Tumour infiltrating lymphocytes in squamous cell carcinoma of the oro- and hypopharynx: Prognostic impact may depend on type of treatment and stage of disease

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Introduction

$CD4^+$ $CD25^+$ regulatory T cells (Treg) represent a unique population of lymphocytes capable of powerfully suppressing immune responses. In transplantable tumour models, the administration of antibodies to CD4 or CD25, which effectively antagonize Treg function, established a critical role for Treg-mediated immune suppression at both early and late-stages of disease, as these manipulations evoked impressive tumour regression and protection against subsequent tumour challenges.[1] Studies on the prognostic significance of intratumoural infiltration of Treg in different human tumour types, however, have produced conflicting data.[2–7] In both Hodgkin and non-Hodgkin's lymphoma as well as in several solid tumours, a favorable outcome was seen in association with a more intense infiltration.[2,3,7] By contrast in ovarian cancer a pronounced infiltration by Treg seemed to be associated with a poor prognosis in a very consistent fashion.[4,6]

Curiel and associates[4] were the first to identify the prognostic significance of regulatory T cell reactions in patients with epithelial ovarian cancer who underwent surgery and chemotherapy. An accumulation of $CD4^+$ $CD25^+$ T cells expressing Foxp3 was documented in ascites and primary tumour sites. Larger numbers of intratumoural regulatory T cells were strongly associated with inferior survival rates. This regulatory T cell recruitment was shown to occur as a consequence of CCL22 production in macrophages and tumour cells that triggered a CCR4-mediated chemotactic response. We have recently identified a subset of Granzyme B^+ cytotoxic T cells as a significant prognostic factor in anal squamous cell carcinoma following chemoradiation. Large numbers of tumour infiltrating Granzyme B^+ cytotoxic cells had a significant negative prognostic effect ($p = 0.008$), whereas no effect was observed for Treg.[5]

In the circulation of patients with squamous cell carcinoma of the head and neck region a significantly higher percentage of $CD4^+$ $CD25^+$ Treg were detected as compared to normal control persons ($10 \pm 4.7\%$ vs. $5.4 \pm 2.7\%$).[8] More specifically, the number of peripheral Treg inversely correlated with that of total $CD8^+$ T

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cells as well as the $CD8^+$ T cell subset representing Tc1 and Tc2 cells which are capable of lysing tumour cells.[9] This would support the notion of a possible crosstalk between different immune cells and consequently the balance between them may have substantial influence on tumour control and survival. It remains unclear whether the type of treatment (surgery vs. radiation vs. chemotherapy) or the stage of disease may correlate with the prognostic influence of tumour infiltrating lymphocytes.

The aim of this study was to evaluate the prognostic influence of various subpopulations of tumour infiltrating lymphocytes (TIL). In particular, we investigated the potential influence of regulatory $CD4^+$ $CD25^+$ $Foxp3^+$ cells (Treg) in head and neck cancer in relation to stage and treatment modalities.

Materials and methods

Patient selection

A total of 115 patients with squamous cell carcinomas of the oro- and hypopharynx received curative treatment at this University Hospital between 1992 and 2000 and were selected for analysis. A low-risk group of 62 patients with early disease (T1–T2, N0–N1) was treated by primary surgery with or without selective neck-dissection followed by external radiotherapy (RT). A high-risk group of 53 inoperable patients with advanced disease (T3–T4, N2–N3) was treated by primary radiochemotherapy. Table 1 gives a detailed analysis of the clinical and pathologic patient characteristics.

T and N categories were retrospectively assigned according to the UICC 1997 classification on the basis of the pathology reports.

Treatment protocols

As for the low-risk group of patients, surgery was performed according to standard procedures and included the resection of the primary tumour and a modified radical ipsi- or bilateral neck-dissection. All 62 patients were routinely scheduled for postoperative RT which was initiated within 4–6 weeks after surgery. In daily fractions of 2 Gy a median total dose of 60 Gy was applied to the primary tumour and pathologically involved neck region using 6 MV photons.[10]

As for the high-risk group, all 53 patients were required to have normal liver, pulmonary and haematologic functions and absence of distant metastases to qualify for aggressive radiochemotherapy.[11] Details of this treatment protocol are given elsewhere.[12] Briefly, total radiation dose to uninvolved nodal areas (PTV3), high-risk nodal areas (PTV2), and involved primary and nodal disease (PTV1) was 50, 60 and 72 Gy, respectively. PTV3 was treated by conventionally fractionated external radiation using a single fraction size of 2 Gy, PTV1 and PTV2 were treated by a hyperfractionated accelerated protocol that included two daily fractions of 1.4 Gy after 30 Gy given once daily in 2 Gy fractions. Concurrent

Table 1
Patient characteristics.

Treatment group	Low-risk group		High-risk group	
	N	(%)	*N*	(%)
All patients	62	(100)	53	(100)
Gender				
Male	51	(82)	43	(81)
Female	11	(18)	10	(19)
Age				
Median (years)	51		56	
28–40	5	(8)	1	(2)
41–50	22	(35)	14	(26)
51–60	29	(47)	20	(38)
61–75	6	(10)	18	(34)
Tumour site				
Hypopharynx	0	(0)	16	(30)
Base of tongue	14	(23)	13	(25)
Tonsils	33	(53)	5	(9)
Oropharynx	15	(24)	19	(36)
T category				
T1	13	(21)	0	(0)
T2	30	(48)	4	(8)
T3	14	(23)	26	(49)
T4	5	(8)	23	(43)
N category				
N0	23	(37)	5	(9)
N1, NX	14	(23)	4	(7)
N2a	7	(11)	0	(0)
N2b	16	(26)	13	(25)
N2c	2	(3)	29	(55)
N3	0	(0)	2	(4)
Grading (WHO)				
G1/2	37	(60)	38	(72)
G3/4	25	(40)	15	(28)
UICC stage				
1	6	(10)	0	(0)
2	13	(21)	0	(0)
3	17	(27)	7	(13)
4A	26	(42)	43	(81)
4B/C	0	(0)	3	(6)

chemotherapy consisted of fluorouracil given as continuous intravenous infusion over 5 days during the first week and mitomycin C given as a single intravenous bolus injection at a dose of 10 mg/m^2 on days 1 and 29.

Tissue microarrays and immunohistocytochemistry

Paraffin-embedded resection samples of the primary tumour available from all 115 patients with oro- and hypopharyngeal squamous cell carcinoma were processed into a tissue microarray (BioCat, Heidelberg, Germany) using a core diameter of 2 mm (Fig. 1A–D). Between 2 and 5 cores were taken per patient (mean 3.1) resulting in a total of 357 cores. Immunohistochemistry of paraffin sections was carried out using a standard streptavidin biotinylated alkaline phosphatase (ABC–AP, DakoCytomation, Hamburg, Germany) method or tyramide signal amplification followed by ABC–AP (only for FoxP3). The following antibodies were used: CD4 (Novocastra, Newcastle upon Tyne, UK), CD3, CD8, CD20, CD79, Granzyme B (DakoCytomation, Hamburg, Germany) and FoxP3 (abcam, Cambridge, UK).[13] Using a standard light microscope, images were acquired by a CCD-camera, transferred to a PC and counted using the image analysis program COUNT (Biomas, Erlangen, Germany).[14,15] Numbers of labelled tumour infiltrating cells were determined in relation to 100 tumour cells (/100 TC, labeling index, LI) as described previously.[5]

Statistical methods

The relationship between variables was assessed using the Spearman rank correlation coefficient (r_s). TIL-subgroups between the low-risk and the high-risk group were compared statistically by use of the *T*-test.[16] Rates for overall survival, disease-free survival and locoregional control were calculated according to the method of Kaplan and Meier.[17] The 66.6‰ was used as cut-off value according to Sato et al. No multivariate analysis was attempted due to the relatively small number of events.

Patient follow-up

Follow-up examinations including ENT evaluation and ultrasound of both neck sides were performed in three month intervals during the first 3 years and every six months thereafter. Rates of overall survival, disease-free survival and locoregional tumour control were calculated as the period from the day of the surgical

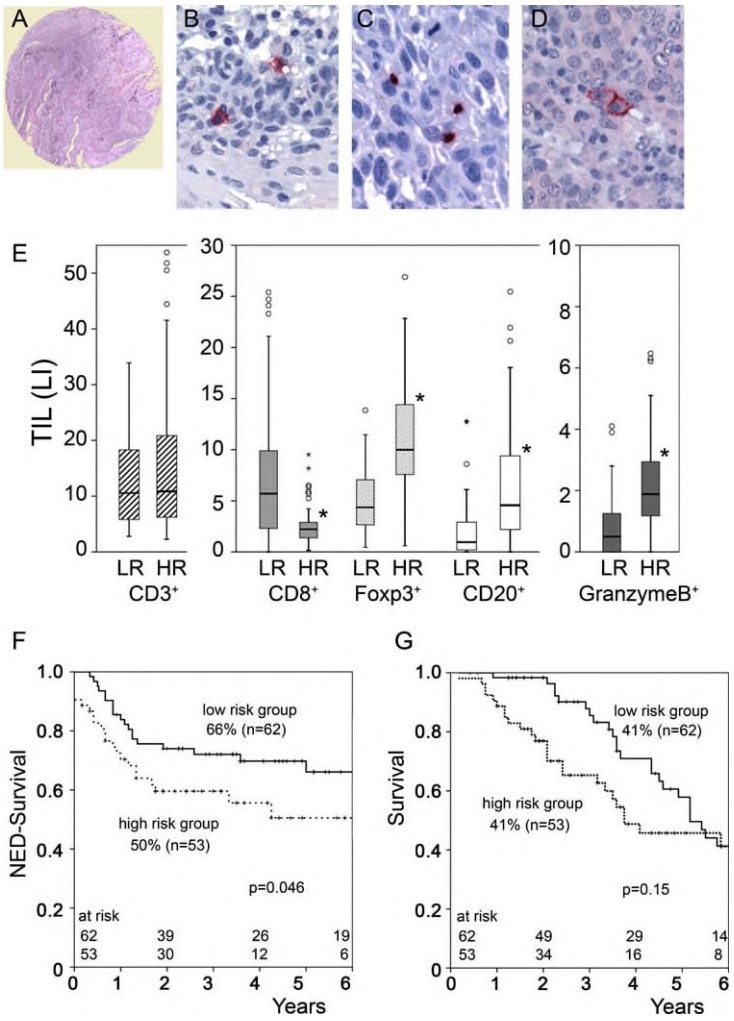

Figure 1 Biopsy specimens were processed into tissue microarrays using a 2-mm needle core (A). Immunohistochemistry was used to identify CD3$^+$ tumour infiltrating lymphocytes (TILs) (B), Foxp3$^+$ regulatory TILs (C, red nuclear labeling), and CD20$^+$ TILs (D). Box plot diagram of the smallest value, lower quartile, median, upper quartile, and largest value and, in addition, the outliers of tumour infiltrating lymphocytes (TIL), i.e. labeling index for CD3$^+$-, CD8$^+$-, Foxp3$^+$-, CD20$^+$- and Granzyme B$^+$-TIL for the different patient groups (E). Labeling index (LI), low-risk group (LR), high-risk group (HR). Using the *T*-test the two groups were found to be significantly different on the $p = 0.05$ level and were marked by an asterisk. NED (F) and overall survival rates (G) according to Kaplan–Meier for the low-risk group ($n = 62$) treated by surgery and postoperative radiation (upper curves) and the high-risk patient group with advanced disease that was treated by primary radiochemotherapy ($n = 53$, lower curves).

procedure until the date of death or the first local, regional, distant or combined relapse.

Results

T cell infiltration

An overview of the results, i.e. median, 25‰ and 75‰, extremes and outliers, for various T- and B cell subtypes of TILs is given in Figure 1E. Comparing the two patient groups, it seems noteworthy that the mean LI of Foxp3$^+$ regulatory T cells differed significantly ($p < 0.001$) with 5% vs. 11.8%, i.e. representing 40% and 76% of all CD3$^+$TIL for low-risk and high-risk patients, respectively. Conversely, a reduced number of CD8$^+$ TIL was seen in the high-risk patients as compared to low-risk patients (mean LI 2.6% vs. 7.4%, $p < 0.001$). Clearly increased numbers of CD20$^+$ and Granzyme B$^+$-TIL were noted in the high-risk patients ($p < 0.001$). No differences were observed between both groups for CD3$^+$ TIL and the CD3$^+$/Foxp3$^+$ ratio ($p > 0.2$). The low-risk and the high-risk group patients overall survival and NED-survival rates were 66% vs. 50% ($p = 0.046$) and 41% vs. 41% ($p = 0.15$) at 5 years, respectively (Fig. 1F and G).

TILs and prognosis

Low-risk group

Results of univariate analysis of NED-survival and the influence of different tumour infiltrating lymphocytes are given in Table 2. It was observed that infiltration by CD8$^+$ TIL (Fig. 2), CD20$^+$ TIL and the quotient of CD3$^+$/Foxp3$^+$ (Fig. 3) had significant influence on NED-survival in the low-risk group. Higher numbers of CD8$^+$ TIL (>66.6‰) within the invasive tumour led to improved NED-survival rates of 95% vs. 52% ($p = 0.005$). Similar results (“more is better”) were seen for CD20$^+$ TIL for the endpoint NED-survival ($p = 0.021$) and these data were supported by a second B cell marker CD79$^+$ with a good correlation to the CD20$^+$ TIL ($r_s = 0.76$, $p < 0.001$) and similar influence on NED-survival ($p = 0.036$).

Influence of tumour infiltrating lymphocytes on locoregional control rates was only noted for CD8$^+$ and CD20$^+$ TIL in the low-risk group. Both a high CD8$^+$ LI ($p = 0.004$) and a high CD20$^+$ LI ($p = 0.04$) were associated with significantly better tumour control rates (Table 2). Higher numbers of CD8$^+$ TIL (>66.6‰) within the invasive tumour led to improved locoregional control of 100% vs. 58% ($p = 0.004$).

High-risk group

In the high-risk group, CD3$^+$ and CD20$^+$ TIL had an influence on NED-survival. Low numbers of TIL were associated with a better survival. Intratumoural CD3$^+$ TIL infiltration below the 66th‰ was associated with a 5-year-NED-survival rate of 72% as compared to 38% for patients with higher CD3$^+$ numbers ($p = 0.045$) (Fig. 2). Corresponding results (“less is better”) were noted for CD20$^+$ TIL (Fig. 3), where patients with an infiltration rate below vs. above the 66th‰ had NED-survival rates of 71% vs. 39% ($p = 0.03$).

Foxp3$^+$–TIL interactions

Infiltration by Foxp3$^+$ TIL alone had no direct impact on prognosis (Fig. 2E and F). However, in the low-risk group the numbers of

Table 2
Univariate analysis of NED-survival and locoregional tumour control in low-risk and high-risk groups and the influence of clinical factors as well as different tumour infiltrating lymphocytes. The 66.6‰ was used for TILs as cut-off value.

Clinical factors	Low-risk group		High-risk group	
		p		*p*
NED-survival				
T category (T1–2 vs. T3–4)	67% vs. 63%	0.4	56% vs. 59%	0.7
N category (N0–1 vs. N2–3)	66% vs. 62%	0.3	69% vs. 51%	0.3
Age (< vs. ⩾ median)	52% vs. 82%	0.07	40% vs. 76%	**0.01**
Gender (male vs. female)	65% vs. 68%	0.7	60% vs. 52%	0.6
Tumour site (tongue base vs. tonsils vs. soft palate)	50% vs. 82% vs. 66%	**0.03**	66% vs. 60% vs. 47%	0.5
TIL-subtype				
CD3$^+$	67% vs. 66%	0.99	72% vs. 38%	**0.045**
CD8$^+$	52% 52% vs. 95%	**0.005**	56% vs. 71%	0.4
CD20$^+$	53% vs. 90%	**0.021**	71% vs. 39%	**0.036**
Granzyme B$^+$	66% vs. 69%	0.9	62% vs. 57%	0.6
Foxp3$^+$	63% vs. 75%	0.7	62% vs. 59%	0.5
CD3/Foxp3	77% vs. 46%	**0.031**	71% vs. 44%	0.4
CD8/Foxp3	64% vs. 72%	0.2	66% vs. 50%	0.6
CD20/Foxp3	54% vs. 85%	0.09	65% vs. 53%	0.3
Granzyme B/Foxp3	66% vs. 70%	0.9	57% vs. 69%	0.3
Locoregional control				
T category	72% vs.74%	0.7	76% vs.87%	0.8
N category	66% vs.81%	0.7	84% vs.77%	0.8
Age	57% vs.89%	**0.04**	71% vs.86%	0.4
Gender	70% vs.76%	0.5	84% vs.72%	0.7
Tumour site	44% vs. 88% vs. 66%	**0.05**	85% vs. 50% vs. 88%	0.3
TIL subtype				
CD3	74% vs. 70%	0.8	81% vs. 79%	0.7
CD8	58% 58% vs.100%	**0.004**	79% vs. 82%	0.8
CD20	60% vs. 90%	**0.04**	84% vs. 72%	0.2
Granzyme B	70% vs. 77%	0.8	79% vs. 83%	0.8
Foxp3	69% vs. 78%	0.8	83% vs. 76%	0.2
CD3/Foxp3	80% vs. 58%	0.2	89% vs. 68%	0.4
CD8/Foxp3	69% vs. 81%	0.1	88% vs. 64%	0.3
CD20/Foxp3	63% vs. 90%	0.1	84% vs. 74%	0.4
Granzyme B/Foxp3	70% vs. 79%	0.7	77% vs. 87%	0.5

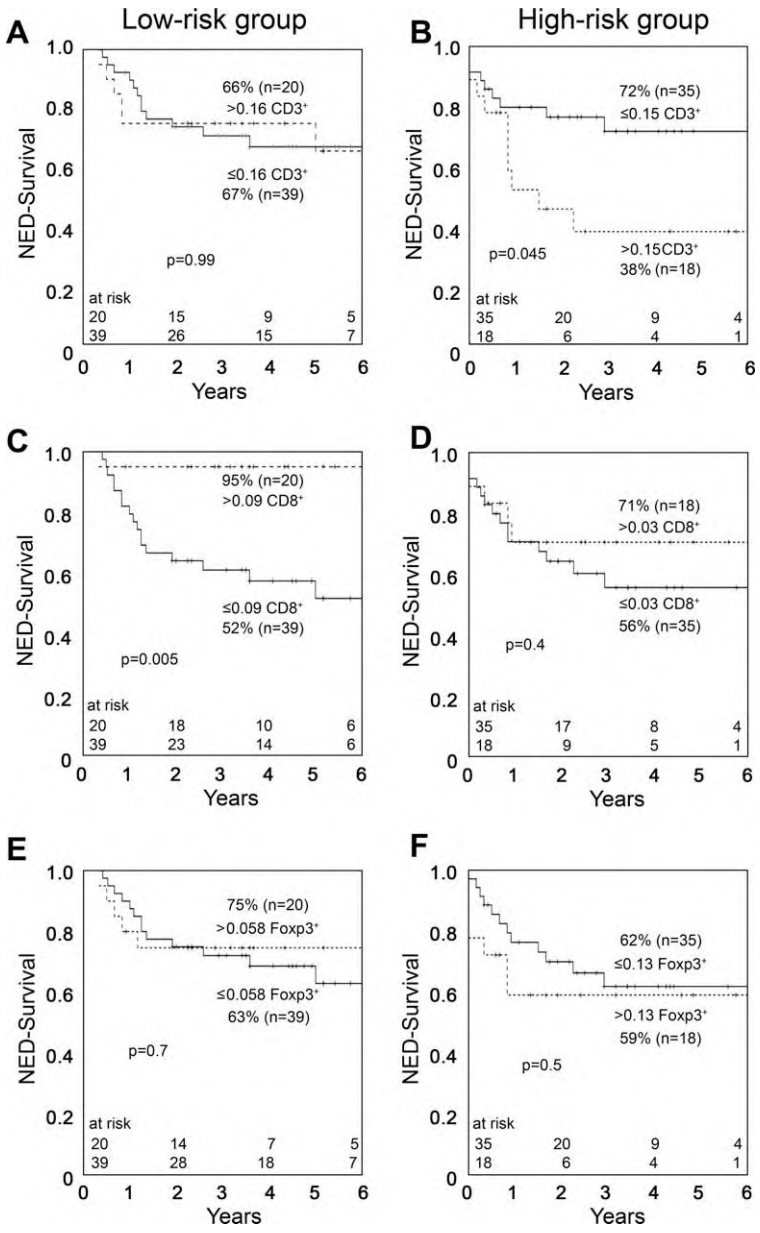

Figure 2 NED-survival rates according to the intratumoural labeling index of $CD3^+$ TILs (A and B), $CD8^+$ TILs (C and D) and $Foxp3^+$ TILs (E and F). Left panel shows patients of the low-risk group and right panel of the high-risk group.

$Foxp3^+$ TILs were highly correlated with all other TILs ($p < 0.001$). Additional categorization of all TILs according to the $Foxp3^+$ TIL infiltration (cut-off: 66‰) lead to a significant difference (Fig. 4A) between the two groups ($p < 0.05$). Contrary, among the high-risk group no correlation was found ($p > 0.17$) and there was no statistically significant difference (Fig. 4B) between both groups ($p > 0.38$).

Clinical factors and prognosis

Among the numerous clinical factors (gender, age, T category, N category, grading) tested for influence on NED-survival, in the low-risk group only "tumour site" did impact NED-survival (Table 2). Five-year-NED-survival rates for patients with squamous cell carcinoma of tonsilar fossa, soft palate and base of tongue were 83%, 66% and 50%, respectively ($p = 0.03$). Among the high-risk patients younger age was associated with poor NED-survival rates of 40% vs. 76% for older patients ($p = 0.01$). For the endpoint locoregional tumour control an impact was noted for age ($p = 0.04$) and tumour site ($p = 0.05$) only in the low-risk group (Table 2).

Discussion

In the present study, we performed a detailed immunohistochemical evaluation of TILs in two distinctly different groups of head and neck cancer patients treated for squamous cell carcinoma of the oro- and hypopharynx. A low-risk group defined as having primarily resectable small volume disease treated by surgery and postoperative radiation and on the other hand a typical high-risk group with advanced disease not amenable to surgery consistently treated by primary radiochemotherapy. Firstly, it appeared that the infiltration pattern of TIL comparing both patient groups was profoundly different with higher numbers of regulatory T cells and B cells and lower numbers of $CD8^+$ cells in the more advanced cases. Secondly, a number of different TIL-subgroups were associated

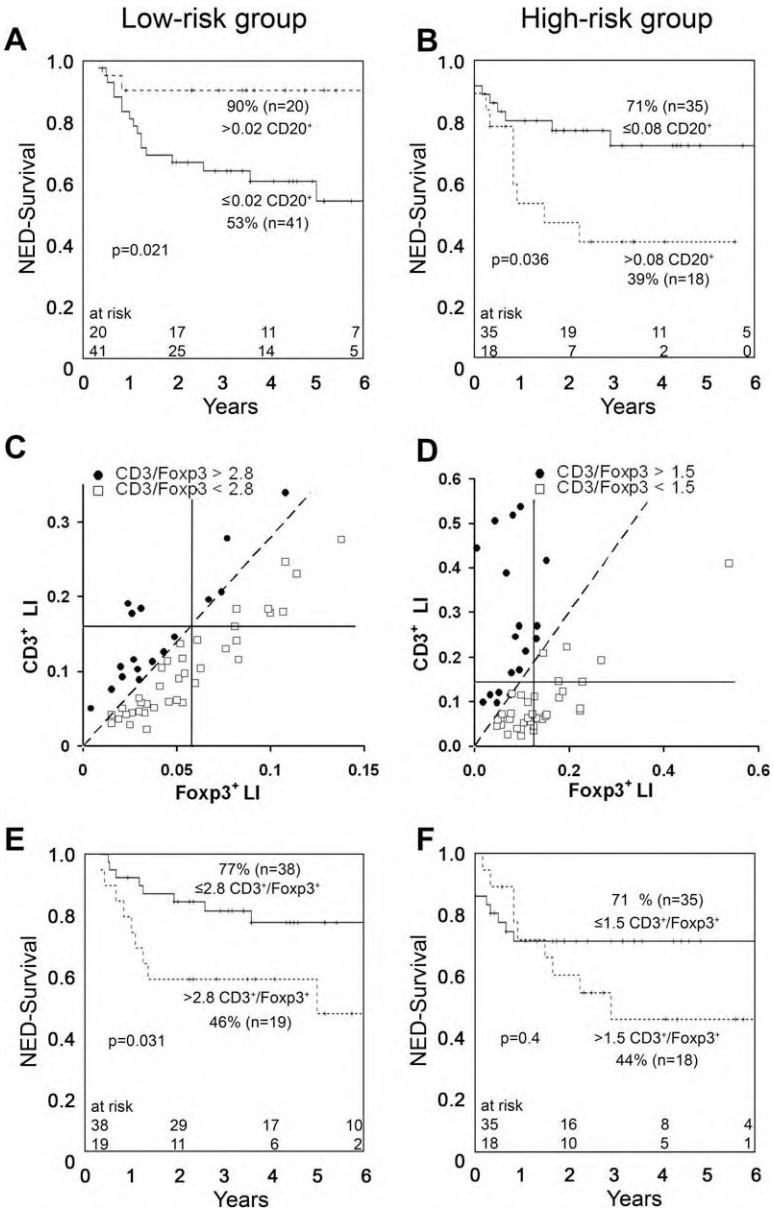

Figure 3 NED-survival rates according to the intratumoural accumulation of $CD20^+$ B-cells (A and B). Scatter plot showing the labeling index for $Foxp3^+$ cells and $CD3^+$ cells of all patients. Horizontal line represents 66.6‰ for $CD3^+$ cells, vertical line the 66.6‰ for $Foxp3^+$ cells and the stripped line the 66.6‰ for the $CD3^+/Foxp3^+$ TIL ratio (C and D). Filled circles represent patients above the 66.6‰ and open squares below. NED-survival rates according to the intratumoural accumulation of $CD3^+/FoxP3^+$ ratio (E and F). Left panel shows patients of the low-risk group and right panel patients of the high-risk group.

with clinically significant impact both on survival and on locoregional control.

The most striking result was the significant finding with regard to $CD20^+$ TIL for the endpoint NED-survival. In the low-risk group, higher numbers of $CD20^+$ TIL (“more is better”) were consistently associated with improved locoregional tumour control ($p = 0.02$). In contrast, for the high-risk group of patients higher numbers of $CD20^+$ TIL were a negative prognostic factor (“less is better”) ($p = 0.04$). To our knowledge this finding with one prognostic marker that is strongly dependent on the type of treatment and stage of disease in a specific tumour site was shown here for the first time. Additionally, this specific finding suggests that both tumour stage and type of treatment may have to be taken into consideration when interpreting data on TIL in head and neck cancer.

In the low-risk group the tumour was treated by surgery and postoperative radiotherapy. Here the bulk of the tumour was removed surgically, and only minimal residues had to be eliminated by subsequent ionizing radiation. It is conceivable that $CD20^+$ cells with their antigen presenting properties and their ability to proliferate and differentiate into antibody-secreting plasma cells may exert some anti-tumour efficacy against minimal residual disease. Reports from the literature describe a protective function of B-lymphocytes in primary tumour tissue of lung cancer[18] and breast cancer.[19] A higher number of $CD20^+$ B-lymphocytes was also reported by Gannon et al.[20] in lymph nodes involved by prostate cancer as compared to uninvolved nodes.

Contrary, in the high-risk group of patients the combination of radiation and chemotherapy had to eradicate the total tumour mass. It remains, however, unclear why a stronger infiltration pattern by B-cells in the context of advanced disease was associated with a less favorable outcome. One can speculate whether the B-cell infiltrate represents tumour specific immune response or

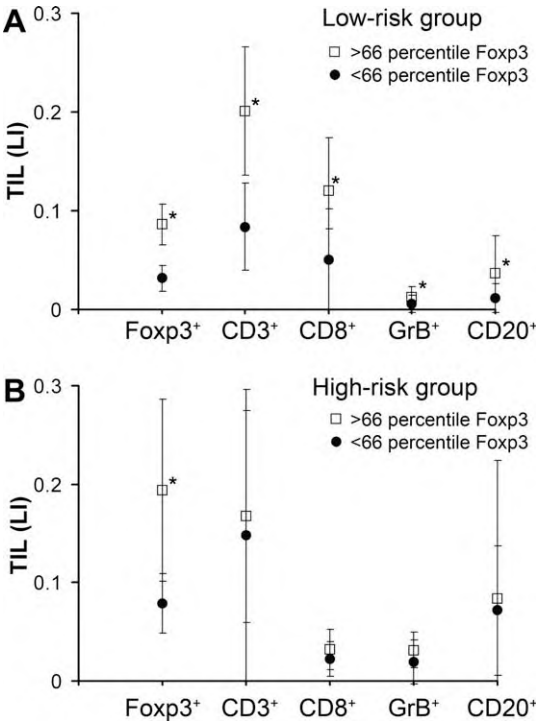

Figure 4 Labeling index was categorized according to the 66‰ for Foxp3$^+$ cells. CD3$^+$, CD8$^+$, Granzyme B$^+$ and CD20$^+$ TILs were categorized according to the Foxp3$^+$ 66‰ below (filled circles) and above (open squares). (A) Low-risk group and (B) high-risk group. Using the *T*-test the two groups were tested to be significantly different on the $p = 0.05$ level. Significantly different groups were marked by an asterisk.

non-specific lymphocyte recruitment by inflammatory and chemotactic cytokines with the ability to promote tumour growth and progression.[21–23]

In the low-risk group of patients intratumoural CD8$^+$ TIL were the only lymphocyte subtype being clearly and consistently associated with improved locoregional tumour control and survival, i.e. CD8$^+$ TIL (>66.6‰) within the invasive tumour led to improved locoregional control of 100% vs. 58% ($p = 0.004$) and NED-survival rates of 95% vs. 52% ($p = 0.005$). Infiltration with CD3$^+$ TIL above the 66.6‰ was associated with an NED-survival rate of 38% as compared to 72% for patients with less CD3$^+$ TIL within the tumour biopsy.

Consistent with our observations, Foxp3$^+$ T cells (Treg), a population of T cells with immunosuppressive properties, have been shown to account for approximately 2/3 of all intratumoural CD4$^+$ TILs in head and neck cancer. Unexpectedly, these were associated with improved survival and tumour control in a recent series reported by Badoual et al.[24] We examined intratumoural Treg infiltration in the current study by detection of Foxp3$^+$-expression among TILs. Our results indicate that intratumoural Treg infiltration by itself does not have an impact on tumour control or survival rates and thus are in contrast to the results reported by Badoual et al.[24] Interestingly, in our group of patients with advanced disease no direct influence of Treg could be demonstrated. However, in the low-risk group there was a distinct interrelation of Tregs with all other TILs. Additionally, the CD3$^+$/Foxp3$^+$ ratio had a clear impact on NED-survival with a low ratio being associated with a better prognosis. These findings nicely correspond to recent data on ovarian carcinoma given by Sato et al. [25] where a clear prognostic impact of the CD8$^+$/FoxP3$^+$ TIL ratio was described. In the high-risk group neither an interrelation between different TILs nor an impact on survival for the CD3$^+$/Foxp3$^+$ ratio was seen. Again, obviously both tumour stage and type of treatment may lead to different impact of TILs on prognosis.

In conclusion, the impact of TIL on prognosis in patients with head and neck cancer may strongly be affected by type of treatment and stage of disease.

Conflict of Interests Statement

None declared.

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