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ORIGINAL RESEARCH

Human papilloma virus load and PD-1/PD-L1, CD8+ and FOXP3 in anal cancer patients treated with chemoradiotherapy: Rationale for immunotherapy

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ABSTRACT

We examined the prognostic role of immune markers programmed cell death protein-1 (PD-1) and its ligand (PD-L1), CD8+ tumor-infiltrating lymphocytes (TILs), FOXP3+ Tregs and phosphorylated Caspase-8 (T273) in patients with anal squamous cell cancer (ASCC) treated with standard chemoradiotherapy (CRT). The baseline immunohistochemical expression of immune markers was correlated with clinicopathologic characteristics, and cumulative incidence of local failure, disease-free survival (DFS) and overall survival (OS) in 150 patients, also in the context of human papilloma virus 16 (HPV16) DNA load and p16^{INK4a} expression. After a median follow-up of 40 mo (1–205 mo), the 5-y cumulative incidence of local failure and DFS was 19.4% and 67.2%, respectively. Strong immune marker expression was significantly more common in tumors with high HPV16 viral load. In multivariant analysis, high CD8+ and PD-1+ TILs expression predicted for improved local control ($p = 0.023$ and $p = 0.007$, respectively) and DFS ($p = 0.020$ and $p = 0.014$, respectively). Also, high p16^{INK4a} ($p = 0.011$) and PD-L1 ($p = 0.033$) expression predicted for better local control, whereas high FOXP3+ Tregs ($p = 0.050$) and phosphorylated Caspase-8 ($p = 0.031$) expression correlated with superior DFS. Female sex and high HPV16 viral load correlated with favorable outcome for all three clinical endpoints. The present data provide, for the first time, robust explanation for the favorable clinical outcome of HPV16-positive ASCC patients harboring strong immune cell infiltration. Our findings are relevant for treatment stratification with immune PD-1/PD-L1 checkpoint inhibitors to complement CRT and should be explored in a clinical trial.

Introduction

Anal squamous cell cancer (ASCC) is a relatively rare malignancy.[1] With standard chemoradiotherapy (CRT),[2-4] complete and durable remission can be achieved in the majority of patients, but local and/or distant relapse still occur in 15–30%. Randomized trials identified T stage, N stage and sex as independent prognostic factors. Better molecular stratification and new therapies beyond standard CRT are needed for treatment escalation and de-escalation strategies.

ASCC mostly arises from infection with high risk oncogenic human papilloma virus (HPV16,18) via inactivation of tumor suppressor proteins TP53 and pRB by the viral oncoproteins E6 and E7. The prevalence of HPV DNA, and its associated surrogate marker p16^{INK4a} (p16), ranges from 85% to 100% in ASCC.[5,6,7] With such high detection rates, a comparison between sole HPV-positive and -negative cancer with respect to oncological outcomes is challenging. In patients with cervical cancer, Kim et al. recently reported that the semi-quantitative measured HPV DNA load was an independent prognostic

factor for disease-free survival (DFS) after radical radiotherapy.[8] We confirmed the prognostic value of a high HPV16 DNA quantitative load as independent prognostic factor for local control after standard CRT for ASCC patients,[9] but the mechanisms behind this phenomenon remain largely unclear.

Programmed cell death protein-1 (PD-1) and its ligand, programmed death ligand 1 (PD-L1) mediate immune tolerance.[10,11] PD-1 is expressed on activated T cells, whereas PD-L1 is found on cancer cells, parenchymal and myeloid cells. Activation of the PD-1/PD-L1 axis leads to tumor-infiltrating lymphocytes (TILs) dysfunction,[10,11] and occurs in several malignancies via either activated oncogenic signaling (innate resistance), or an inflammatory process (adaptive resistance).[10,11,12]

Although few studies have investigated the prognostic impact of CD8+ TILs and FOXP3 Tregs in ASCC,[13,14] the prognostic role and correlation with PD-1+ TILs and PD-L1+ cells in this disease remains unknown. Also, the prognostic role of PD-1/PD-L1 has not been investigated in ASCC. Here, we assessed CD8+, PD-1, PD-L1, FOXP3, pCasp-8 alone, and also

in correlation with HPV16 DNA viral load and p16 in a large patient cohort treated homogeneously with primary CRT.

Results

Immune markers staining characteristics

The results of immune marker scoring, HPV16 viral load, p16 expression in pretreatment biopsies and correlation with clinicopathologic characteristics are shown in Table 1 and Tables S1 and S2. Higher CD8$^+$ TILs expression was more common in patients with early N stage ($p = 0.012$), high HPV16 viral load ($p = 0.043$), and also high expression of PD-1 ($p = 0.016$), PD-L1 ($p = 0.006$), FOXP3 ($p < 0.001$) and pCaspase-8 ($p = 0.039$). High PD-1+ TILs expression correlated with high HPV16 load ($p < 0.001$), high p16 expression ($p = 0.015$), high PD-L1 ($p < 0.001$) and high FOXP3+ Tregs ($p = 0.001$). PD-L1+ cells and FOXP3+ Tregs were found more often in patients with high CD8$^+$ and PD-1+ TILs expression, whereas only FOXP3 correlated with high HPV16 load ($p = 0.006$) and high p16 expression ($p = 0.035$). Representative images of all immune cells and markers with high and low expression is shown in Fig. S1. Additionally, high HPV16 load was more common in females ($p = 0.009$) and in patients with early T stage ($p = 0.017$). The expression of immune cell populations and markers was comparable between HIV-positive and HIV-negative patients (Table S3).

Immune markers and treatment outcome

After a median follow-up of 40 (range, 1–205) mo, locoregional failure occurred in 26 (17.3%) patients, whereas distant metastases were encountered in 17 (11.3%) patients. A total of 36 (24%) patients died during follow-up: 18 (12%) of ASCC, 16 (10.7%) of intercurrent, non-malignant disease and 2 (1.3%) due to treatment-related complications. Complete remission occurred in 123 (82%) patients, 20 (13.3%) had lack of remission. The 5-y and 10-y cumulative incidence of locoregional failure for the entire cohort was 19.4% and 20.9%, respectively. The 5-y and 10-y DFS were 67.2% and 58.3%, respectively. The 5-y and 10-y overall survival (OS) rates amounted to 87.1% and 82.8%, respectively.

In univariant analysis, patients with high total CD8$^+$ TILs expression had a significantly better local control ($p = 0.007$), DFS ($p = 0.008$) and OS ($p = 0.040$) (Fig. 1A; Table 2). Patients with high PD-1+ TILs expression had a significantly better local control ($p = 0.003$), DFS ($p = 0.007$) and OS ($p = 0.039$) (Fig. 1B; Table 2). Similarly, high PD-L1 expression correlated

Table 1. Correlation of CD8$^+$, PD-1 and PD-L1 with clinicopathologic parameters in the entire cohort.

Parameter	No.	CD8 low n (%)	CD8 high n (%)	p value	PD-1 low n (%)	PD-1 high n (%)	p value	PD-L1 low n (%)	PD-L1 high n (%)	p value
Age										
≤ 59 y	75	48 (48)	27 (53)	0.730	38 (49)	37 (51)	1	45 (45)	30 (60)	0.119
> 59 y	75	51 (52)	24 (47)		39 (51)	36 (49)		55 (55)	20 (40)	
Gender										
male	66	46 (46)	20 (39)	0.488	36 (47)	30 (41)	0.514	45 (45)	21 (42)	0.862
female	84	53 (54)	31 (61)		41 (53)	43 (59)		55 (55)	29 (58)	
T-stage										
T1/2	105	66 (66)	40 (78)	0.133	53 (69)	51 (71)	0.859	71 (71)	34 (68)	0.709
T3/T4	45	34 (34)	11 (22)		24 (31)	21 (29)		29 (29)	16 (32)	
N-stage										
N0	97	57 (58)	40 (78)	**0.012**	50 (65)	47 (64)	1.00	64 (64)	33 (66)	0.858
N1–3	53	42 (42)	11 (22)		27 (35)	26 (36)		36 (36)	17 (34)	
Grading										
G1/2	114	72 (76)	42 (82)	0.429	60 (79)	54 (77)	0.881	80 (82)	34 (71)	.232
G3	25	19 (20)	6 (12)		13 (17)	12 (17)		15 (15)	10 (21)	
Gx	7	4(4)	3 (6)		3 (4)	4 (6)		3 (3)	4 (8)	
HPV-16 load										
HPV-16 low	67	51 (56)	16 (36)	**0.043**	47 (68)	20 (30)	**< 0.001**	50 (55)	17 (39)	.098
HPV-16 high	68	40 (44)	28 (64)		22 (32)	46 (70)		41 (45)	27 (61)	
p16^{INK4a}										
p16 low	73	54 (55)	19 (37)	0.058	45 (58)	28 (38)	**0.015**	48 (48)	25 (50)	0.863
p16 high	77	45 (45)	32 (63)		32 (42)	45 (62)		52 (52)	25 (50)	
CD8$^+$										
CD8 low					58 (75)	41 (56)	**0.016**	74 (74)	25 (50)	**.006**
CD8 high					19 (25)	32 (44)		26 (26)	25 (50)	
PD-1										
PD-1 low	77	58 (59)	19 (37)	**0.016**				63 (63)	14 (28)	**< 0.001**
PD-1 high	73	41 (41)	32 (63)					37 (37)	36 (72)	
PD-L1										
PD-L1 low	100	74 (75)	26 (51)	**0.006**	63 (82)	37 (51)	**< 0.001**			
PD-L1 high	50	25 (25)	25 (49)		14 (18)	36 (49)				
FOXP3										
FOXP3 low	77	62 (63)	15 (29)	**< 0.001**	50 (65)	27 (37)	**0.001**	62 (62)	15 (30)	**< 0.001**
FOXP3 high	73	37 (37)	36 (71)		27 (35)	46 (63)		38 (38)	35 (70)	
pCasp-8										
pCasp-8 low	80	59 (60)	21 (41)	**0.039**	46 (60)	34 (47)	0.14	53 (53)	27 (54)	1.00
pCasp-8 high	70	40 (40)	30 (59)		31 (40)	39 (53)		47 (47)	23 (46)	

Abbreviations: HPV, human papilloma virus; pCasp-8: phosphorylated Caspase-8 (pT273).
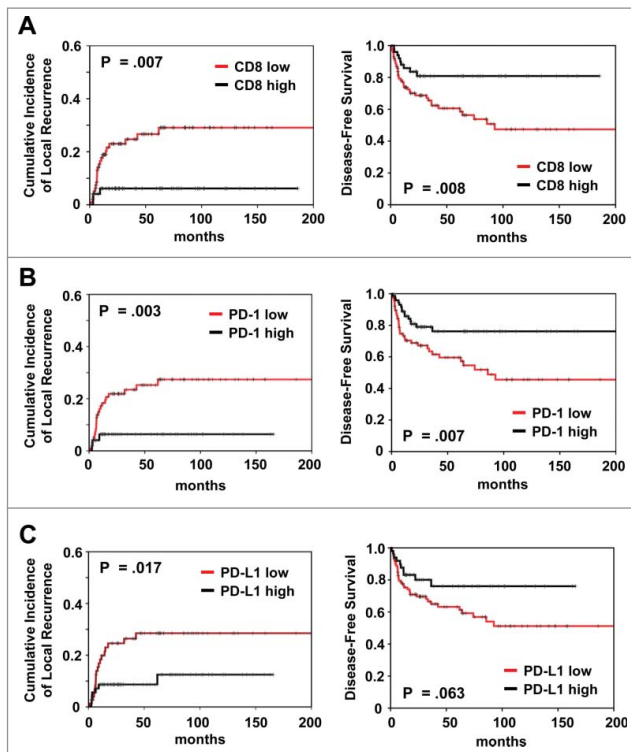Significant p values have been marked with bold.

Figure 1. Prognostic impact of (A) CD8$^+$ and (B) PD-1 and (C) PD-L1 expression on cumulative incidence of local recurrence and disease-free survival, as indicated. Analysis was based on the dichotomized total score in patient tumor samples (cut-off according to median value of total score).

with better local control ($p = 0.017$) (Fig. 1C; Table 2). Also, high FOXP3+ Tregs and intensity of Caspase-8 phosphorylation predicted for improved local control ($p = 0.025$ and $p = 0.013$, respectively) and DFS ($p = 0.013$ and $p = 0.001$, respectively) (Fig. S2; Table S2). Female sex and high HPV16 viral load correlated with better outcome for all three clinical endpoints, whereas high p16 expression only predicted for superior local control. Distant metastasis was more common in patients with advanced T stage ($p < 0.001$). Advanced N stage was associated with worse local control ($p = 0.004$), DFS ($p = 0.010$) and OS ($p = 0.022$).

Subsequently, we conducted a multivariant analysis by including factors that showed significance in univariant analysis (Table 2). Of note, due to multicollinearity and the low number of local failure events ($n = 26$), multivariant analysis was performed separately for each of the immune cell markers, always including the standard clinicopathologic parameters (sex, T/N stage and age, if significant in univariant analysis). In the Cox model, female sex and high HPV16 viral load retained their significance for improved outcome for all three clinical endpoints. High CD8$^+$ and PD-1+ TILs expression predicted for improved local control ($p = 0.023$ and $p = 0.007$, respectively) and DFS ($p = 0.020$ and $p = 0.014$, respectively). Local failure was significantly lower in patients with strong p16 ($p = 0.011$) and PD-L1 ($p = 0.033$) expression. Strong FOXP3+ Tregs ($p = 0.050$) and Caspase-8 phosphorylation ($p = 0.031$) only correlated with better DFS in multivariant analysis.

Table 2. Univariate and multivariate analyses of prognostic factors in patients with ASCC.

Parameter	Univariate	Multivariate		
	p value	HR	95% CI	*p* value
Cumulative incidence of local recurrence				
Age (>/≤ 59 y)	0.710			
Sex (female/male)	**0.004**	0.39	0.16 to 0.96	**0.040**
T-category (T3/4 / T1/2)	**0.010**	1.48	0.59 to 3.72	0.404
N-category (N1–3/N0)	**0.004**	2.29	0.99 to 5.30	0.054
HPV16 load (>/≤ median)*	**0.002**	0.28	0.10 to 0.77	**0.014**
p16 (>/≤ median)*	**0.002**	0.32	0.14 to 0.77	**0.011**
CD8 (>/≤ median)*	**0.007**	0.25	0.07 to 0.83	**0.023**
PD-1 (>/≤ median)*	**0.003**	0.29	0.12 to 0.72	**0.007**
PD-L1 (>/≤ median)*	**0.017**	0.27	0.08 to 0.90	**0.033**
FOXP3 (>/≤median)*	**0.025**	0.44	0.18 to 1.05	0.065
pCasp-8 (>/≤ median)*	**0.013**	0.48	0.20 to 1.17	0.105
Disease-free survival				
Age (>/≤ 59 y)	0.269			
Sex (female/male)	**0.004**	0.44	0.23 to 0.82	**0.010**
T-category (T3/4 / T1/2)	0.060			
N-category (N1–3/N0)	**0.010**	1.56	0.84 to 2.87	0.156
HPV16 load (>/≤ median)*	**0.001**	0.40	0.20 to 0.79	**0.009**
p16 (>/≤ median)*	0.088			
CD8 (>/≤ median)*	**0.008**	0.42	0.20 to 0.87	**0.020**
PD-1 (>/≤ median)*	**0.007**	0.46	0.25 to 0.86	**0.014**
PD-L1 (>/≤ median)*	0.063			
FOXP3 (>/≤median)*	**0.013**	0.54	0.29 to 1.00	**0.050**
pCasp-8 (>/≤ median)*	**0.001**	0.49	0.26 to 0.94	**0.031**
Overall survival				
Age (>/≤ 59 y)	**0.050**	2.32	1.13 to 4.73	**0.021**
Sex (female/male)	**0.002**	0.32	0.15 to 0.68	**0.003**
T-category (T3/4 / T1/2)	**0.012**	1.62	0.81 to 3.24	0.171
N-category (N1–3/N0)	**0.022**	1.11	0.51 to 2.43	0.789
HPV16 load (>/≤ median)*	**0.005**	0.44	0.20 to 0.95	**0.036**
p16 (>/≤ median)*	0.674			
CD8 (>/≤ median)*	**0.040**	0.52	0.23 to 1.19	0.120
PD-1 (>/≤ median)*	**0.039**	0.54	0.26 to 1.10	0.090
PD-L1 (>/≤ median)*	0.250			
FOXP3 (>/≤median)*	0.114			
pCasp-8 (>/≤ median)*	**0.011**	0.51	0.24 to 1.07	0.073

*Due to multicollinearity for HPV16, p16, CD8, PD-1, PD-L1, FOXP3 and caspase-8, and a limited number of events, multivariate analyses were performed separately including only one molecular parameter in conjunction with sex, T- and N-category each time. Only parameters found to be significant in the univariate analysis were included in the multivariate one. HRs and CIs originate from the respective, separate multivariate analysis.
Significant *p* values have been marked with bold.

Moreover, we assessed the prognostic impact of CD8$^+$ TILs, PD-1+ TILs and FOXP3+ Tregs according to tumor compartment (intraepithelial and stromal; Table S4; Fig. S3). High intraepithelial but not stromal CD8$^+$ and PD-1+ TILs expression predicted for superior local control ($p = 0.005$ and $p = 0.005$, respectively), DFS ($p = 0.013$ and $p = 0.002$, respectively), and OS ($p = 0.041$ and $p = 0.030$, respectively). Improved local control ($p = 0.031$) and DFS ($p = 0.020$) was observed for stromal rather than intraepithelial compartment FOXP3+ Tregs expression (Table S4).

We also investigated the prognostic impact of CD8^{high}/PD-L1^{high} vs. CD8^{low}/PD-L1^{low} vs. CD8^{high}/PD-L1^{low} vs. CD8^{low}/PD-L1^{high})[15,16] based on both, the total CD8$^+$ and PD-L1 score (Fig. 2A). Patients with CD8^{high}/PD-L1^{low} expression had both better local control ($p = 0.002$) and better DFS ($p = 0.022$) for the comparison between all groups. Similar effects were observed for the combined CD8$^+$/PD-1 expression (Fig. 2B).
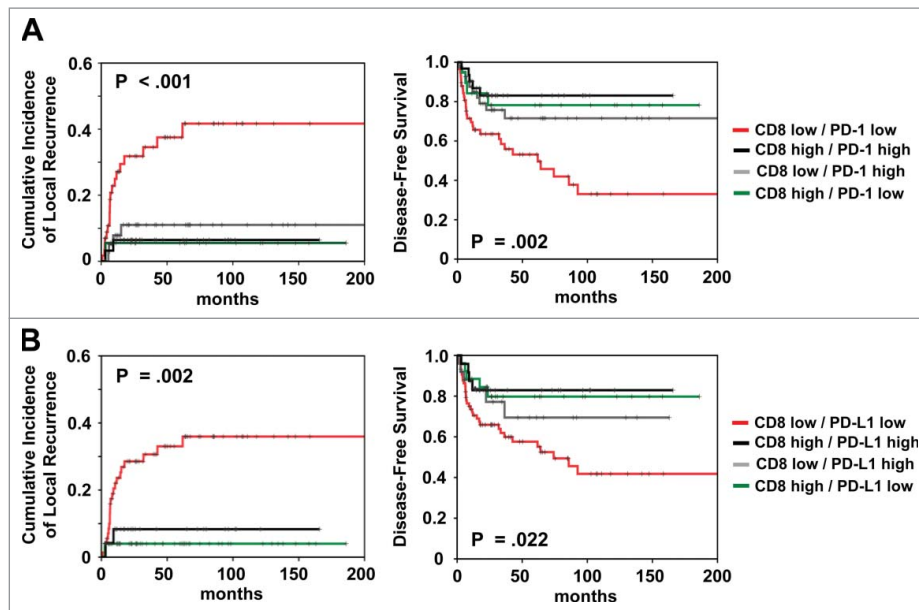
Figure 2. Prognostic impact of (A) combined total CD8$^+$/PD-1 and (B) combined CD8$^+$/PD-L1 expression on cumulative incidence of local recurrence and disease-free survival, as indicated. Analysis was based on the dichotomized total score in patient tumor samples (cut-off according to median value of total score).

The correlation of immune markers with HPV

Patients with high HPV16 viral load presented a significantly improved outcome compared with patients with low load in univariant analysis (cumulative incidence of local recurrence, $p = 0.002$; DFS, $p = 0.001$ and OS, $p = 0.005$) (Fig. 3; Table 2). High p16 expression correlated significantly with better local control ($p = 0.002$) but not DFS ($p = 0.088$).

Because HPV is directly linked with tumor immunogenicity, we analyzed the prognostic impact of CD8$^+$ combined with HPV16 viral load and p16 (Fig. 4; Fig. S4). Patients with HPV16^{high}/CD8^{high} and p16^{high}/CD8^{high} tumors had superior local control ($p = 0.003$ and $p = 0.002$, respectively). Regarding DFS, patients with high HPV viral load and p16 expression presented a favorable outcome ($p = 0.001$ and $p = 0.029$, respectively), both in case of low and high CD8$^+$ TILs expression. Similar data were observed for the combination of HPV16 and p16 with PD-1, PD-L1 and FOXP3 expression (Fig. 4; Fig. S4).

Discussion

Patients with HPV16/p16 positive ASCC have a more favorable outcome with better response to CRT compared with HPV16/p16-negative patients.[17,18] Several mechanisms, including impaired repair of DNA damage, cell cycle arrest and upregulation of TP53 have been proposed to explain the better outcome after CRT.[19] The classical "5 Rs" of radiobiology[20] cannot explain, why ASCC often show a delayed response over a period of months after completion of CRT.[4] It becomes increasingly recognized that the higher immunogenicity of HPV-positive tumors might determine the improved response. Also, radiotherapy has profound immunomodulatory effects and can, via induction of an immunogenic cancer cell death activate cytotoxic T cells.[21] Therefore, the slow regression often observed in ASCC might represent an activated immune response that unfolds progressively over a period of weeks after completion of CRT.

The role of the immune system in ASCC, however, remains largely unexplored.[22] In that context, we demonstrated a strong association between high HPV16 viral load and high CD8$^+$ and PD-1+ TILs expression in our series, supporting the notion that HPV can render tumors more immunogenic.[22,23] Also, phosphorylated Caspase-8, a marker of apoptosis[23] that is closely linked to immunogenic cell death,[24] correlated linearly with HPV16 viral load and CD8$^+$ TILs infiltration.

Although few studies have examined the impact of TILs and Tregs in human ASCC,[13,14] the prognostic value of PD-1 and PD-L1, and their association with TILs and Tregs remain unknown. Here, patients with strong CD8$^+$ TILs and PD-1+
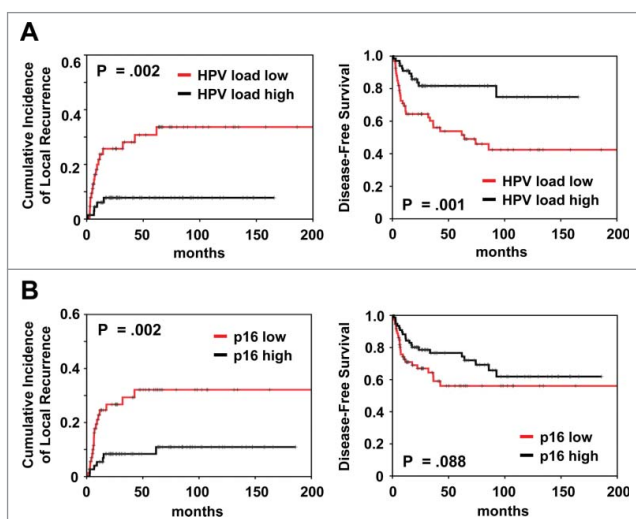

Figure 3. Prognostic impact of (A) HPV viral load and (B) p16 expression on cumulative incidence of local recurrence and disease-free survival, as indicated. Analysis was based on the dichotomized total score in patient tumor samples (cut-off according to median value).
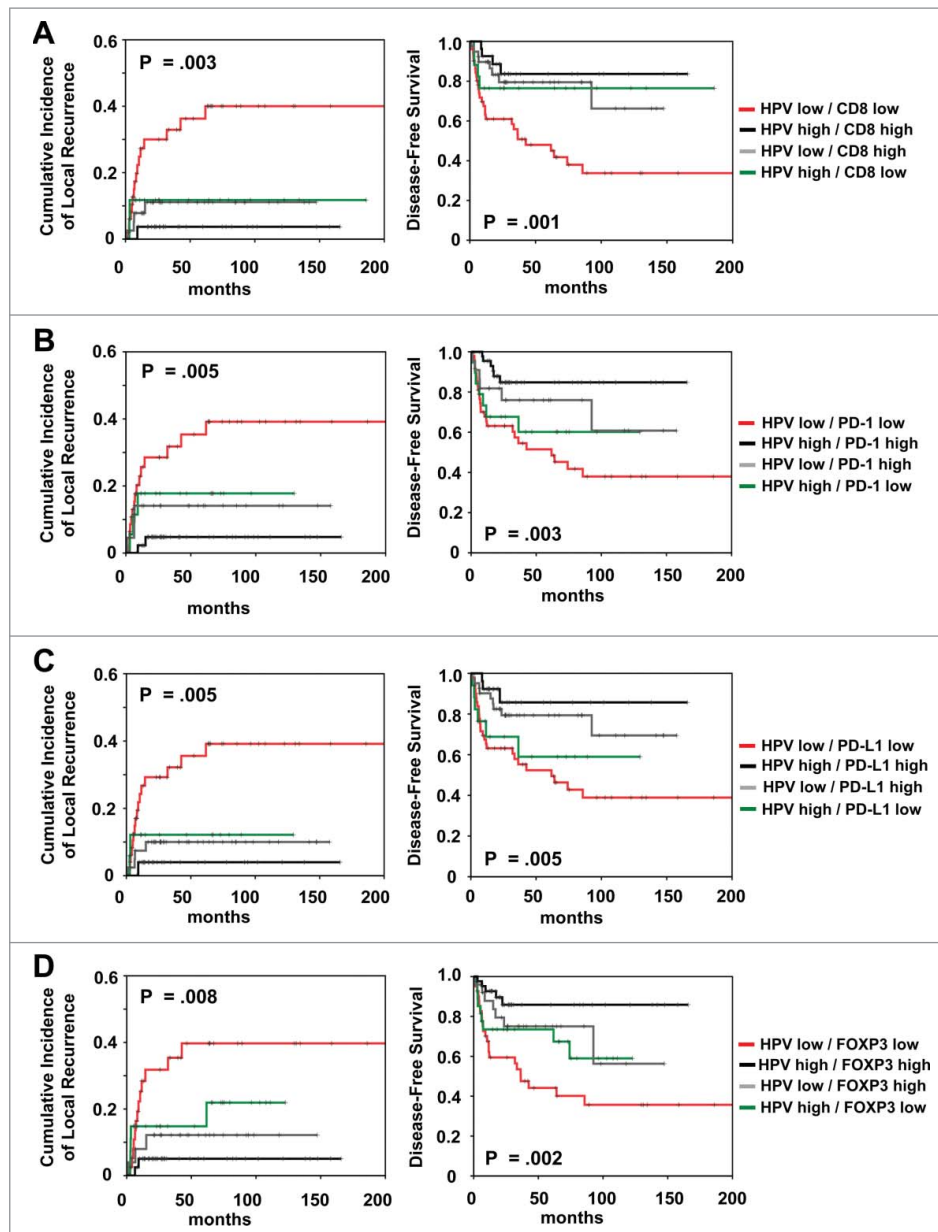
Figure 4. Prognostic impact of (A) combined HPV viral load/CD8$^+$ expression and (B) combined HPV viral load/PD-1 expression and (C) combined HPV viral load/PD-L1 expression and (D) combined HPV viral load/FOXP3 expression on cumulative incidence of local recurrence and disease-free survival, as indicated. Analysis was based on the dichotomized total score in patient tumor samples (cut-off according to median value of total score).

TILs infiltration had a significantly superior local control and DFS compared with patients with low expression. Similar findings were observed for PD-L1 positivity and FOXP3+ Tregs for local recurrence and DFS, respectively. Accumulating evidence highlights the importance of TILs in mediating response to RT/CRT.[25] CD8$^+$ binds to the major histocompatibility complex class I molecule together with the T-cell receptor to elicit the cytotoxic effect of TILs on cancer cells.[26,27] In agreement with our findings, numerous reports have revealed superior clinical outcomes in patients with high CD8$^+$ TILs expression in esophageal, colorectal, head and neck, breast, ovarian, renal, pancreatic and lung cancer,[28,29] whereas only two studies have confirmed the favorable prognostic role of TILs in ASCC.[13,30]

Strong intratumoral FOXP3+ Tregs infiltration was associated with HPV16-positivity and better local control in our

work. Tregs can promote immune evasion but also exert anti-inflammatory effects that limit tumor progression.[31] A meta-analysis in over 15,000 patients showed that high FOXP3+ Tregs expression predicted for worse survival in breast, cervical, melanoma and renal cancers, but better outcome in colorectal, esophageal and oropharyngeal cancers.[32] Several groups have found higher Tregs infiltration in HPV-positive oropharyngeal cancer,[33-35] whereas reports on the correlation of Tregs and HPV in ASCC are lacking. A plausible explanation for the unexpected positive role of Tregs in our analysis may also rely on the co-infiltration with effector T cells.

Regarding PD-1+ TILs, our data are in agreement with previous work demonstrating a positive prognostic impact for these cells in head and neck, ovarian cancer, pancreatic and colorectal cancer, among others.[36-41] Albeit surprising due to its immunosuppressive function, several mechanisms could

explain the paradox of better outcome in patients with PD-1+ TILs. Antigen-specific immune activation following T-cell receptor stimulation can lead to upregulation of PD-1 on TILs.[42] Badoual et al. observed upregulation of the immune activation markers HLA-DR and CD38 in PD-1+ TILs as compared with PD-1- TILs. Thus, PD-1+ TILs could represent a previous endogenous antitumor immune response that decelerated tumor growth, albeit it failed to induce regression due to TILs dysfunction.[11]

The prognostic impact of CD8+ and PD-1+ TILs is tumor compartment-dependent.[15] Indeed, high intratumoral but not stromal compartment CD8+ and TILs and PD-1+ TILs predicted for better local control and DFS in our cohort. Mixed findings have been reported regarding the clinical impact of TILs according to the tumor compartment in different malignancies that could be attributed to the heterogeneous patient cohorts and treatments.[43-47]

This analysis also provides more insight into other aspects of ASCC. In accordance to the literature,[4] HIV-positivity was more common in young males, whereas we failed to detect a difference in immune marker expression according to HIV status. The comparable response and survival between HIV-positive and HIV-negative patients with ASCC indicates that antiretroviral treatment facilitates adequate immune response in this group.[4] Also, consistent with numerous reports, female sex predicted for superior outcome in our series. Interestingly, female sex was strongly correlated with a higher HPV-16 viral load that could explain the well-known clinical observation of favorable prognosis in female patients with ASCC, at least in part.

A four group classification according to the combined TILs/PD-L1 status has been proposed in melanoma to guide future immunotherapy decisions.[15,16,48] These included type I (TILs^{high}/PD-L1^{high} mediating adaptive immune resistance), type II (TILs^{low}/PD-L1^{low} mediating immunologic ignorance), type III (TILs^{low}/PD-L1^{high} mediating intrinsic induction) and type IV (TILs^{high}/PD-L1^{low} mediating tolerance).[15,16] In our cohort, type I ASCC (CD8^{high}/PD-L1^{high}) showed better local control and DFS compared with the other groups. Similar findings were observed for CD8^{high}/PD-1^{high} tumors. Examination of the immuno-genomic properties in the whole Cancer Genome Atlas ($n = 9677$ cases) revealed a strong association between type I tumors and high mutational burden[23] that correlates with better response to immunotherapies.[49] Indeed, melanoma responders to immune checkpoint inhibitors had higher baseline expression of CD8+ and PD-1+ TILs and PD-L1 upregulation due to release of IFNγ by PD-1+ TILs.[11] Such adaptive immune resistance have also been reported in breast cancer[50] and Merkel cell carcinoma.[51] The efficacy of immune checkpoint inhibitors appears to be higher in patients with pre-existing immunity suppressed by the PD-1/PD-L1 pathway that can be reinvigorated with these drugs.[38] Our analysis thus provides an important framework for future combination strategies with CRT in ASCC since type I malignancies are most likely to benefit from immune checkpoint blockade.

Our work has limitations. First, the retrospective design of this analysis could have resulted in selection bias. Second, the number of events observed was small that limited multivariant analyses. Third, the median follow-up was relatively short.

Clearly, our findings warrant validation in other data sets and possibly in a prospective cohort.

In conclusion, we here showed that high tumor HPV16 viral load and associated infiltration with CD8+ and PD-1+ TILs may, in conjunction with PD-L1, identify patients with favorable prognosis after standard CRT for ASCC. The response to CRT can potentially be further enhanced by immune checkpoint inhibitors as these agents appear to be more effective in patients with immunogenic tumors, and high CD8+ and PD-1+ TILs expression in baseline. In that context, nivolumab a monoclonal antibody against PD-1, demonstrated impressive response rates in 37 heavily pre-treated patients with PD-L1+ metastatic ASCC.[52] Our data provide a strong rationale for testing the efficacy of immune checkpoint inhibitors or other forms of immunotherapy with CRT to improve the outcome in patients with ASCC.

Patients and methods

Patient and treatment characteristics

In total, 150 patients were treated with primary CRT for ASCC at the Departments of Radiotherapy at the University Hospital of Frankfurt/Main and at the University Medical Center of Göttingen. Written consent and approval from the institutional review board had been previously obtained, also in accordance with the Helsinki Declaration of 1975. The eligibility criteria for this analysis were histological confirmation of anal SCC, curative intent of 5-fluorouracil (5-FU)/mitomycin C-based CRT and lack of previous malignancies. As part of disease staging, patients received clinical examination, CT/MRI of the abdomen and pelvis, chest X-ray, proctoscopy with biopsy, complete blood count and serum chemistry.

Radiotherapy was applied using linear accelerators (Elekta, Crowley, UK; Varian, Palo Alto, USA) with either 3-D conformal RT or intensity-modulated RT with a median dose of 53.4 Gy (range 46.8–64.8 Gy, including the boost) using daily fractions of 1.8–2 Gy. Two patients received a brachytherapy-boost of 10 Gy. An external boost to the primary tumor and/or enlarged lymph nodes was applied in 71 patients using a median dose of 7.2 Gy (range 3.6–19.8 Gy). Chemotherapy consisted of two cycles of 5-fluorouracil (1.000 mg/m^2/24 h) either as 4- or 5-d continuous infusion in the first and fifth week of RT, whereas mitomycin C (10 mg/m^2) was applied as intravenous bolus on day one of each cycle.

Follow-up examination

Patients were initially assessed 8–10 weeks after completion of therapy and thereafter every 3 mo for the first 2 y followed by 6-mo intervals. Follow-up examination included rectal-digital examination, proctoscopy (with biopsies taken in case of suspicious residual tumor), and pelvic CT/MRI-scan.

Immunohistochemical staining and scoring of immune cell populations and markers

Slides from 150 patients were subjected to an automatic staining procedure with standardized DAKO EnVision^{TM} FLEX

Peroxidase Blocking reagent (K8000, DAKO, Hamburg, Germany) on a DAKO Autostainer Link 48 (DAKO). Antigen retrieval was performed via pretreatment of the paraffin sections (SuperFrost Plus, Thermo Scientific) using either an Epitope Retrieval Solution (Trilog, Cell Marque, Rocklin, CA) or Citrate Buffer 6.0 (Abcam) for 20 min. Slides were stained with the primary antibodies for either CD8$^+$ (1:100, clone C8/144B; Dako M7103), FOXP3 (1:300, clone 236A/E7; Abcam AB20034), PD-1 (1:100, clone NAT105; Abcam AB52587), PD-L1 (1:50, clone E1L3N(R); Cell Signaling Technology) and T273 phosphorylated Caspase-8 antibodies23 for 120 min at room temperature. Following this, dextran polymer conjugated horseradish peroxidase and 3,3′-diamino-benzidine (DAB) chromogen or LSAB Detection system (PD-1, K5005, Dako) was used for visualization of the epitope–antibody reaction product and hematoxylin solution (Gill 3, Sigma Aldrich, Munich, Germany) for counterstaining. Negative control slides in the absence of primary antibodies were included. The expression of CD8$^+$ TILs, PD-1+ TILs, PD-L1 tumor (and myeloid cells) and FOXP3+ Tregs was scored semi-quantitatively via measurement of cell density as described before.[43] Scoring was as follows: (1) no, or sporadic cells; (2) moderate numbers of cells; (3) abundant occurrence of cells and (4) highly abundant occurrence of cells. Cell density was assessed in both the intra-epithelial compartment and stromal compartment. The total score was calculated by adding the separate scores from both compartments (range, 2–8). The median score was used as cut-off to classify patients into two groups: low or high CD8$^+$, PD-1+, PD-L1 and FOXP3+ cells expression. We did not score PD-L1 separately in the intra-epithelial and stromal compartment. Analysis of intensity Caspase-8 T273 phosphorylation as well as PCR-based HPV16 viral load detection and histochemical p16^{INK4a} (CINtec histology Kit, Roche) expression were reported in detail before.[9,53] Images were acquired with the AxioImager Z1 microscope using the Axiovision 4.6 software (Zeiss, Germany). To minimize interobserver variability, two investigators (PB and FR) without knowledge of the clinicopathologic data performed scoring. In cases of discrepancy, a final decision was made after additional examination of the specimens.

Statistical analysis

The Spearman's coefficient assessed the correlation between the different parameters. The cumulative incidence of locoregional failure was calculated from the beginning of CRT to non-complete response at restaging or locoregional tumor detection after initial complete response. Data from patients, who were alive and free of recurrences or who died without having a recurrence were censored for these endpoints. DFS was measured from the beginning of CRT to the day of locoregional failure or distant recurrence, or death from any cause. OS was calculated from the beginning of CRT to death for any reasons or to cancer-related death, or the day of the last follow-up. The Kaplan–Meier method was used to plot the clinical endpoints. Univariable and multivariant analyses were conducted using the log-rank test and the Cox proportional hazard model, respectively. Due to multicollinearity for HPV16, p16, CD8$^+$, PD-1, PD-L1, FOXP3 and pCaspase-8, and a limited number of events, multivariable

analyses were performed separately for each immune marker, including only one each time, in conjunction with sex, T- and N-stage. Only parameters found to be significant in the univariant analysis were included in the multivariable one. A $p < 0.05$ was considered statistically significant. Statistical analyses were performed using the IBM SPSS Version 21.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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