlin *et al*, 2011). CD4+ T cells respond to antigens presented by HLA class II proteins to generate effective antitumour immune responses by promoting proliferation of CD8+ cytotoxic T lymphocytes (Whiteside, 2012). In addition, T regulatory cells (Tregs; defined as Foxp3+ lymphocytes) infiltrate tumours following stimulation by inflammatory chemokines and macrophages and can lead to the suppression of effective immunosurveilance (Whiteside, 2012); however, this effect appears to tumour histology-dependent. The large meta-analysis by Gooden *et al* (2011) failed to detect a significant association between either CD4 or FOXP3 expression and clinical outcome. In HNSCC, the role of CD4 and FOXP3 remains controversial as mixed findings have been reported. Distel *et al* (2009) found no correlation between CD4 or FOXP3 expression and clinical outcome of patients with head and neck cancer, whereas Badoual *et al* (2006) reported better locoregional control and longer survival in 51 HNSCC patients with high CD4 and FOXP3 expression. We did not observe any correlation for either CD4 or FOXP3 and the clinical outcome in our series.

Several mechanisms have been proposed regarding tumour–immune interaction in response to cancer treatment in malignant tumours (Denkert *et al*, 2010; Deschoolmeester *et al*, 2010; Dahlin *et al*, 2011; Gooden *et al*, 2011). These investigations have hypothesised that the pretreatment of host immune response may augment the potential of cancer therapies to eliminate cancer cells. This hypothesis is strongly supported by our analysis. We are tempted to speculate that the killing of tumour cells by CRT might release tumour-associated antigens and chemokines that stimulate an immune response against the tumour cells – that is, will be highly potent in patients with immunosensitization already present before the initiation of cancer treatment. Moreover, the significant correlation of high CD3 and CD8 expression with better DMFS could indicate the presence of a systemic immunosurveillance mechanism that suppresses the development of micrometastases in distant organs. Milas and colleagues (Stone *et al*, 1979) have previously demonstrated reduced tumour response to radiotherapy in mice that lacked a normal T-cell repertoire. In a preclinical study, Burnette *et al* (2011) have previously shown that the efficacy of radiotherapy largely relies upon the induction of interferon-dependent innate and adaptive immunity. This effect was mediated by CD8, as the depletion of CD8+ T cells drastically reduced treatment efficacy, whereas alteration of CD4 did not affect tumour growth (Burnette *et al*, 2011). Strategies that induce T-cell activation, for instance by using an antibody targeting the cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) have been previously explored in combination with radiotherapy (Formenti and Demaria, 2013). In two carcinoma models growing in syngeneic mice, the addition of anti-CTLA-4 significantly and dose-dependently enhanced response to radiotherapy that resulted in complete regression of the majority of irradiated tumours

(Dewan *et al*, 2009). Stimulation of T cells via the inhibition of programmed-death-1 (PD-1), a surface receptor that leads to immunosuppression via the blockade of CD8 cell motility (Zinselmeyer *et al*, 2013), significantly enhanced radiotherapy response in an orthotopic model of glioblastoma (Zeng *et al*, 2013). Illidge and colleagues (Dovedi *et al*, 2013) demonstrated that CD8+ T-cell-mediated tumour eradication in experimental mouse models of T- and B-cell lymphomas following treatment with the Toll-like receptor 7 agonist, R848 in combination with radiation therapy. Hence, the use of immunotherapies that promote T-cell activation in combination with radiotherapy might be useful in patients with low CD8 expression in their pretherapeutic biopsy specimens to maximise irradiation outcome.

Liu and colleagues have previously demonstrated the value of p16 immunostaining in assessing the expression of p16 (HPV16) in patients with HNSCC (Shi *et al*, 2009). Here, we employed HPV16 immunostaining to examine the prognostic role of TILs according to the HPV status in our cohort. The HPV16 status did not affect clinical outcome in our series. Although this is not consistent with the literature as several studies have demonstrated better survival in HPV-positive patients compared with HPV-negative individuals following CRT (Fakhry *et al*, 2008; Shi *et al*, 2009; Ang *et al*, 2010; Rischin *et al*, 2010), this discrepancy can be attributed to the high percentage of older patients ($\sim$50% of patients were $>$61 years of age) and to the low number of patients with HPV16 in our cohort. Moreover, smoking history was less common in HPV16-positive patients (data not shown).

Interestingly, high expression of CD3 and CD8 was strongly associated with improved clinical outcome only in HPV16-negative patients in our study, whereas no significance was detected for TILs in HPV16-positive patients. Importantly, the degree of lymphocytic infiltration was comparable between HPV-positive- and HPV-negative patients in our cohort. Only few studies have examined the prognostic impact of TIL expression in correlation with the HPV status in HNSCC patients. Nasman *et al* (2012) found that high CD8 expression was correlated to a good clinical outcome in both HPV-positive- and HPV-negative patients. Similar findings were reported from Nordfors *et al* (2013), and the authors hypothesised that the increased expression of CD8+ TILs could contribute to the better response of HPV-positive patients to therapy. In contrast, Wansom *et al* (2012) did not detect any difference in TIL expression or prognostic impact by the HPV status, which is in line to our observations. Kong *et al* (2009) reported the significance for high counts of CD3+ T cells only in HPV-negative tumours, similarly to our work. In addition, mechanisms other than T-cell infiltration, such as gene upregulation in the TP53 pathway that results in increased tumour-cell killing (Kimple *et al*, 2013) and differential expression of cell cycle-related genes (Pyeon *et al*, 2007), are known to contribute to the better response of HPV-positive tumours to radiotherapy. Hence, this issue remains controversial, and more studies with higher number of patients, preferably treated within randomized trials, are required to better elucidate the expression pattern and prognostic significance of TILs in HPV-positive- and HPV-negative patients.

Common risk factors for HNSCC include smoking and alcohol history and HPV infection. Aviles-Jurado and Leon (2013) have previously identified the N-stage as an important prognostic factor in a retrospective study in 3373 patients with HNSCC, which is in accordance to our observations.

We acknowledge that the retrospective evaluation of TIL is a limitation of our study, and potential calculation bias cannot be excluded. The median follow-up in our study (25 months; range, 2.3–63 months) is also relatively short, as studies with a follow-up of several years in HNSCC have been previously reported (Lassen *et al*, 2009; Ang *et al*, 2010). Moreover, our observations warrant validation in large, independent cohorts.

In summary, CD3+ and CD8+ TIL represent strong markers to identify a subgroup of HNSCC patients with higher probability of disease progression and shorter survival after definitive CRT. The use of TIL markers, such as CD3 and CD8, to predict recurrence and cancer-related death is of particular clinical interest in advanced stage HNSCC, as high-risk patients may potentially benefit from the implementation of novel immunotherapy to stimulate T cells in combination with CRT.

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