Infiltrating immune cells are associated with radiosensitivity and favorable survival in head and neck cancer treated with definitive radiotherapy

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Objectives. The aim of this study was to investigate the influence of $CD4^+$, $CD8^+$ and Forkhead box protein 3 ($FoxP3^+$) tumor-infiltrating lymphocytes, as well as $CD1a^+$ tumor-infiltrating dendritic cells on the radiosensitivity and survival of primarily chemoirradiated advanced head and neck squamous cell carcinomas.

Study Design. Immunohistochemical staining for CD4, CD8, FoxP3 and CD1a was performed in 82 primarily chemoirradiated head and neck squamous cell carcinomas. Associations with clinicopathologic data, programmed cell death protein-1 (PD-1), programmed cell death ligand-1 (PD-L1), p16, radiation response, and survival were examined.

Results. High CD4 expression was associated with complete response after radiation (P = .006) and high CD1a expression (P = .024). High $CD8^+$ tumor-infiltrating lymphocyte counts were associated with absence of tumor relapse (P = .032) and better disease-free survival (P = .051). Strong overall T-cell infiltration was found more often in tumors with high-grade differentiation (P = .004), complete response after radiation (P = .022), and better overall survival and disease-specific survival (each P = .052). Tumors with high $FoxP3^+$ T regulatory (T_{reg}) infiltration more often showed high-grade tumor differentiation (P = .017), advanced patient age (P = .02), high PD-1 (P = .007), high CD4 (P = .002), and high CD8 expression (P = .002), as well as better disease-free survival (P = .019).

Conclusions. T-cell activation (high CD4, CD8 and FoxP3 expression) is associated with radio response and favorable survival in advanced head and neck cancer treated with definitive chemoradiation. (Oral Surg Oral Med Oral Pathol Oral Radiol 2020;129:612−620)

With more than half a million diagnoses per year, head and neck squamous cell carcinoma (HNSCC) is the sixth most common tumor in the world, representing about 6% of all cases.[1] Surgery remains the gold standard therapy for limited tumor extension, and regionally advanced tumor stages often require adjuvant chemoradiation. In cases of nonresectable tumors or tumors with distant metastases, definitive radiotherapy (RT) or concurrent chemoradiation (CRT) is the treatment of choice.[2] In tumor cells, radiation causes direct cytotoxic effects that can lead to apoptosis or senescence.[3] However, it may also induce immune effects that play an indirect role in tumor cell death.[4,5]

The role of the immune response in carcinogenesis is complex. It is mediated by the innate immune system as well as the adaptive immune system, which includes T cells and B cells.[6,7] Several studies have shown that the number of tumor-infiltrating lymphocytes (TILs) in HNSCCs is associated with the patients' outcomes and survival rates, however, leading to discrepant results.[7-11]

Cytotoxic T cells ($CD8^+$ T cells) are major histocompatibility I−restricted T cells that mediate apoptosis via cytokines and Fas ligands. In HNSCCs, both better and worse patient outcomes have already been described as being associated with high levels of $CD8^+$ TILs.[8,9,12] Further controversial findings have been shown for the $CD4^+$ T-cell population. $CD4^+$ T cells are known to regulate the response of different lymphocyte populations.[13] Previous studies in HNSCCs showed $CD4^+$ T cells to be associated with better overall survival (OS) and locoregional control but otherwise to be predictive of poor outcomes.[8,10,14] These contradictory findings may be a result of the heterogeneity of the $CD4^+$ T-cell subsets.[14] The best-characterized $CD4^+$ T cells are T helper 1 (Th1) and T helper 2 (Th2) cells which produce interferon-γ (INF-γ) and interleukin-4 (IL-4) respectively.[13] Besides those classic $CD4^+$ T-cell subpopulations, a number of further $CD4^+$ T-cell subsets have been found, with each pro-

Statement of Clinical Relevance

Definitive radiotherapy or concurrent chemoradiation is the treatment of choice for nonresectable head and neck cancer. Nevertheless, radiation response differs among patients. Pretreatment immune infiltrate assessment might help choose the right treatment modality.

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ducing its individual cytokine profile.[8,13] One of these subsets is the Forkhead box protein 3 (FoxP3) expressing a population of T regulatory (T_{reg}) cells, which commonly suppress the immune system by expressing cytotoxic T lymphocyte−associated protein 4 (CTLA-4) and secreting immunosuppressive cytokines, such as transforming growth factor (TGF-β) and IL-10.[4,11,13] Thus, T_{reg} cells may have a key role in the escape mechanism of human cancers against the immune response.[8]

A further important group of cells in antitumor immunity may be the group of immature dendritic cells (DCs). Immature DCs are known to express CD1a, a molecule responsible for presenting glycolipid antigens.[15] Among others, DCs are likely to play an important functional role in presenting tumor-derived glycolipid antigens to T cells, resulting in an effective antitumor immune response.[15] Earlier studies have reported that worse survival rates and higher rates of recurrence were associated with low levels of $CD1a^+$ DCs in tumors, including oral squamous cell carcinomas (OSCCs).[15,16]

The purpose of this study was to investigate the immunohistochemical (IHC) expressions of TILs ($CD4^+$, $CD8^+$, $FoxP3^+$) and $CD1a^+$ DCs in tumor samples of patients with HNSCCs treated with definitive irradiation or chemoradiation and to analyze the associations with clinicohistopathologic data, programmed cell death protein-1 (PD-1) expression, programmed cell death ligand-1 (PD-L1) expression, p16 expression, treatment response, and survival.

MATERIAL AND METHODS

Patient data collection

The study cohort comprised 82 patients (73 men, 9 women) with advanced HNSCC, and this cohort was already used in earlier studies.[17] Patients with nasopharyngeal carcinomas were excluded because of their strong associations with Epstein-Barr virus (EBV) infections.[18] All patients were diagnosed and staged in the Departments of Oral and Maxillofacial Surgery or Otorhinolaryngology at the University Hospital Regensburg (Germany), between 2004 and 2013. The treatment was performed in the Department of Radiotherapy at the University Hospital Regensburg or, in some cases, in regional radiotherapeutic departments. The treatment modalities consisted of primary single radiation or CRT. Single radiation was favored in cases of comorbidities or if the patient was older than 70 years of age.[19] The clinicohistopathologic data were collected from the patients' medical records, and follow-up data were obtained from the tumor registry of Regensburg. The study was performed according to the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments and was approved by the local ethics committee (No. 15-101-0336).

Immunohistochemistry

IHC sample preparation and staining. For all patients, formalin-fixed, paraffin-embedded tumor samples were available from the Department of Pathology, University Hospital Regensburg. Tissue microarray (TMA) construction was performed as previously described;[17,20] 1.5 mm−diameter punch cores were taken from 82 pretreatment tumor samples and 21 paraffin wax-embedded relapsed tumor samples, and 3-μm sections were cut from each block. Then, the slides were dewaxed, rehydrated, and washed. IHC staining was performed according to the institutional standard protocol of the Department of Pathology at the University Hospital Regensburg.

The incubation was performed at 72°C for 30 minutes, and the slides were rehydrated by using xylene and a series of graded alcohol. For antigen retrieval, the slides were heated in TRIS/EDTA buffer in the Decloaking Chamber (Biocare Medical, Concord, CA) at 15 bars and 120°C for 5 minutes. Subsequently, the endogenous peroxidase was blocked with Peroxidase-Blocking Solution (Dako, Glostrup, Denmark). Antibody incubation for CD4 (anti-CD4 [SP35] Rabbit Monoclonal Primary Antibody; Ventana Medical Systems, Inc., Tucson, AZ;), CD8 (anti-CD8 [SP57] Rabbit Monoclonal Primary Antibody; Ventana Medical Systems, Inc., Tucson, AZ), FoxP3 (eBioscience FOXP3 Monoclonal Antibody [236 A/E7]; Thermo Fisher Scientific, San Diego, CA ;) and CD1a (Novocastra Liquid Mouse Monoclonal Antibody CD1a; Leica Biosystems Newcastle Ltd., UK) was performed for 30 minutes at room temperature. For detection, the Dako REAL Envision Detection System, Peroxidase/DAB+, Rabbit/Mouse (Dako, Glostrup, Denmark) was used. Counterstaining was conducted with a hematoxylin solution.

IHC staining assessment. CD4, CD8, FoxP3, and CD1a IHC evaluation was performed by an experienced pathologist (W. F.), who was blinded to clinical patient data. For CD4, CD8, FoxP3, and CD1a, the absolute numbers of intratumoral membranous stained cells were counted in a high-power field (HPF) at × 400 magnification. The assessed area at × 400 magnification equals 1.25 mm^2 at a diameter of 0.625 mm per HPF for the microscope used in this study. Counting was done manually to ensure reproducibility; the area with the highest number of cells of interest was counted in each core of the TMA. Besides analyzing the absolute TIL numbers, sums of $CD4^+$ and $CD8^+$ cells were calculated for each individual tumor sample and subsequently assessed.

The median value of all tumor samples was used as the cutoff point, to dichotomize the biomarker expressions (CD4, CD8, FoxP3, CD1a, and CD4+CD8) in the

groups of low or high expression (low expression ≤ median) (Figure 1).[7,10]

PD-1, PD-L1, and p16 immunostaining and the corresponding evaluations have already been performed in previous studies.[17]

Statistical analysis

Statistical analyses were performed with SPSS software version 23 (IBM, Ehningen, Germany). If the variables were dichotomized, Pearson's χ^2 test ($P \leq .05$) or Fisher's exact test ($P \leq .05$) was used for examining the association between parameters. Univariate survival analyses for OS, disease-specific survival (DSS), and disease-free survival (DFS) were carried out by using the Kaplan-Meier method. Survival distributions were compared by using the log-rank test ($P \leq .05$). OS was defined as the time from diagnosis to all-cause death. DSS was defined as the time from diagnosis to tumor-related death. DFS was defined as the time from therapy completion to relapse or death, whichever occurred first.

RESULTS

Patient characteristics

The study cohort consisted of 73 men and 9 women. The patients' age at diagnosis ranged from 43.4 to 83.6 years (mean age 60.5 years). Of the study patients, 34 (41.5%) had tumors of the oropharynx; 17 (20.7%) of the larynx; 16 (19.5%) of the oral cavity; 13 (15.9%) of the hypopharynx; and 2 (2.4%) of the maxillary sinus. The exact locations of the oral cavity carcinomas were the floor of mouth (n12; 14.6%), the tongue, the alveolar ridge, the buccal mucosa, and the jaw angle (each 1; 1.2%). There were no significant associations between the primary tumor localization and treatment response (Table I). Laryngeal carcinomas showed the highest treatment response rates (13 of 17; 76.5%), followed by hypopharyngeal carcinomas (8 of 13; 61.5%) and oropharyngeal carcinomas (20 of 34; 58.8%). In contrast, carcinomas of the floor of the mouth presented the lowest response rates (4 of 12; 33.3%). The distribution of the treatment modalities was as follows: 65 patients (79.3%) received CRT, and 17 patients (20.7%) received primary radiation. Radiation schedules were normofractionated in 42 cases and hyperfractionated in 36 cases. The single radiation doses ranged from 1.4 to 2.7 Gy and amounted to total doses of 46 to 72.6 Gy (mean 67.7 Gy). Chemotherapy was performed according to the standard protocol (mostly 6 cycles) or to the study protocols in cases of study participation. Because of the appearance of comorbidities, the chemotherapy was cancelled ahead of schedule in a few cases. Fifty-five patients received platinum-based chemotherapy with either cisplatin weekly or carboplatin medication. Within the frame of study protocols,

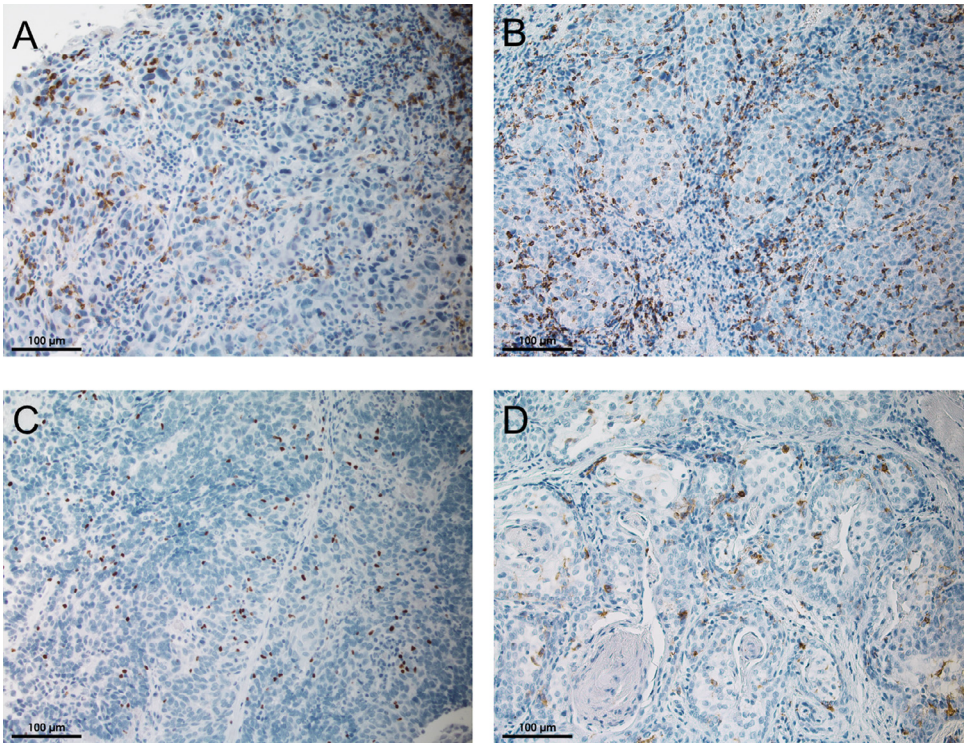

Fig. 1. Examples of biopsy specimens from patients with head and neck squamous cell carcinomas with high CD4 expression **(A)**, high CD8 expression **(B)**, high Forkhead box protein 3 (FoxP3) expression **(C)**, and high CD1a expression **(D)** (magnification × 200).

Table I. Relation of primary tumor location and treatment response

	Treatment response		
Primary tumor location	*Complete response*	*Incomplete response**	*P = .299*
Oropharynx	20 (58.8%)	14 (41.2%)	
Hypopharynx	8 (61.5%)	5 (38.5%)	
Larynx	13 (76.5%)	4 (23.5%)	
Floor of mouth	4 (33.3%)	8 (66.7%)	
Tongue	1 (100.0%)	0 (0%)	
Alveolar ridge	1 (100.0%)	0 (0%)	
Buccal mucosa	1 (100.0%)	0 (0%)	
Jaw angle	1 (100.0%)	0 (0%)	
Maxillary sinus	2 (100.0%)	0 (0%)	

Absolute number and percentage (vertical column).
*Incomplete response = partial response or no response.

paclitaxel (Taxol) or cetuximab was additionally administered in 7 and 6 cases, respectively. Non−platinum-based chemotherapy was chosen in 7 cases.

Six weeks after completion of RT, an initial local as well as locoregional tumor response control was performed with computer tomography and clinical investigations. Further evaluations were carried out in 3-month intervals. Tumor response was complete (CR) in 51 patients (62.2%) and incomplete (IR) in 31 patients (37.8%). IR was defined as no or partial tumor response.

Post-treatment tumor evaluations showed tumor recurrence in 31.7% of all patients (n = 26). Tumor recurrence was local (n = 24), loco-regional at the neck (n = 4), or distant (n = 4). Mean follow-up interval after therapy completion was 17.4 months (range 0−120.9 months).

Biomarker expressions of the primary tumor and associations with clinicohistopathologic parameters

As mentioned above, the median value was used as the cutoff to dichotomize lymphocyte expressions. Median counts were calculated as follows: 68.5 for $CD4^+$ TILs; 43 for $CD8^+$ TILs; 122.5 for the sum of $CD4^+$ and $CD8^+$ TILs; 47 for $FoxP3^+$ T_{reg} cells, and 12 for $CD1a^+$ DCs. In 3 cases, the evaluation of FoxP3 and CD1a was not possible.

The results of the statistical analyses of the biomarker expressions and clinicohistopathologic parameters are summarized in Table II.

High CD4 expression was associated with CR (78.0% vs 46.3% for low CD4 expression; $P = .006$). Furthermore, CD4 was more often expressed in smokers (100.0% vs 89.5% for low CD4 expression) without reaching significance ($P = .052$). Analyses of the $CD8^+$ T-cell subpopulation revealed significant association of high $CD8^+$ TIL counts with absence of tumor relapse ($P = .032$).

Strong T-cell infiltration (sum of $CD4^+$ and $CD8^+$ TILs) occurred more often in high-grade tumors (G3) ($P = .004$) and was associated with carcinomas that showed CR after RT or CRT ($P = .022$).

Tumors with high CD1a expression were located more often in the hypopharynx (11 of 13; 84.6%) or larynx (9 of 17; 52.9%) than in the oropharynx (13 of 32; 40.6%) or the floor of the mouth (3 of 11; 27.3%) ($P = .022$). Moreover, high levels of CD1a were found more often in patients with distant recurrence (4 of 4) ($P = .044$).

Further associations were revealed between high FoxP3 expression and advanced patient age (> 70 years) ($P = .02$), high-grade differentiation (G3) ($P = .017$), and absence of distant metastases ($P = .051$). No significant results were found for subgroup analyses of OSCCs.

Associations of TILs and CD1a

High $CD4^+$ TIL expression was associated with high PD-1 expression (70.7% vs 24.4% for low CD4 expression; $P < .001$); high CD8 expression (70.7% vs 29.3% for low CD4 expression; $P < .001$); high FoxP3 expression (65.0% vs 28.2% for low CD4 expression; $P = .002$); and high CD1a expression (60% vs 33.3% for low CD4 expression; $P = .024$).

In addition, high-grade $CD8^+$ lymphocyte tumor infiltration was associated with high PD-1 expression (65.9% vs 29.3% for low CD8 expression; $P = .002$); high $PD\text{-}L1^+$ TIL expression (73.2% vs 45.0% for low CD8 expression; $P = .013$); and high $FoxP3^+$ T_{reg} infiltration (65.0% vs 28.3% for low CD8 expression; $P = .002$).

High T-cell infiltration (sum of $CD4^+$ and $CD8^+$ TILs) was significantly associated with high PD-1 levels (78.0% vs 17.1% for CD4+CD8 expression; $P \leq .001$); high PD-L1 expression of TILs (73.2% vs 45.0% for low CD4+CD8 expression; $P = .013$); and high FoxP3 expression (70.0% vs 23.1% for low CD4 +CD8 expression; $P \leq .001$). In subgroup analyses of OSCCs, high levels of T-cells (CD4+CD8) were also associated with high PD-1 expression ($P = .036$).

Furthermore, 24 of 37 samples with high FoxP3 expression showed high PD-1 expression. In contrast, only 14 of 42 samples with low FoxP3 expression showed high PD-1 expression ($P = .007$).

Biomarker expressions of the primary tumor and associations with survival

Univariate survival analyses, performed by using the Kaplan-Meier method, showed that high FoxP3 expression was predictive of better DFS in the overall HNSCC group (Figure 2; $P = .019$), as well as in the subgroup of oral cavity cancer (53.6% vs 0% 5-year-survival-rate) ($P = .016$). In the oral cavity cancer group, high FoxP3 expression was a predictor for better OS (85.7% vs 0% 5-year-survival-rate) and DSS (see

Table II. Relationship between clinicopathologic characteristics and CD4, CD8, sum of CD4 and CD8, FoxP3 and CD1a

Parameter N = 82	*CD4*		*n*	*P*	*CD8*		*n*	*P*	*CD4+CD8*		*n*	*P*	*FoxP3*		*n*	*P*	*FoxP3*		*n*	*P*
	Low	*High*			*Low*	*High*			*Low*	*High*			*Low*	*High*			*low*	*high*		
Age (mean 60.5355 [43.36−83.60] years)			82	.519			82	.519			82	.519			79	.020			79	1.000
≤ 70 years	37 (90.2)	34 (82.9)			37 (90.2)	34 (82.9)			37 (90.2)	34 (82.9)			40 (95.2)	28 (75.7)			36 (85.7)	32 (86.5)		
> 70 years	4 (9.8)	7 (17.1)			4 (9.8)	7 (17.1)			4 (9.8)	7 (17.1)			2 (4.8)	9 (24.3)			6 (14.3)	5 (13.5)		
Gender			82	.482			82	1.000			82				79	.271			79	1.000
Male	35 (85.4)	38 (92.7)			37 (90.2)	36 (87.8)			36 (87.8)	37 (90.2)			36 (85.7)	35 (94.6)			38 (90.5)	33 (89.2)		
Female	6 (14.6)	3 (7.3)			4 (9.8)	5 (12.2)			5 (12.2)	4 (9.8)			6 (14.3)	2 (5.4)			4 (9.5)	4 (10.8)		
Primary tumor			82	.222			82	.453			82	.351			79	.435			79	**.015**
Oral cavity	9 (22.0)	7 (17.1)			8 (19.5)	8 (19.5)			10 (24.4)	6 (14.6)			7 (16.7)	7 (18.9)			11 (26.2)	4 (10.8)		
Oropharynx	21 (51.2)	13 (31.7)			18 (43.9)	16 (39.0)			19 (46.3)	15 (36.6)			20 (47.6)	13 (35.1)			19 (45.2)	13 (35.1)		
Hypopharynx	5 (12.2)	8 (19.5)			7 (17.1)	6 (14.6)			6 (14.6)	7 (17.1)			6 (14.3)	7 (18.9)			2 (4.8)	11 (29.7)		
Larynx	5 (12.2)	12 (29.3)			6 (14.6)	11 (26.8)			5 (12.2)	12 (29.3)			7 (16.7)	10 (27.0)			8 (19.0)	9 (24.3)		
Maxillary sinus	1 (2.4)	1 (2.4)			2 (4.9)	0 (0)			1 (2.4)	1 (2.4)			2 (4.8)	0 (0)			2 (4.8)	0 (0)		
T stage (n = 79)			79	1.000			79	.471			79	1.000			77	.705			77	1.000
T1+2	4 (10.3)	4 (10.0)			5 (13.2)	3 (7.3)			4 (10.5)	4 (9.8)			3 (7.5)	4 (10.8)			4 (9.8)	3 (8.3)		
T3+4	35 (89.7)	36 (90.0)			33 (86.8)	38 (92.7)			34 (89.5)	37 (90.2)			37 (92.5)	33 (89.2)			37 (90.2)	33 (91.7)		
N stage (n = 80)			80	.713			80	.476			80	1.000			78	.466			78	.466
N0	3 (7.7)	5 (12.2)			5 (12.8)	3 (7.3)			4 (10.3)	4 (9.8)			3 (7.3)	5 (13.5)			3 (7.3)	5 (13.5)		
N+	36 (92.3)	36 (87.8)			34 (87.2)	38 (92.7)			35 (89.7)	37 (90.2)			38 (92.7)	32 (86.5)			38 (92.7)	32 (86.5)		
M stage (n = 80)			80	.063			80	.063			80	.063			78	.051			78	.199
M0	30 (76.9)	38 (92.7)			30 (76.9)	38 (92.7)			30 (76.9)	38 (92.7)			32 (78.0)	35 (94.6)			33 (80.5)	34 (91.9)		
M1	9 (23.1)	3 (7.3)			9 (23.1)	3 (7.3)			9 (23.1)	3 (7.3)			9 (22.0)	2 (5.4)			8 (19.5)	3 (8.1)		
UICC stage (n = 79)			79	.241			79	.228			79	.228			77	.228			77	.496
I+II	2 (5.1)	0 (0)			2 (5.3)	0 (0)			2 (5.3)	0 (0)			0 (0)	2 (5.4)			2 (4.9)	0 (0)		
III+IV	37 (94.9)	40 (100)			36 (94.7)	41(100)			36 (94.7)	41 (100)			40 (100)	35 (94.6)			39 (95.1)	36 (100)		
Tobacco use (n = 78)			78	.052			78	.616			78	.352			75	1.000			75	1.000
Yes	34 (89.5)	40 (100)			37 (97.4)	37 (92.5)			35 (92.1)	39 (97.5)			37 (94.9)	34 (94.4)			36 (94.7)	35 (94.6)		
No	4 (10.5)	0 (0)			1 (2.6)	3 (7.5)			3 (7.9)	1 (2.5)			2 (5.1)	2 (5.6)			2 (5.3)	2 (5.4)		
Histologic grade			82	.441			82	.198			82	**.004**			79	**.017**			79	.606
G1+G2	33 (80.5)	29 (70.7)			34 (82.9)	28 (68.3)			37 (90.2)	25 (61.0)			37 (88.1)	24 (64.9)			33 (78.6)	27 (73.0)		
G3	8 (19.5)	12 (29.3)			7 (17.1)	13 (31.7)			4 (9.8)	16 (39.0)			5 (11.9)	13 (35.1)			9 (21.4)	10 (27.0)		
Local recurrence			82	1.000			82	.088			82	.467			79	.628			79	.808
No	29 (70.7)	29 (70.7)			25 (61.0)	33 (80.5)			27 (65.9)	31 (75.6)			28 (66.7)	27 (73.0)			30 (71.4)	25 (67.6)		
Yes	12 (29.3)	12 (29.3)			16 (39.0)	8 (19.5)			14 (34.1)	10 (24.4)			14 (33.3)	10 (27.0)			12 (28.6)	12 (32.4)		
Response to radiation		82	**.006**			82	.361			82	**.022**			79	**.038**			79	1.000	
CR	19 (46.3)	32 (78.0)			23 (56.1)	28 (68.3)			20 (48.8)	31 (75.6)			22 (52.4)	28 (75.7)			26 (61.9)	23 (62.2)		
NR/PR	22 (53.7)	9 (22.0)			18 (43.9)	13 (31.7)			21 (51.2)	10 (24.4)			20 (47.6)	9 (24.3)			16 (38.1)	14 (37.8)		

Absolute number and percentage (vertical column).
Statistically significant values ($p < 0.05$) are in bold.
FoxP3, Forkhead box protein 3; *CR*, complete response; NR/PR, no response/partial response; *T*, tumor size; *N*, lymph nodes; *M*, distant metastasis, *UICC*, Union for International Cancer Control.

Figure 2) (each $P = .010$). A further nonsignificant predictor for better DFS was high $CD8^+$ T-cell infiltration (see Figure 2; $P = .051$). In addition, high overall T-cell infiltration was associated with better OS (83.3% vs 20.0% 5-year-survival-rate) and DSS (see Figure 2) in the oral cavity cancer group, but without reaching significance (each $P = .052$).

Biomarker expression of relapsed tumors

IHC staining was performed on 21 samples of relapsed tumors. The evaluation of CD4, CD8, and CD1a was impossible in 1 case. FoxP3 evaluation was not possible in 3 cases.

High $CD4^+$ TIL expression was found in 25% (5 of 20) of all relapsed samples. CD8 expression was high in 4 samples (4.9%). The sum of $CD4^+$ and $CD8^+$ TILs resulted in 3 high-expression cases (3.7%). The expression of CD1a and FoxP3 was high in 8.5% (7 of 20) and 6.1% (5 of 18), respectively.

DISCUSSION

In the last few years, it has been realized that an immune infiltrate assessment can play a prognostic role in various types of tumors.[8] In the present study, we investigated the IHC expression of different T-cell subtypes as well as DCs in patients with advanced HNSCCs treated with definitive RT or CRT.

In accordance with findings from previous studies, the current results showed that high T-cell infiltration was a prognostic factor for a better outcome.[8,21] A recent meta-analysis correlated high $CD3^+$ T-cell infiltration with a favorable prognosis for both human papillomavirus−positive (HPV^+) and human papillomavirus−negative (HPV^-) HNSCCs.[21] CD3 is a general T-cell marker that exists on both $CD4^+$ and $CD8^+$ T-cells.[21] In the present investigation, high TIL expression was associated with CR after RT or CRT in HNSCCs, as well as better OS and DSS in the OSCC subgroup. Instead of using CD3, general T-cell infiltration was estimated by using the sum of $CD4^+$ and $CD8^+$ T cells. Another finding in the present study was that TIL expression is associated with more aggressive tumor behavior because it is more frequent in poorly differentiated (G3) tumors. This finding agrees with those of recent investigations in cutaneous squamous cell carcinomas (SCCs), which showed that $CD4^+$ and $CD8^+$ T cells were highly expressed in G2 and G3 differentiated carcinomas.[22] One explanation for the association of high TIL expression with more aggressive tumor behavior as well as with better outcome may be that poorly differentiated tumors go along with higher cell proliferation, which generally results in better radiation response.[23]

Earlier studies have reported significantly better outcomes in patients with HPV^+ HNSCCs, which may reflect a different immune response directed against the viral antigens.[8] For example, Ward et al. found an association between HPV^+ oropharyngeal cancer and increased T-cell infiltration.[24] Because of these earlier findings, analyses of T-cell infiltration and p16 were performed in the present study, but without reaching any significant results.

$CD8^+$ T cells, also referred to as *cytotoxic T-cells*, are supposed to be the most powerful anticancer cells of the immune system.[8] A well-established finding is that $CD8^+$ T cells are capable of targeting and destroying tumor cells by binding major histocompatibility complex class I molecules.[21,25] Nevertheless, a prognostic role of $CD8^+$ TILs in cancer is still unclear. In accordance with previous studies, the present results also showed that high $CD8^+$ TIL expression was associated with a favorable outcome.[8,9,26] The results indicated that high $CD8^+$ T-cell infiltration was associated with better DFS as well as absence of tumor relapse. However, other studies have linked high cytotoxic T-lymphocyte expression also with a poor outcome. For example, Wolf et al. showed that high $CD8^+$ T-cell levels were associated with tumor recurrence in OSCCs treated with surgery.[12] In addition, Wolf et al. could not find any association between $CD8^+$ T-cell infiltration and patient survival.[12] One reason for the contrasting results from previous studies may be the examination of different primary tumor sites. Therefore, we also performed subgroup analyses of OSCCs. In line with Wolf et al., the examinations did not demonstrate a significant association between CD8 expression and patient survival in the OSCC group.[12] On the scale of things, previous studies mostly focused on oropharyngeal and oral cavity cancers treated with surgery or with surgery combined with adjuvant treatment modalities.[8,9,12,26] This study and a few others included HNSCCs originating from different sites. Moreover, the only treatment modality was definitive chemoradiation.[7,8] Therefore, the findings may be a result of better radiosensitivity mediated through high CD8 expression.

The prognostic role of cytotoxic T cells is still being debated, and examinations of $CD4^+$ T cells have led to conflicting results in previous studies. Investigations in HNSCCs as well as esophageal SCCs and pancreatic cancers have shown that $CD4^+$ T cells are independently predictive of a favorable outcome.[8,10] However, high $CD4^+$ T-cell infiltration has been associated with worse survival in OSCCs.[14] The current results did not show any associations between $CD4^+$ T-cell infiltration and survival in the overall HNSCC group or in the OSCC group. However, high CD4 expression was associated with CR after chemoradiotherapy. Radiation has the ability to beneficially trigger immune cell activation.[27,28] Preclinical studies have suggested that localized radiation has immunomodulatory effects, which may increase tumor recognition.[28] High pretreatment tumor T-cell infiltration may reflect better

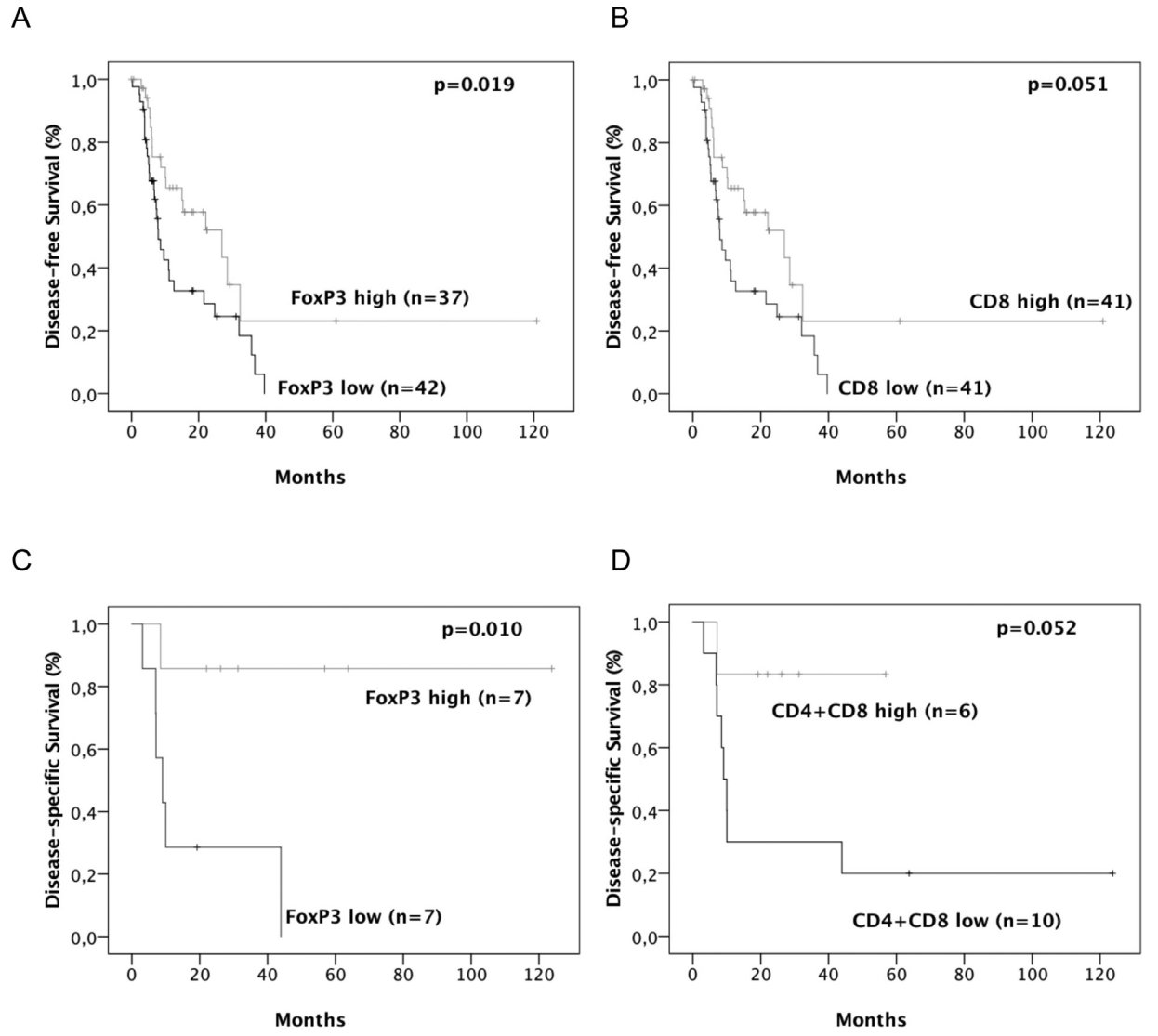

Fig. 2. The influence of Forkhead box protein 3 (FoxP3) **(A)** and CD8 **(B)** on disease-free survival in the overall head and neck squamous cell carcinoma group. The influence of FoxP3 **(C)** and overall T-cell infiltration (CD4+CD8) **(D)** on disease-specific survival in the oral cavity cancer group.

antitumor immune response after irradiation because there are more T cells to be activated. Radiation primarily induces DNA damage and endoplasmic reticulum (ER) stress by reactive oxygen species, resulting in tumor cell cycle arrest and tumor cell death.[27,29] DNA from dying tumor cells induces the stimulator of the IFN genes pathway of DCs, which is responsible for IFN-I production.[29,30] In turn, IFN-I is necessary for DC recruitment, and DCs are part of the T-cell activation by cross-presentation of tumor-derived antigens.[29,31,32] In the present study, high $CD1a^+$ DC levels were significantly associated with high intratumoral CD4 expression. Indeed, there was no direct association between high intratumoral DC expression and response to radiation. However, high pretreatment CD1a and CD4 expression may represent a group of tumors capable of an intense antitumor immune response.

A possible explanation for the prognostic discrepancies in previous studies of $CD4^+$ TILs may be the existence of several $CD4^+$ T-cell subsets. Besides the classic Th1 and Th2 $CD4^+$ T-cell subsets, other subtypes, such as the group of T_{reg} cells, are known to exist.[8] The staining for CD4 expression alone does not differentiate among those groups.[21] FoxP3 encodes a transcription factor and is specifically expressed in naturally arising $CD4^+$ T_{reg} cells.[33] Therefore, FoxP3 is used as a specific marker for T_{reg} expression. T_{reg} cells are known to have an immune-suppressing effect in the tumor microenvironment. They are known to express immune checkpoint receptors, such as CTLA-4 and PD-1, as well as immunosuppressive molecules, such

as TGF-1, which results in poor outcome in most solid tumors.[34,35] However, a recent meta-analysis reported better prognosis for high T_{reg} infiltration in HNSCCs.[21] The present study also connected high FoxP3 levels with favorable survival in the overall HNSCC group as well as in the OSCC subgroup. Furthermore, high FoxP3 expression was also associated with high PD-1 expression. One possible explanation for the contradictory findings of worse and favorable outcomes is that T_{reg} cells may help suppress the current, ineffective inflammatory response that has been suggested to promote tumor growth by inflammatory cytokine and growth factor−producing immune cells, such as macrophages and DCs.[21,35,36] Another possible explanation is that high FoxP3 expression may reflect high overall tumor T-cell infiltration resulting in a more successful antitumor immune response.[21,37] The beneficial effects of $CD8^+$ T cells may outweigh the immune-suppressing effects of T_{reg} cells.[21,37] This argument is strengthened by the present study, which showed significant associations of high FoxP3 levels with high CD4 levels as well as high CD8 levels. Another finding of the current investigation was that high FoxP3 expression seems to be more frequent in poorly differentiated tumors (G3). Earlier studies in cutaneous SCC, breast cancer, and prostate cancer have already linked high T_{reg} infiltration with more aggressive tumor behavior.[22,38,39] Nevertheless, in these studies high T_{reg} levels were linked to poor prognoses.[38,39] Therefore, the results of the present study were rather unusual, in that high T_{reg} infiltration is linked to a favorable prognosis as well as poor tumor differentiation, which is a general factor for more aggressive tumor behavior.

The present investigation has several limitations. As already mentioned in our previous publication, the population was very heterogeneous with regard to primary tumor sites, radiation schedules, and chemotherapeutics.[17] Furthermore, the results might be underpowered because of the small cohort. We used TMAs that showed only excerpts of the whole tumor. First, this might underestimate or overestimate IHC marker expressions because of intratumoral heterogeneity.[40] Second, we could only determine the intratumoral immunohistochemical marker expression because of the small tumor excerpts. Indeed, the prognostic role of $CD8^+$ T cells seems to differ among tumor compartments.[8]

In summary, high overall T-cell infiltration was associated with better survival and radio response in patients with advanced HNSCCs that were treated with definitive RT or chemoradiotherapy. Furthermore, TIL subgroup analyses revealed that high $CD4^+$, $CD8^+$ and $FoxP3^+$ T-cell infiltrations were associated with better survival and outcome.

CONCLUSIONS

Pretreatment immune infiltrate assessment might help determine the right treatment option and, thus, improve patient outcomes.

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