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ORIGINAL ARTICLE

Morphomolecular analysis of the immune tumor microenvironment in human head and neck cancer

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Abstract

Immunotherapy is effective in head and neck squamous cell carcinoma (HNSCC), but only a minority of patients responds to immune checkpoint blockade (ICB). To contribute to a better understanding of the underlying immune biology, we combined histomorphological evaluation and molecular analysis of the HNSCC immune microenvironment in the TCGA cohort. Analyzing digital HE-stained slides, a method for classification of tumor infiltrating lymphocytes (TILs) in the intra-epithelial compartment (ieTILs, present vs. absent) and the stromal compartment (strTILs, high vs. low) was established. We also analyzed the abundance of eight immune cell populations (estimated from RNAseq data) and PD-L1 mRNA expression. Status of ieTILs and status of strTILs were concordant for 61%, but discordant for 39% of tumors. In univariate survival analysis, ieTILs were a positive prognostic marker for DFS in the study cohort (HR = 0.66, $p = 0.015$) and in the HPV− subcohort (HR = 0.68, $p = 0.04$), but not in the HPV + subcohort. T cells were a positive prognostic marker for DFS in the study cohort (HR = 0.80, $p = 0.03$) and in the HPV + subcohort (HR = 0.20, $p = 0.001$), but not in the HPV− subcohort. In univariate survival analysis, PD-L1 mRNA expression was neither associated with DFS nor with OS. However, in bivariate and multivariate analyses including both PD-L1 mRNA levels and T cells, PD-L1 was a negative prognostic marker of DFS and OS, while T cells remained a positive prognostic marker. In conclusion, ieTILs and strTILs were non-redundant biomarkers in HNSCC and should be evaluated separately. The identified prognostic markers should be evaluated for predictivity in ICB-treated patients.

Keywords Head and neck squamous cell cancer (HNSCC) · Immune checkpoint blockade · Tumor infiltrating lymphocytes (TILs) · T cells · PD-L1

Abbreviations

CTLA4	Cytotoxic T-lymphocyte associated protein 4
DFS	Disease-free survival
HNSCC	Head and neck squamous cell carcinoma
HR	Hazard ratio
ieTILs	Intra-epithelial TILs
MATH	Mutant allele tumor heterogeneity
OS	Overall survival

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PD1	Programmed cell death 1, official gene symbol: PDCD1
PD-L1	Programmed cell death 1 ligand 1, official gene symbol: CD274
strTILs	Stromal TILs
TILs	Tumor infiltrating lymphocytes
TMB	Tumor mutational burden

Introduction

Each year, more than 550,000 people develop head and neck cancer worldwide and around 300,000 die from disease progression [1, 2]. Male to female ratio ranges from 2:1 to 4:1. About 90% of all head and neck cancers are squamous cell carcinomas (HNSCC) [3]. HNSCC is the sixth leading cancer entity by worldwide incidence with a 5-year overall survival rate of about 40–50% [4]. Recurrent and metastatic disease is currently incurable in most cases, underscoring the need for more effective therapies. Most HNSCCs arise from the epithelial lining of the oral cavity, oropharynx, larynx and hypopharynx [2, 5]. HNSCCs are known to be strongly associated with environmental risk factors including tobacco smoking, alcohol consumption and infection with human papillomavirus (HPV) [6, 7]. HPV+ tumors represent 5–20% of all HNSCC cases and 40–90% of those arise from the oropharynx [8].

Immunotherapy harnesses the body's own immune system to fight against cancer and can be combined with other standard therapy modalities like surgery, radiotherapy or chemotherapy to achieve better outcomes [9–11]. Over the last years, the introduction of immune checkpoint inhibitors has greatly enhanced treatment options for advanced cancers [12]. Immune checkpoint blockade in HNSCC is currently investigated in numerous clinical trials with the predominant targets being PD-1/PD-L1 and CTLA-4 [13].

In 2016, both pembrolizumab and nivolumab were approved by the FDA for patients with HNSCC that progressed or has recurred or metastasized after first-line platinum-based chemotherapy [14]. Nivolumab was shown to significantly prolong overall survival (OS) in the CheckMate 141 trial, while exhibiting a favorable safety profile. Of note, OS benefit was observed irrespectively of PD-L1 expression and HPV status [15–17]. KEYNOTE-012 was a multicohort study designed to investigate efficacy and safety of pembrolizumab in advanced solid tumors of four different cancer types. Patients with recurrent/metastatic HNSCC showed a response rate of 18%, pembrolizumab was well tolerated and exhibited durable antitumor activity [18]. Some patients received 2 years of treatment and have shown clinical response for more than 30 months. The durable antitumor activity and tolerable safety profile observed with long-term follow-up support the use of pembrolizumab as a treatment for recurrent/metastatic HNSCC [19]. In the ECHO-202/KEYNOTE-037 and ECHO-204 trials, HNSCC patients responded well to the combinations of epacadostat plus pembrolizumab and epacadostat plus nivolumab [20]. So far, no full phase III clinical trial data evaluating the PD-L1 inhibitors atezolizumab and durvalumab have been published [21]. but a phase II study of durvalumab has demonstrated antitumor activity [22].

Despite the encouraging results for immune checkpoint blockade in HNSCC including durable responses and significantly prolonged overall survival, it should be noted that only a limited number of patients responds to immunotherapy: Overall response rates were 13% in the CheckMate 141 trial and 18% in the KEYNOTE-012 as well as in the KEYNOTE-040 trial [23]. Thus, biomarkers are urgently needed to identify HNSCC patients who are most likely to benefit from immunotherapy given potentially severe toxicities and high treatment costs. Moreover, a deeper understanding of the HNSCC tumor immunology is paramount to identify and finally overcome mechanisms of immune therapy resistance. Histomorphological and molecular parameters of the tumor microenvironment (TME) and their role in prognosis still await comprehensive analysis in HNSCC [24, 25].

Here, we combined histomorphological evaluation and molecular analysis of the immune microenvironment in the TCGA HNSCC cohort. Analyzing digital HE-stained slides, a method for classification of tumor infiltrating lymphocytes (TILs) in the intra-epithelial compartment (ieTILs, present vs. absent) and the stromal compartment (strTILs, high vs. low) was established. Additionally, using the bioinformatics method MCP-counter [26], the abundance of eight specific immune cell populations was estimated from RNAseq data. TIL levels, immune cell populations and PD-L1 mRNA expression levels were evaluated for correlation with clinicopathological tumor characteristics and clinical outcome.

Materials and methods

Study cohort

The study cohort comprised 518 treatment-naïve HNSCC tumors that were molecularly profiled in the TCGA project (Suppl. Figure 1). Digital slides were available and suitable for evaluation for 451 tumors, while gene expression data were available for 510 tumors. Clinical data of the tumors were downloaded from the GDAC Firehose portal (gdac.broadinstitute.org). Molecular data were downloaded from the cBioPortal (www.cbioportal.org). Digital slides were viewed and evaluated in the digital slide archive (cancer.digitalslidearchive.net). HPV status of the tumors (96 HPV+, 420 HPV− and 2 intermediate/unknown cases) was available from the clinical data table. Samples were classified as

HPV+ using an empiric definition of > 1000 mapped RNA sequencing (RNA-Seq) reads, primarily aligning to viral genes E6 and E7 as described in the corresponding TCGA publication [27]. The TCGA HNSCC data are freely available without restrictions for their use in publications.

Quantification of TILs

The HNSCC digital slides were examined for the presence of intra-epithelial TILs (ieTILs) and of stromal TILs (strTILs) by an expert pathologist. The densities of both kinds of TILs were separately scored semi-quantitatively and categorized into the four levels "absent", "low", "intermediate" and "high" (Suppl. Figure 2). To this end, the complete tissue section was evaluated and subdivided into regions that were homogeneous with respect to the level of TIL infiltration. Corresponding to the four levels of TIL infiltration, we obtained four percentage values ×1, ×2, ×3 and ×4 summing up to 100% of the tumors area (ieTILs) or the stromal area (strTILs), respectively. Subsequently, we scored the total number of ieTILs and strTILs as $S = (0 * x_1 + 1 * x_2 + 2 * x_3 + 4 * x_4)/4$. Both, the scores of ieTILs and the scores of strTILs showed clear bimodal distributions. Thus, we introduced cut-points to separate between the two modes of the distributions and analyzed both markers as binary variables rather than as continuous variables (cf. Suppl. Figure 2): For ieTILs, we introduced a cut-point of 0 to separate cases with intra-epithelial TILs ($n = 277$) from cases without intra-epithelial TILs ($n = 176$). For strTILs, we introduced a cut-point of 40 to separate cases with high stromal TILs ($n = 236$) from cases with low stromal TILs ($n = 215$).

Molecular tumor parameters

The abundance of ten different cell populations in the tumor-stromal microenvironment (T cells, CD8 T cells, Cytotoxic lymphocytes, B lineage, NK cells, Monocytic lineage, Myeloid dendritic cells, Neutrophils, Endothelial cells and Fibroblasts) was estimated from RNAseq data using MCP-counter [26]. For some of the analyses, levels of the cell populations and of PD-L1 mRNA expression were dichotomized ("high" vs. "low") using the median level as cut-point.

Cytolytic activity (CYT) was defined as the average of GZMA and PRF1 expression [28]. Tumor mutational burden (TMB) was calculated as the number of the exonic, non-synonymous somatic mutations in the WES data (included variant classes: Missense_Mutation, Nonsense_Mutation, Frame_Shift_Del, Frame_Shift_Ins, In_Frame_Del, In_Frame_Ins, Translation_Start_Site, Nonstop_Mutation, Splice_Site). Clonal diversity was quantified by the Mutant Allele Tumor Heterogeneity (MATH) [29].

Statistical analysis

Statistical modeling and calculation of p-values was performed using SPSS 25. Correlations of molecular parameters and the clinicopathological cohort characteristics were assessed for significance using Fisher's exact test and Welch's t test. Univariate survival analysis was performed using Kaplan–Meier curves, Cox proportional hazard models and the log-rank test. Bivariate and multivariate survival analyses were performed using Cox proportional hazard models and Wald's test. Multivariate survival analysis included correction for age (continuous variable), HPV status, smoking status, tumor stage (stages I–III vs. IV), tumor grade (G1/G2 vs. G3/G4) and tumor localization (all categorical variables). Figures 3 and 4 were generated using the statistical language R. p-values < 0.05 were considered significant.

Results

The study cohort comprised 518 treatment-naïve primary HNSCC (Suppl. Figure 1, Suppl. Table 1). HPV status was positive for 96 tumors, negative for 420 tumors and could not be determined for two tumors. For statistical analysis, tumor stages were grouped to I–III or IV, tumor grade was grouped to G1/G2 or G3/G4, while tumor topography was categorized into six main sites. To correct for a high content of immune cells at sites including immune organs, "tonsil" and "base of tongue" were part of the topography stratification. TIL evaluation was possible for 451 tumors where HE slides were available. TILs were evaluated separately in the intra-epithelial compartment (present vs. absent) and in the tumor stroma (high vs. low; Fig. 1). RNA-Seq data were available for 510 tumors and were used to estimate the abundance of ten cell populations (endothelial cells, fibroblasts and eight types of immune cells) in the tumor microenvironment.

Immunological and clinicopathological tumor characteristics

Intra-epithelial TILs (ieTILs) were present in 277 tumors (61.4%), while stromal TILs (strTILs) were high in 236 cases (52.3%). Correlation between ieTILs and strTILs was positive and highly significant ($p < 0.001$). Test of ieTILs and strTILs coincided for 274 cases (60.8%), but in 109 cases (24.2%) ieTILs were present, while strTILs were low and in 68 cases (15.1%) strTILs were high, while ieTILs were absent. A significant association of ieTILs and strTILs was also observed in the subgroup of HPV− tumors (62.3%, $p < 0.001$), but not in the subgroup of HPV + tumors (coincidence = 51.5%).

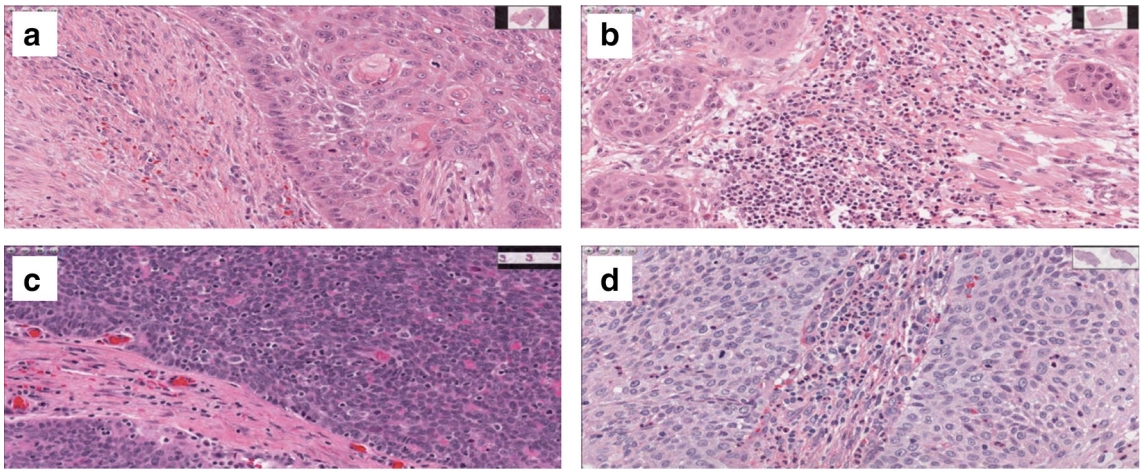

Fig. 1 Immune cells in the microenvironment of HNSCC (examples from the TCGA cohort, magnification: 64×). **a** Tumor with absent ieTILs and low strTILs. **b** Tumor with absent ieTILs and high strTILs. **c** Tumor with present ieTILs and low strTILs. **d** Tumor with present ieTILs and high strTILs

We correlated ieTIL, strTIL, T cell, and PD-L1 status with clinicopathological characteristics (Table 1). A higher abundance of ieTILs and T cells was detected in HPV+ tumors compared to HPV− tumors (76.8% vs. 58.2%, $p=0.01$ and 69.1% vs. 45.7%, $p<0.001$), while abundance of strTILs was not associated with HPV status. PD-L1 mRNA was highly expressed more often in non-smoking compared to smoking patients (58.8% vs. 45.6%, $p=0.005$). Lower stage tumors (I–III) exhibited more often high strTIL levels (59.9% vs. 47.4%, $p=0.01$) and showed more often high PD-L1 mRNA expression (55.8% vs. 46.3%, $p=0.046$) compared to stage IV tumors. Presence of ieTILs as well as high T cell levels correlated positively with high tumor grade ($p=0.025$ and $p=0.04$). T cells and PD-L1 mRNA correlated significantly ($p<0.0001$ and $p=0.035$) with tumor localization, while no significant correlations were observed for ieTILs and strTILs with tumor localization. In particular, T cells infiltration was often high in tumors located in the tonsils (82.5%) and at the base of the tongue (70.8%).

Univariate survival analysis

Next, we analyzed the prognostic impact of ieTIL, strTIL, T cell and PD-L1 status (Fig. 2). Tumors with high T cells had a significantly better DFS (HR = 0.8, $p=0.03$) as well as a significantly better OS (HR = 0.82, $p=0.001$) compared to tumors with low T cells. Tumors with detectable ieTILs had a better DFS (HR = 0.66, $p=0.015$) compared to tumors without ieTILs, but impact on OS was not significant. Neither strTIL status nor PD-L1 status were associated with a significant difference in DFS or OS.

We further investigated the prognostic relevance of the immune markers separately in HPV− and HPV+ tumors (Fig. 3a, b). In this analysis, ieTILs remained a positive prognostic factor of DFS in the HPV− subgroup (HR = 0.68, $p=0.04$), but not in the HPV+ subgroup. Stromal TILs showed no prognostic impact in HPV− or in HPV+ tumors. High T cell levels were positively associated with DFS and OS in HPV + tumors (HR = 0.2, $p=0.001$ and HR = 0.21, $p<0.001$) and showed a trend to better OS in HPV− tumors (HR = 0.79, $p=0.1$).

Furthermore, we analyzed the prognostic impact of 14 continuous variables including the levels of 10 specific cell populations estimated by MCP-counter (Fig. 3c, d; HR per doubling of the levels). Most of the immune cell populations tended to be positive prognostic markers in HNSCC, in the HPV− tumors as well in the HPV+ tumors. Among these, T cells were the most significant markers in the entire HNSCC cohort (DFS: HR = 0.8, $p=0.003$; OS: HR = 0.82, $p<0.001$). T cells were also significantly prognostic in HPV+ HNSCC (DFS: HR = 0.65, $p=0.004$; OS: HR = 0.58, $p<0.001$), but not in HPV− HNSCC. Cytolytic activity (CYT) and PD-L1 mRNA expression levels were positive prognostic markers in HPV + HNSCC, but not in the entire HNSCC cohort and not in HPV− HNSCC. The abundance of endothelial cells was a positive prognostic factor in HPV− HNSCC (DFS: HR = 0.67, $p=0.002$; OS: HR = 0.83, $p=0.08$), but a negative prognostic factor in HPV+ HNSCC (DFS: HR = 1.7, $p=0,09$; OS: HR = 2.06, $p=0.01$).

The analysis also included two genetic variables which were only borderline significantly associated with prognosis: Tumor mutational burden (TMB) was a borderline-significant prognostic factor for OS in HPV− HNSCC (HR = 1.12, $p=0.1$), while mutant-allele heterogeneity (MATH) reflecting subclonal variability was a borderline-significant negative prognostic factor in HNSCC

Table 1 Association of ieTILs, strTILs, T cells and PD-L1 mRNA with clinico-pathological cohort characteristics

Parameters	ieTILs		strTILs		T cells		PD-L1 mRNA	
	Absent	Present	Low	High	Low	High	Low	High
Age								
≤61 years	91 (38.4%)	146 (61.6%)	113 (47.9%)	123 (52.1%)	146 (53.7%)	126 (46.3%)	144 (52.9%)	128 (47.1%)
>61 years	84 (39.3%)	130 (60.7%)	101 (47.4%)	112 (52.6%)	109 (46.2%)	127 (53.8%)	109 (46.2%)	127 (53.8%)
	$p=0.92$		$p=0.93$		$p=0.11$		$p=0.13$	
HPV status								
Negative	160 (41.8%)	223 (58.2%)	183 (47.9%)	199 (52.1%)	225 (54.3%)	189 (45.7%)	212 (51.2%)	202 (48.8%)
Positive	16 (23.2%)	53 (76.8%)	32 (47.1%)	36 (52.9%)	29 (30.9%)	65 (69.1%)	43 (45.7%)	51 (54.3%)
	$p=0.01$		$p=0.63$		$p<0.001$		$p=0.23$	
Tobacco smoking								
Non-smoker[a]	58 (34.9%)	108 (65.1%)	72 (43.6%)	93 (56.4%)	86 (46%)	101 (54%)	77 (41.2%)	110 (58.8%)
Smoker[b]	114 (41.9%)	158 (58.1%)	135 (49.8%)	136 (50.2%)	161 (52.1%)	148 (47.9%)	168 (54.4%)	141 (45.6%)
	$p=0.15$		$p=0.2$		$p=0.196$		$p=0.005$	
Tumor stage								
I–III	64 (36%)	114 (64%)	71 (40.1%)	106 (59.9%)	95 (47.7%)	104 (52.3%)	88 (44.2%)	111 (55.8%)
IV	112 (40.7%)	163 (59.3%)	144 (52.6%)	130 (47.4%)	160 (51.4%)	151 (48.6%)	167 (53.7%)	144 (46.3%)
	$p=0.31$		$p=0.01$		$p=0.47$		$p=0.046$	
Tumor grade								
G1/G2	138 (42.3%)	188 (57.7%)	147 (45.2%)	178 (54.8%)	194 (53.6%)	168 (46.4%)	184 (50.8%)	178 (49.2%)
G3/G4	35 (30.4%)	80 (69.6%)	62 (54.4%)	52 (45.6%)	57 (43.2%)	75 (56.8%)	65 (49.2%)	67 (50.8%)
	$p=0.025$		$p=0.09$		$p=0.04$		$p=0.76$	
Tumor localization								
Mouth	53 (47.3%)	59 (52.7%)	52 (46.4%)	60 (53.6%)	71 (62.8%)	42 (37.2%)	53 (46.9%)	60 (53.1%)
Tongue (other parts)	45 (38.8%)	71 (61.2%)	47 (40.5%)	69 (59.5%)	68 (53.5%)	59 (46.5%)	61 (48%)	66 (52%)
Base of tongue	7 (33.3%)	14 (66.7%)	8 (38.1%)	13 (61.9%)	7 (29.2%)	17 (70.8%)	11 (45.8%)	13 (54.2%)
Tonsil	6 (25%)	18 (75%)	11 (47.8%)	12 (52.2%)	7 (17.5%)	33 (82.5%)	17 (42.5%)	23 (57.5%)
Pharynx	5 (26.3%)	14 (73.7%)	9 (47.4%)	10 (52.6%)	9 (45%)	11 (55%)	13 (65%)	7 (35%)
Larynx	41 (39.8%)	62 (60.2%)	60 (58.8%)	42 (41.2%)	56 (49.1%)	58 (50.9%)	71 (62.3%)	43 (37.7%)
Other	18 (31.6%)	39 (68.4%)	27 (47.4%)	30 (52.6%)	37 (52.1%)	34 (47.9%)	28 (39.4%)	43 (60.6%)
	$p=0.23$		$p=0.22$		$p<0.001$		$p=0.035$	

[a]Including former smokers, since at least 15 years

[b]Including former smokers, since less than 15 years

(DFS: HR = 1.34 (0.97–1.84), $p=0.076$; OS: HR = 1.16 (0.89–1.52), $p=0.27$).

Multivariate survival analysis

Using multivariate proportional hazard models, ieTILs, strTILs, T cells and PD-L1 expression were analyzed for their prognostic impact independent of classic clinicopathological tumor characteristics (Table 2; HRs from multivariate models include correction for age, HPV status, smoking status, tumor stage, tumor grade and tumor localization). ieTILs which were significantly prognostic in univariate analysis of DFS showed a non-significant trend towards better prognosis in multivariate analysis of DFS (HR = 0.77, $p=0.15$). Stromal TILs which showed a trend towards better prognosis in univariate analysis of OS were significantly prognostic in the multivariate analysis of OS (HR = 0.74, $p=0.04$). T cells which were positively prognostic in the univariate analyses of OS and DFS remained borderline significant in the multivariate analysis of DFS (HR = 0.87, $p=0.08$) and significant in the multivariate analysis of OS (HR = 0.85, $p=0.01$). PD-L1 mRNA levels did not show significant associations with DFS or OS in any univariate or multivariate analysis.

To analyze the contributions of the presence of immune cell infiltrates and of PD-L1 expression to prognosis, we performed bivariate analysis of ieTILs, strTILs or T cells and PD-L1 mRNA levels (Table 3). While PD-L1 mRNA levels were not significantly prognostic when analyzed in univariate analyses (Figs. 2 and 3), it was a negative marker of DFS (HR = 1.13, $p=0.03$) and of OS (HR = 1.12, $p=0.02$) when analyzed together with T cells. PD-L1 remained significant in

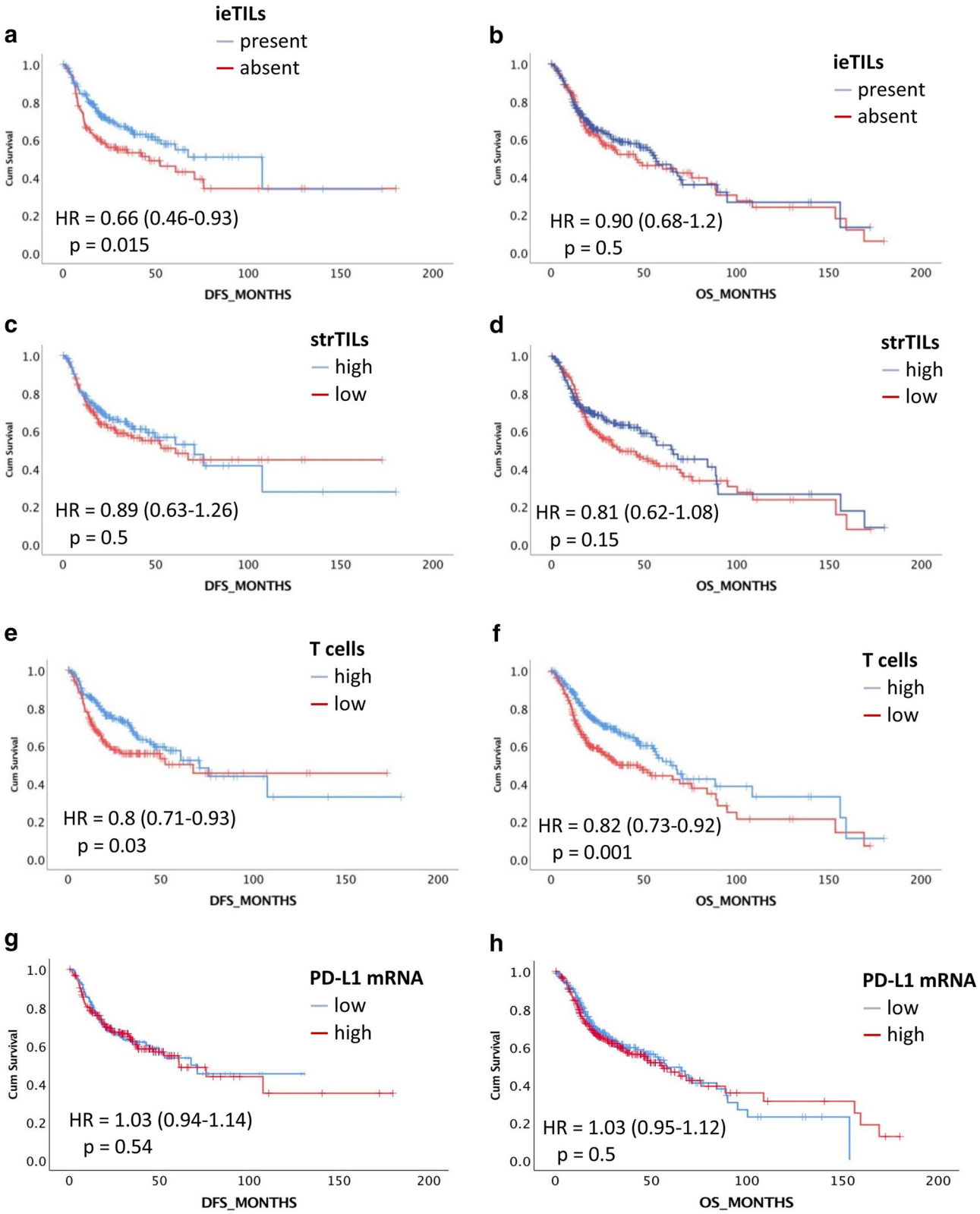

Fig. 2 Immune cell infiltrates as positive prognostic marker for HNSCC (TCGA cohort). **a**, **b** Intra-epithelial TILs were a significant positive prognostic marker for DFS, but not for OS. **c**, **d** Stromal TILs showed a trend to better prognosis for OS, but not for DFS. **e**, **f** T cells (estimated from mRNA expression data using MCP-counter, cut-point: median) were a significant positive prognostic marker for both DFS and OS. **g**, **h** PD-L1 mRNA expression (cut-point: median) did not correlate with DFS and OS

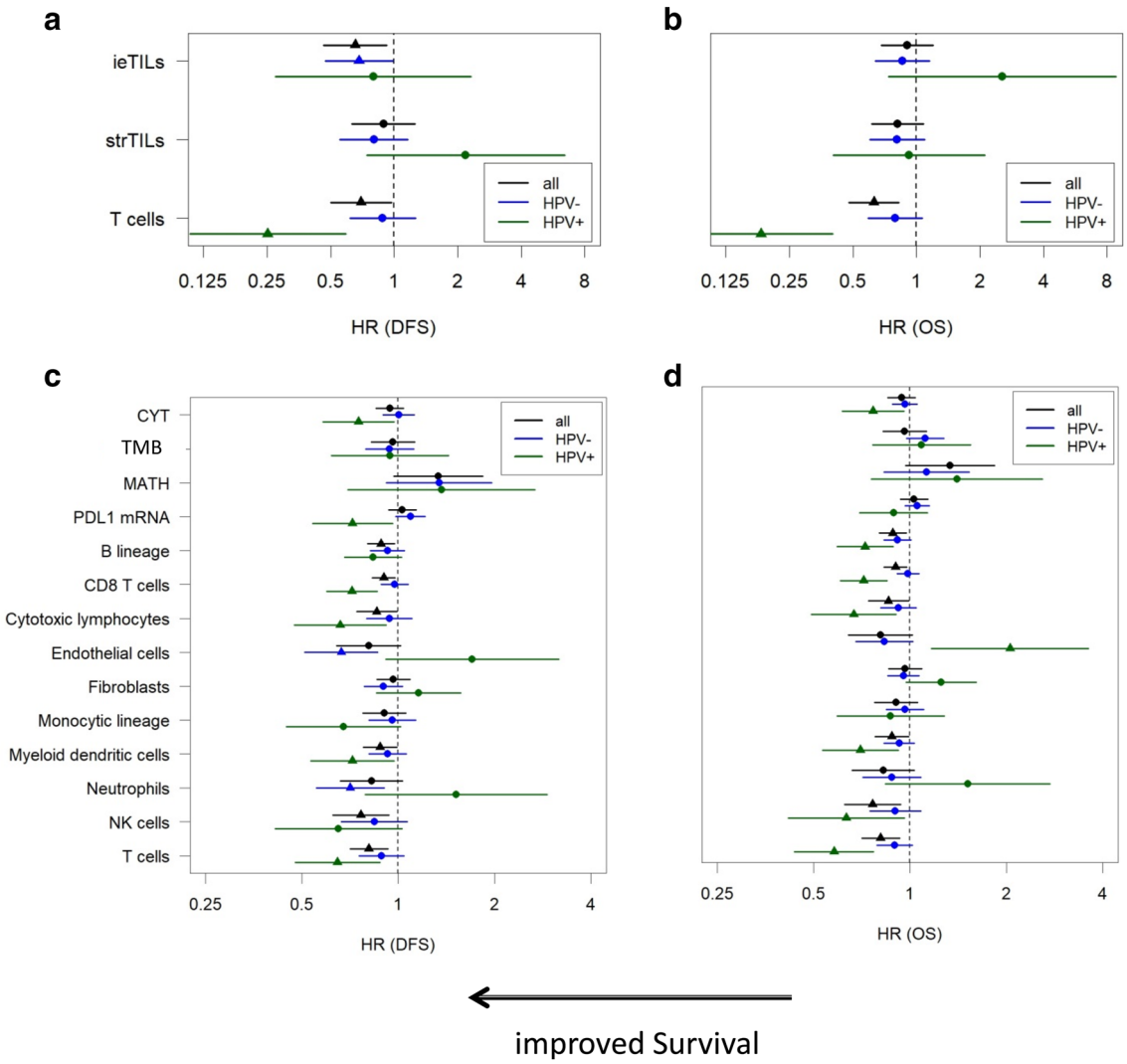

Fig. 3 Immune cells as prognostic markers in HPV + and HPV − HNSCC (TCGA cohort). **a**, **b** Analysis of ieTILs (present vs. absent), strTILs (high vs. low) and T cells (above vs. below median) as binary variables. **c**, **d** Analysis of cytolytic activity (CYT), TMB, MATH, PD-L1 mRNA and ten cell populations as continuous variables (log scale, HR per doubling). Triangle: significant result ($p < 0.05$), circle: non-significant result

Table 2 Univariate and multivariate survival analysis of ieTILs, strTILs, T cells and PD-L1 mRNA. The multivariate models include correction for age, HPV status, smoking status, tumor stage, tumor grade and tumor localization

Marker	DFS		OS	
	Univariate	Multivariate	Univariate	Multivariate
ieTILs	HR = 0.66 (0.46–0.93) $p = 0.015$	HR = 0.77 (0.53–1.10) $p = 0.15$	HR = 0.90 (0.68–1.2) $p = 0.48$	HR = 1.03 (0.76–1.39) $p = 0.87$
strTILs	HR = 0.89 (0.63–1.26) $p = 0.51$	HR = 0.89 (0.62–1.28) $p = 0.54$	HR = 0.81 (0.62–1.08) $p = 0.15$	HR = 0.74 (0.55–0.99) $p = 0.04$
T cells	HR = 0.8 (0.71–0.93) $p = 0.003$	HR = 0.87 (0.75–1.02) $p = 0.08$	HR = 0.82 (0.73–0.92) $p < 0.001$	HR = 0.85 (0.75–0.97) $p = 0.01$
PD-L1 mRNA	HR = 1.03 (0.94–1.14) $p = 0.54$	HR = 1.08 (0.97–1.21) $p = 0.14$	HR = 1.03 (0.95–1.12) $p = 0.5$	HR = 1.05 (0.96–1.15) $p = 0.27$

multivariate analyses including T cells as well as age, HPV status, smoking status, tumor stage, tumor grade and tumor localization (DFS: HR = 1.17, $p = 0.01$; OS: HR = 1.14, $p = 0.01$). In all of the bivariate and the multivariate analyses including PD-L1 expression, T cells remained a strong positive prognostic marker like in the univariate analyses (Table 3).

Correlation analysis

Finally, we investigated the associations between the immune biomarkers (Fig. 4). T cells were increased in HNSCC with high strTIL infiltrates (fold change = 1.4, $p = 1.8e{-}06$) and in tumors with present ieTILs (fold change = 1.4, $p = 0.00012$). PD-L1 expression was increased in tumors with high strTIL infiltrates (fold change = 1.6, $p = 6.8e{-}05$), but did not depend on the presence of ieTILs. PD-L1 expression correlated significantly with the level of T cell infiltration (R = 0.44, $p = 1e{-}22$). This correlation was also highly significant in the subgroup of HPV + tumors (R = 0.38, $p = 0.0014$) and in the subgroup of HPV− tumors (R = 0.47, $p = 6.2e{-}22$). Levels of T cell infiltrates were higher in HPV+ compared to HPV− tumors (fold change = 1.73, $p = 2.7e{-}05$), while PD-L1 mRNA expression was not significantly different between HPV+ and HPV− tumors.

Discussion

We analyzed the relation of clinical outcome and immune cell infiltration in a large cohort of clinicopathologically and molecularly well-characterized HNSCC. To our best knowledge, this is the first study integrating histomorphological patterns of immune cell infiltration (TILs) and mRNA expression data of immune genes in HNSCC. TILs were analyzed by histomorphological evaluation of digital HE-stained slide images and their spatial distribution was assessed separately for ieTILs and strTILs. Status of ieTILs and strTILs were consistent for the majority of tumors (61%), but different for the remaining 39% of cases. Therefore, in HNSCC, ieTILs and strTILs are non-redundant markers and should be analyzed separately. The abundance of eight types of immune cells and two stromal cell types in the TME was estimated from RNA-Seq data using the bioinformatics method MCP-counter [26]. In particular, the abundance of T cells was estimated as mean value of the expression of sixteen genes including CD3D, CD3G and CTLA4. Additionally, PD-L1 mRNA expression was analyzed.

A higher level of immune activity in HPV + tumors in comparison to HPV− tumors became apparent with a higher number of tumors with ieTILs (77% vs. 58%) and with high T cell levels (69% vs. 46%). In contrast, strTILs levels and PD-L1 mRNA expression were not significantly different between HPV+ and HPV− tumors. The high immune activity in HPV+ tumors is in line with an immune response due to viral infection and was reported before [30–35]. PD-L1 mRNA levels were more often higher in non-smoking patients (59%) compared to smoking patients (46%), while the other immune markers where not associated with smoking history. T cell levels showed a strong association with tumor localization ($p < 0.001$) and had the highest levels in tumors located in the tonsils (83% "T cell high") and at the base of the tongue (71% "T cell high") compared to tumors located elsewhere (less than 56% "T cell high" for all other localizations).

Table 3 Bivariate survival analysis combining one of the immune cell markers (ieTILs, strTILs or T cells) and PD-L1 mRNA level. The multivariate models include correction for age, HPV status, smoking status, tumor stage, tumor grade and tumor localization

	DFS		OS	
Model	Bivariate	Multivariate	Bivariate	Multivariate
ieTILs and PD-L1_mRNA	ieTILs: HR = 0.65 (0.46–0.9), $p = 0.01$ PD-L1: HR = 1.04 (0.9–1.16), $p = 0.41$	ieTILs: HR = 0.76 (0.53–1.1), $p = 0.15$ PD-L1: HR = 1.09 (0.97–1.2), $p = 0.14$	ieTILs: HR = 0.92 (0.69–1.2), $p = 0.56$ PD-L1: HR = 1.03 (0.95–1.1), $p = 0.48$	ieTILs: HR = 1.03 (0.76–1.4), $p = 0.86$ PD-L1: HR = 1.05 (0.96–1.2), $p = 0.31$
strTILs and PD-L1 mRNA	strTILs: HR = 0.87 (0.6–1.2), $p = 0.44$ PD-L1: HR = 1.05 (0.95–1.2), $p = 0.38$	strTILs: HR = 0.87 (0.6–1.25), $p = 0.44$ PD-L1: HR = 1.09 (0.98–1.22), $p = 0.12$	strTILs: HR = 0.8 (0.6–1), $p = 0.12$ PD-L1: HR = 1.04 (0.96–1.1), $p = 0.34$	strTILs: HR = 0.7 (0.53–0.97), $p = 0.03$ PD-L1: HR = 1.07 (0.98–1.18), $p = 0.15$
T cells and PD-L1 mRNA	T cells: HR = 0.75 (0.65–0.88), $p < 0.001$ PD-L1: HR = 1.13 (1.01–1.26), $p = 0.03$	T cells: HR = 0.79 (0.66–0.9), $p = 0.006$ PD-L1: HR = 1.17 (1.04–1.32), $p = 0.01$	T cells: HR = 0.77 (0.68–0.87), $p < 0.001$ PD-L1: HR = 1.12 (1.02–1.23), $p = 0.02$	T cells: HR = 0.78 (0.68–0.9), $p = 0.001$ PD-L1: HR = 1.14 (1.03–1.26), $p = 0.01$

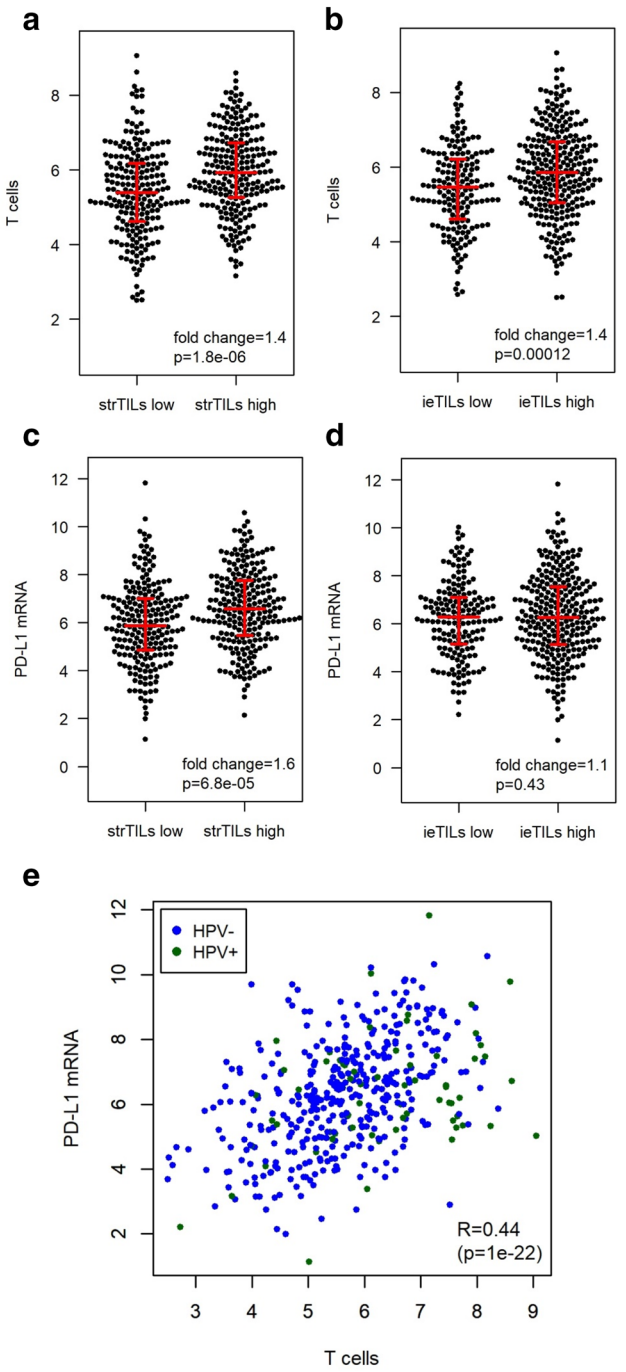

Fig. 4 Relation between TILs, T cell abundance and PD-L1 mRNA expression in HNSCC (TCGA cohort). **a**, **b** T cells were increased when strTILs were high or ieTILs were present. **c**, **d** PD-L1 expression was increased when strTILs were high, but did not depend on the presence of ieTILs. **e** Association of PD-L1 expression with T cell abundance. PD-L1 correlated positively with T cells in HPV− tumors ($R=0.47$), in HPV+ tumors ($R=0.38$) and in all tumors ($R=0.44$). Comparing HPV+ and HPV− tumors, T cells levels were increased (fold change = 1.73, $p=2.7E{-}05$), while PD-L1 mRNA levels were not significantly different

The strength of association with patient survival was compared for the two histomorphological immune markers (ieTILs and strTILs) and for the two mRNA expression-based immune markers (T cells and PD-L1). For all four markers, a dichotomized graduation ("high" vs. "low") was used to facilitate comparison in terms of HRs and *p*-values. In univariate survival analyses, ieTILs and T cells were significant prognostic markers, while strTILs were borderline significant and PD-L1 mRNA levels were not significant at all. Presence of ieTILs was a positive prognostic factor for DFS in the study cohort (HR = 0.66, $p=0.015$) and in the HPV− subcohort (HR = 0.68, $p=0.04$), but not in the HPV+ subcohort. Vice versa, T cells were a positive prognostic factor for DFS in the study cohort (HR = 0.80, $p=0.03$) and in the HPV+ subcohort (HR = 0.20, $p=0.001$), but did not reach significance in the HPV− subcohort. The DFS advantages of tumors with high T cells propagated to OS advantages for both the study cohort and the HPV+ subcohort. The presence of ieTILs was associated with significantly better DFS, but not with OS; while high levels of strTILs were associated borderline significantly with better OS, but not with DFS. This may be related to the different biology of primary, recurrent and metastatic disease as well as different therapy strategies in the adjuvant and the metastatic setting.

The prognostic role of TILs in HNSCC has been widely investigated, reviewed in [36, 37]. While most of the studies used immunohistochemistry (IHC) for TILs evaluation, three studies have evaluated TILs on HE-stained slides only and are therefore comparable to the present study. Two studies have evaluated TILs in the tumor stroma only and found strTILs (defined as ratio of the stromal area covered by TILs to the total stromal area in representative regions of the slides) positively associated with prognosis in HNSCC and in particular in laryngeal carcinoma [38, 39]. In the third study, a three-tier classification based on combined evaluation of tumor and stroma TILs has been shown to be positively prognostic in oropharyngeal SCC [32]. While all these studies agree on a positive influence of TILs on HNSCC prognosis, careful separate evaluation of ieTILs and strTILs was carried out within the current study for the first time.

Histomorphological TIL evaluation represents a cost-effective and straightforward method, which was successfully applied in other cancer entities such as breast cancer [40, 41]. However, in contrast to breast cancer, in our study, ieTIL and strTIL levels were discordant in a large proportion of HNSCC cases and thus represent non-redundant markers in HNSCC. The present study and work by others agree on a prognostic relevance of TILs in HNSCC and the feasibility of TIL evaluation using HE slides. However, methods for TIL evaluation were heterogeneous: Different tumor compartments were evaluated (intra-epithelial and/or stromal TILs), representative areas or the complete slide were taken

into account and different cut-points were used for stratification in "TIL high" vs. "TIL low". Therefore, methods need to be standardized and both ieTILs as well as strTILs should be further analyzed for their prognostic and possibly predictive impact in HNSCC.

In recent years, many studies have investigated the relation of infiltrates of specific immune cell populations to clinical outcome of HNSCC using IHC staining, in which CD3, CD4, CD8 and FOXP3 were the most frequently investigated markers [36, 37]. A recent meta-analysis showed that CD3+ and CD8+ T cell infiltrates represent robust prognostic markers in HNSCC and are associated with favorable survival in HPV− HNSCC as well as HPV+ HNSCC [37]. IHC staining allows identification of specific lymphocytic immune cell populations which is not possible by histomorphological evaluation of HE-stained slides. However, the latter method does not require additional stains and tissue sections and is therefore more cost-effective and easier to implement in routine diagnostics. As studies comparing the predictive value of histomorphological TIL evaluation and IHC markers in HNSCC are lacking, this issue should be addressed in future studies.

We used the bioinformatic method MCP-counter [26] to estimate the abundance of ten cell populations in the TME from gene expression data and found that the levels of T cells were most closely associated with prognosis. T cells in general and—to a slightly weaker extent—CD8+ T cells were associated with favorable DFS and OS in the study cohort. Our results are in line with an earlier report using the bioinformatics method CIBERSORT [42], that also found an association between general T cell infiltration and also more specifically CD8+ T cell infiltration with better survival. CIBERSORT has the advantage to allow estimation of a larger number of specific immune cell populations; while, the MCP-counter method is mathematically simpler and possibly more robust (MCP-counter is based on the expression of marker genes, while CIBERSORT uses machine learning and gene expression signatures). Compared to the earlier report, we had the opportunity to analyze the complete TCGA cohort ($n = 510$ cases with RNA-Seq data available) instead of the subcohort analyzed there ($n = 280$). The larger sample size facilitated stratified analyses by HPV status, in which T cell levels showed an especially high predictive power in the HPV+ subcohort, while association of the T cell levels with survival was not significant in the HPV− subcohort.

In univariate survival analysis, PD-L1 mRNA expression (as dichotomized variable and as continuous variable) was neither predictive of DFS nor of OS in the study cohort. In line with this observation, a recently published meta-analysis did not recommend PD-L1 protein expression for survival prediction in HNSCC patients, because of inconclusive and inconsistent published results in the literature [43, 44]. In the dynamics of tumor progression, expression of the immune checkpoint regulator PD-L1 on tumor cells represents an unfavorable step linked to immune evasion. However, PD-L1 expression is more complex to interpret when analyzing a static snapshot of a tumor such as a tissue sample acquired at a single time point. In the study cohort, PD-L1 mRNA and T cell levels were significantly positively correlated ($R = 0.44$) and in bivariate models including both variables, PD-L1 mRNA levels were a negative prognostic marker of DFS and OS, while T cell levels remained a positive prognostic marker. Moreover, both molecular variables remained prognostic markers of DFS and OS in multivariate models including the two markers and additional clinicopathological parameters (age, HPV status, smoking status, tumor stage, tumor grade and tumor localization). In the meta-analysis of Yang et al. [43] PD-L1 expression was a negative prognostic factor in patients with low CD8+ tumor-infiltrating T cells. In line with this, our results argue that in HNSCC the prognostic relevance of PD-L1 expression should be evaluated together with the overall immunological tumor status including the degree of T cell infiltration.

In summary, levels of ieTILs were associated with favorable outcome in the subcohort of HPV− tumors, while levels of T cells were associated with favorable outcome in the subcohort of HPV+ tumors. PD-L1 expression was not associated with clinical outcome in HNSCC when analyzed alone, but a negative prognostic marker of DFS and OS when analyzed together with T cell levels.

Conclusion

Intra-epithelial and stromal TIL levels represent non-redundant parameters of the immune microenvironment in HNSCC and should be analyzed separately. PD-L1 expression should not be analyzed isolated, but integrated with parameters describing the immunological activity in the tumor. The current study confirms a positive prognostic role of TILs and in particular T cells in conventionally (surgery, radiotherapy and chemotherapy) treated HNSCC. PD-L1 mRNA expression, T cell infiltration, ieTILs and strTILs should be further investigated as possible predictive biomarkers for immunotherapy.

Author contributions JB conceived the study. MB and JB developed the statistical analysis plan. IT and CD contributed the statistical analysis plan. MB and KJ analyzed the images of the histopathological slides. MB and JB analyzed the data. All authors contributed to discussion of the data. MB and JB wrote the manuscript. All authors commented on the manuscript and approved its final version.

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Compliance with ethical standards

Conflict of interest W. Weichert received consultant/advisory board as well as speaker's honoraria from Roche, Merck Sharp & Dohme (MSD), Bristol-Myers Squibb (BMS), AstraZeneca, Pfizer, Merck, Lilly, Boehringer, Novartis and Takeda. He received research funding from Roche, MSD, BMS and Bruker. C. Denkert received consultant/advisory board honoraria from MSD, Amgen and Daiichi Sankyo as well as speaker's honoraria from Teva, Novartis, Pfizer, Roche and Amgen. He has stock and other ownership interests concerning Sividon Diagnostics. P. Schirmacher received consultant/advisory board honoraria from Pfizer, Roche, Novartis and AstraZeneca as well as speaker's honoraria and research funding from Roche, AstraZeneca and Novartis. A. Stenzinger received consultant/advisory board honoraria from AstraZeneca, BMS, Novartis, Thermo Fisher Scientific and Illumina as well as speaker's honoraria from BMS, MSD, Roche, Illumina, AstraZeneca, Novartis and Thermo Fisher as well as research funding from Chugai and BMS. The authors declare that there are no other conflicts of interest.

Ethical approval and ethical standards The TCGA HNSCC data are freely available without restrictions for use in publications.

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