Expression of immune response biomarkers (PD-L1, p16, CD3+ and CD8+ TILs) in recurrent head and neck squamous cell carcinoma within previously irradiated areas

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Abstract. The immune landscape of head and neck squamous cell carcinoma in pretreated areas remains poorly documented. We aimed to assess the tumor microenvironment for biomarkers of antitumor immune responses in tumors in previously irradiated areas compared with *de novo* tumors. This retrospective monocentric study analyzed 100 paraffin-embedded surgical samples of invasive head and neck squamous cell carcinoma (oral cavity, oropharynx, larynx, hypopharynx) from patients who underwent surgery between January 2010 and November 2017. We compared the immune microenvironment in 50 *de novo* tumors and 50 tumors recurring within irradiated areas. We used immunohistochemistry to assess p16 status, CD3+/CD8+ tumor-infiltrating lymphocytes (TILs), and programmed death-ligand 1 (PD-L1) expression on tumor and immune cells in stromal and intratumoral components. CD3+ TIL counts were significantly lower in intratumoral and stromal components (P=0.003 and P=0.020, respectively) in the irradiated area cohort; there was no significant difference between CD8+ TIL counts in the two cohorts. The percentage of tumors with PD-L1+ tumor cells (tumor proportion score ≥1%) was significantly lower within the irradiated area cohort than the *de novo* cohort (56.0% vs. 86.0%, P<0.001). There were also significantly fewer tumors with PD-L1+ immune cells in the irradiated area cohort. Predominantly, tumors from the irradiated area cohort had microenvironments classified as 'adaptive immune resistance'. There was persistence of cytotoxic cells in tumors in the irradiated areas but lower PD-L1 expression and CD3+ TIL counts than in the *de novo* tumors. This offers an initial hypothesis to explain why these lesions are less responsive to immunotherapy, even though they may still have antitumor capacities. Assessment of immune response biomarkers in patients treated with immunotherapy in randomized trials is required.

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Abbreviations: HNSCC, head and neck squamous cell carcinoma; HPV, human papillomavirus; ICIs, immune checkpoint inhibitors; PD-L1, programmed death-ligand 1; TILs, tumor-infiltrating lymphocytes; TPS, tumor proportion score

Key words: head and neck cancer, squamous cell carcinoma, irradiated area, tumor microenvironment, radiotherapy, tumor-infiltrating lymphocytes, TIL, programmed death-ligand 1, PD-L1, human papillomavirus

Introduction

Recurrent head and neck squamous cell carcinoma (HNSCC) in previously irradiated areas and not accessible to local treatment usually have a poor prognosis, with a median overall survival of less than one year in the first-line setting (1-4).

Few therapeutic options are available, mostly as patients that have previously received irradiation are unsuitable candidates for salvage surgery, and often have unstable overall conditions (5). Today, salvage surgery remains the standard of care for selected operable and fit patients, but leads to a high rate of both local and regional failures (1,4).

The recent watershed of immunotherapy, particularly immune checkpoint inhibitors (ICIs), has provided renewed hope for recurrent and metastatic HNSCC; nivolumab treatment has resulted in a 30% reduction in risk of death (CheckMate 141 trial) (3,6) and pembrolizumab treatment in a 20% reduction (KEYNOTE-040 trial) (6,7). ICI use in patients with locoregional recurrence seems to be less satisfying than for metastases (3,7). Inhibition of the programmed death-1/programmed death-ligand 1 (PD-1/PD-L1) pathway by these antibodies and the presence of CD8+ tumor-infiltrating

lymphocytes (TILs) are of the greatest importance in terms of mounting an antitumor immune response (8).

Yet, the immune landscape of pretreated areas remains unclear, mainly because none of the randomized trials assessing the efficacy of ICIs stratified patients according to whether they had previously received radiation or not. Some investigators have already described the complexity of radio-induced alterations in growth factors and proinflammatory, profibrotic, and proangiogenic cytokines (9,10). It is well known that radiotherapy increases the mutational load and improves response to ICIs (11-13). Any changes in the frequency of $CD3^+$ and $CD8^+$ TILs or changes in the expression of PD-L1 by tumor and immune cells remain to be determined and if numbers or expression appear to be reduced, this could support the strategy for introducing an ICI earlier in future therapeutic approaches, as is being assessed in first-line recurrent or metastatic HNSCC and current studies KEYNOTE-048 (14) , KEYNOTE-689 (Clinical Trial NCT03765918), PembroRad (Clinical Trial NCT02707588), KEYNOTE-412 (Clinical Trial NCT03040999), and REACH trial (Clinical Trial NCT02999087).

We focused on HNSCC locoregional recurrences as a recent study found a higher rate of hyperprogression following initiation of an ICI in patients with locoregional recurrence (with or without distant metastases) than in patients with only distant metastases (15), although this may be lower and more variable among solid tumors (16). We aimed to assess whether there is a difference in the expression of antitumor immune response biomarkers in the tumor microenvironment of *de novo* tumors and tumors in irradiated areas.

Materials and methods

Study design and patients. A total of 100 HNSCC tumor tissue specimens from patients who had undergone surgery from January 2010 to November 2017 were analyzed and divided into two cohorts: 50 *de novo* tumors and 50 tumors recurring within previously irradiated areas. The samples in the irradiated area came either from local recurrences at the initial tumor site, or from neck metastases or from second head and neck squamous cell carcinoma. Samples were included if they were paraffin-embedded surgical samples of invasive squamous cell carcinoma, from four locations (oral cavity, oropharynx, larynx, hypopharynx). Samples were excluded if they displayed other histology, carcinoma *in situ*, cancer at stage T1N0M0, tumors from the nasal or paranasal cavity or the nasopharynx, or if they were frozen tissue samples. All cases were recorded at the Lorraine Institute of Oncology and tissue specimens were obtained from the tumor bank of the Institute (tumor bank certification FR17/81842500; norm NF S96-900). According to French regulations, patients were informed of the research performed with tumor tissue specimens and did not object. We compared two parallel cohorts, *de novo* vs. pretreated (irradiated) area.

Histological assessment. Formalin-fixed and paraffin-embedded tumor tissue samples stained by hematoxylin, eosin, and saffron were reviewed by an experienced pathologist to check the quality and the representativity of the specimens selected for supplementary analysis with immunohistochemistry (IHC). IHC preparations were analyzed under microscopic examination. After hematoxylin counterstaining, tumor cells were differentiated from lymphocytes using morphologic criteria.

Immunohistochemical analysis. We used four monoclonal antibodies: Anti-PD-L1 ones, based on previous assessments of PD-L1 expression (17) (SP263, Ventana Medical Systems), anti-CD3 (SP7, Thermo Fisher Scientific, Inc.), anti-CD8 (C8/144B, Agilent Dako) and anti-p16 [anti-p16INK4a (E6H4), Ventana Medical Systems Inc.].

P16 status was assessed as a surrogate marker of human papillomavirus (HPV) association. Threshold positivity was at least 70% of tumor cells stained with at least moderate to strong and nuclear and cytoplasmic staining (18). The density of $CD3^+$ and $CD8^+$ TILs were measured quantitatively by counting each $CD3^+$ or $CD8^+$ cell in intratumoral and stromal compartments separately on photographs at magnification, x20. Values were dichotomized (low/high) according to the median cell number calculated from all samples and considered high if >40 $CD3^+$ cells and >30 $CD8^+$ cells were observed in intratumoral regions and if >160 $CD3^+$ cells and >90 $CD8^+$ cells were observed in stromal regions. PD-L1 IHC expression, defined as membranous and cytoplasmic staining, was assessed on both tumor cells and immune cells. For tumor cells, threshold positivity was a tumor proportion score (TPS) ≥1%, as previously described (19,20). For immune cells, semi-quantitative assessment was carried out in both tumoral (categorized into three classes scored from 0 to 2) and stromal regions (categorized into six classes scored from 0 to 5). The expression of PD-L1 by immune cells was considered high with a score of 1-2 in intratumoral components and 2-5 in stromal components.

We also characterized the immune phenotype of samples of each cohort into four types as described by Teng *et al* based on TIL counts and expression of PD-L1 by tumor cells: Type I (adaptive immune resistance: $PD\text{-}L1^+$/TILs high), type II (immunological ignorance: PD-L1-/TILs low), type III (intrinsic induction: $PD\text{-}L1^+$/TILs low) and type IV (immune tolerance: $PD\text{-}L1^-$/TILs high) (21). We considered TPS to be positive at ≥1% and $CD8^+$ TIL numbers to be high when they were greater than or equal to the median cell number. Fig. 1 presents representative microphotographs of immunohistochemical staining.

Statistical analysis. Quantitative parameters were described as median and interquartile range or as mean and standard deviation according to the normality of the distribution assessed by the Shapiro-Wilk test; qualitative parameters as frequency and percentage. The two groups were compared by the Chi-square test or Fisher's exact test for qualitative parameters; for quantitative parameters, the Student's t-test was performed in case of normal distribution and the Mann-Whitney U test in the other cases. Each quantitative value of immune response biomarkers was dichotomized according to its median value.

In order to adjust the comparisons of immune response biomarkers on confounding factors, the propensity score was computed with the two groups as dependent parameters, and with patient and tumor characteristics with a P-value <0.2 for the comparisons of the two groups. The inverse probability of treatment weighting (IPTW) was computed (22) and immune response biomarkers were compared by weighting analyses.

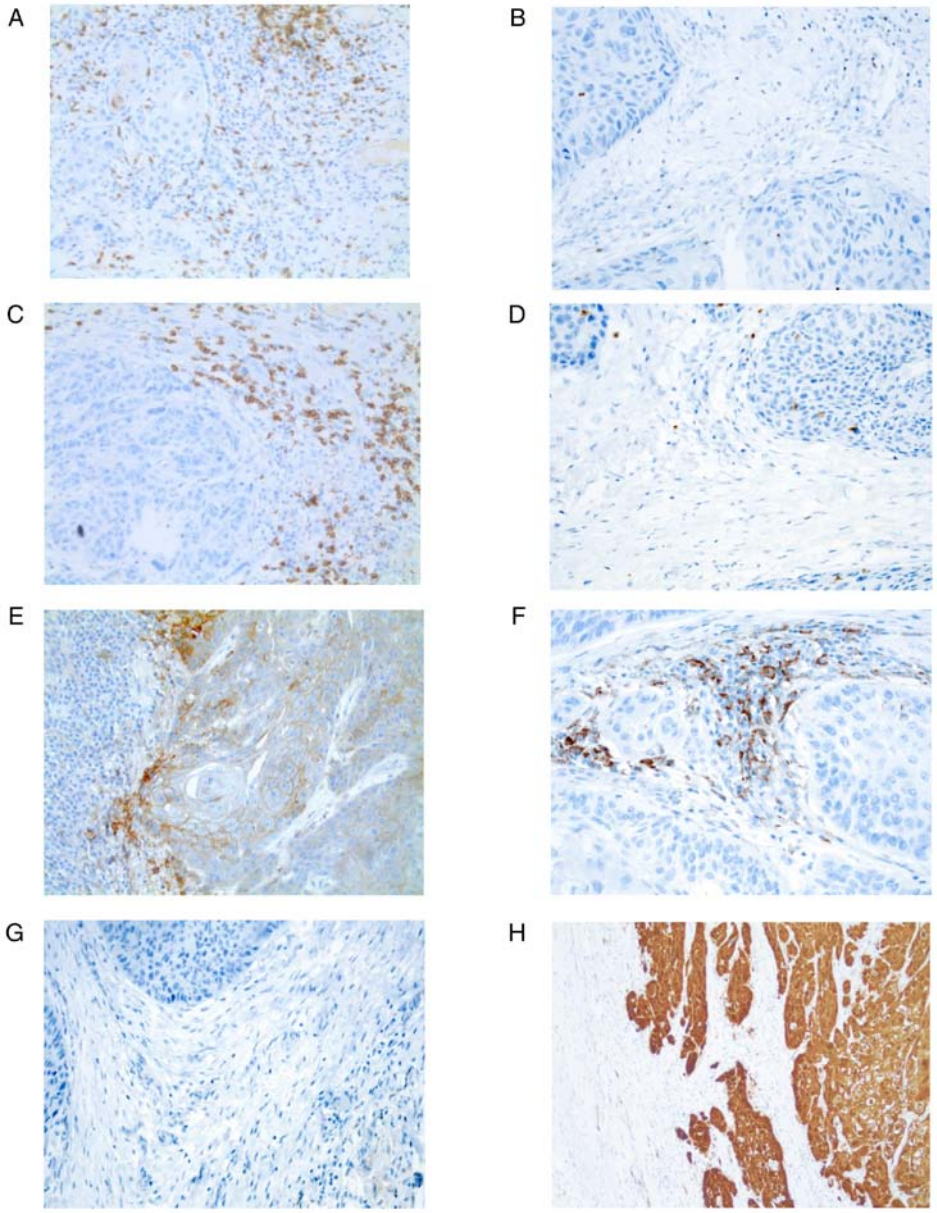

Figure 1. Representative microphotographs of immunohistochemical staining of CD3, CD8, PD-L1 on tumor cells and immune cells and p16 in the irradiated area cohort (magnification, x20). (A) High $CD3^+$ TIL count, (B) low $CD3^+$ TIL count, (C) high $CD8^+$ TIL count, (D) low $CD8^+$ TIL count, (E) $PD\text{-}L1^+$ tumor cells and $PD\text{-}L1^-$ immune cells (F) $PD\text{-}L1^-$ tumor cells and $PD\text{-}L1^+$ immune cells, (G) $PD\text{-}L1^-$ tumor cells and $PD\text{-}L1^-$ IC, and (H) positive P16 status. TIL, tumor-infiltrating lymphocyte; PD-L1, programmed death-ligand 1.

Progression-free survival (PFS) was defined as the time between surgery and relapse, progression, or death (whichever occurred first).

In each group, the prognostic factors of PFS were investigated according to the following process. First, bivariate analyses were performed with the Cox proportional hazard model. Parameters with a P-value <0.1 were introduced in a multivariate Cox proportional hazard model with backward selection. Results are presented as hazard ratio (HR) and its 95% confidence interval (CI). The statistical analyses were performed with SAS, version 9.4 (SAS Institute Inc.) and the significance level was set at 0.05.

Results

Patient and tumor characteristics. Table I presents the patient and tumor characteristics. Mean age was 64.2±8.3 years in the *de novo* cohort and 65.8±10.7 years in the irradiated area cohort (P=0.457). The two groups were well-balanced according to the following criteria: Sex (P=0.806), tumor location (P=0.743), and disease stage (P=0.873). Oropharyngeal tumors exhibited significantly greater p16 expression in the *de novo* cohort (n=7, 43.7%) than in the irradiated area cohort (n=2, 10.5%) (P=0.025). Tumors that had developed in irradiated areas were histologically less differentiated than the *de novo* tumors [n=32 (64.0%) vs. n=37 (74.0%) were well differentiated; P=0.051], and were more frequently stage pNx/N0/N1 [n=38 (76.0%) vs. n=26 (52.0%); P=0.012]. Resection margins were significantly more positive [n=23 (46.0%) vs. n=11 (22.0%); P=0.011]. Lymphovascular [n=21 (58.3%) vs. n=13 (30.2%); P=0.012] or perineural invasion [n=28 (73.7%) vs. n=23 (53.5%); P=0.060] were more frequently observed in the tumors that had developed in irradiated areas.

Table I. Patient and tumor characteristics.

Characteristics	*De novo* (n=50)	Irradiated area (n=50)	P-value
Age (years)	62.1; 64.2±8.3	64.9; 65.8±10.7	0.457[c]
Sex, male	40 (80.0)	39 (78.0)	0.806[d]
Smoking			
Never smoker	7 (14.2)	4 (8.3)	0.289[d]
Current smoker	21 (42.9)	16 (33.2)	
Former smoker	21 (42.9)	28 (58.4)	
Smoking history (pack*year)	40.0; 41.1±15.3	40.0; 42.2±17.8	0.964[c]
Alcohol			
Yes	27 (55.1)	31 (68.9)	0.170[d]
No	22 (44.9)	14 (31.1)	
Former drinker	9 (33.3)	11 (36.7)	0.792[d]
WHO performance status			
0	28 (56.0)	22 (44.0)	0.453[e]
1	21 (42.0)	26 (52.0)	
2	1 (2.0)	2 (4.0)	
Tumor location[a]			
Oral cavity	20 (40.0)	15 (31.9)	0.743[d]
Oropharynx	16 (32.0)	19 (40.4)	
Larynx	6 (12.0)	7 (14.9)	
Hypopharynx	8 (16.0)	6 (12.8)	
Status P16+ (>70%)[b]	7 (43.7)	2 (10.5)	0.025[e]
Differentiation			
Well differentiated	37 (74.0)	32 (64.0)	0.051[d]
Moderately/poorly	5 (10.0)	14 (28.0)	
Undifferentiated	8 (16.0)	4 (8.0)	
pT stage			
Tx-T1-T2	35 (70.0)	28 (56.0)	0.147[d]
T3-T4	15 (30.0)	22 (44.0)	
pN stage			
Nx-N0-N1	26 (52.0)	38 (76.0)	0.012[d]
N2- N3	24 (48.0)	12 (24.0)	
AJCC disease stage (8th edition)			
I/ II	20 (40.0)	18 (36.0)	0.873[d]
III	11 (22.0)	13 (26.0)	
IV	19 (38.0)	19 (38.0)	
Resection margins			
Positive (R1 or R2)	11 (22.0)	23 (46.0)	0.011[d]
Negative (R0 or limit)	39 (78.0)	27 (54.0)	
Lymphovascular invasion	13 (30.2)	21 (58.3)	0.012[d]
Perineural invasion	23 (53.5)	28 (73.7)	0.060[d]
Number of lymphadenopathies	2.0; 4.2±9.8	2.0; 3.2±2.9	0.992[f]
Number of nodes removed	38.0; 41.5±17.7	13.0; 16.3±16.5	<0.001[f]
Extranodal spread	22 (66.7)	11 (73.3)	0.644[e]

Results are expressed as frequency and percentage [n (%)] or as median; mean ± standard deviation. P16+, P16-positive; AJCC, American Joint Committee on Cancer; WHO, World Health Organization. [a]Computed for 97 patients, since three patients in the irradiated area cohort had lymphatic recurrence. [b]Only for patients with oropharyngeal cancer. [c]Student's t-test. [d]Chi-square test. [e]Fisher Exact test. [f]Mann Whitney U test.

Treatment characteristics. Table II details the treatment characteristics. In the irradiated cohort, 22 (44.0%) patients had been treated with surgery and postoperative radiotherapy, 6 (12.0%) by surgery and postoperative chemoradiation, 10 (20.0%) by

Table II. Characteristics of the treatments received by patients in the irradiated area cohort.

Characteristics of the irradiated cohort (n=50)	Data
Type of tumor	
Locoregional recurrence beyond initial treatment[a]	29 (58.0)
>6 months	20 (69.0)
≤6 months	9 (31.0)
New location	21 (42.0)
Time of new location (years)	10.1 (3.4-13.1)
Treatment of the initial tumor	
Type of treatment	
Surgery T/N + RT	22 (44.0)
Surgery T/N + RTCT	6 (12.0)
Definitive RTCT[b]	10 (20.0)
RT only	5 (10.0)
Induction chemotherapy + RTCT	7 (14.0)
Surgery T/N of the initial tumor	28 (56.0)
Type of RT[c]	
3D	22 (51.1)
IMRT	21 (48.8)
Dose on tumor site recurrence (T and/or N) ≥66 Gy[c]	21 (48.8)
Mean dose on tumor site recurrence (T and/or N) ≥66 Gy[c]	19 (44.2)
Overall treatment time (days) >6 weeks[c]	20 (46.5)
Treatment of the tumor in irradiated area	
Salvage surgery	50 (100)
Tumor surgery	47 (97.9)
Neck dissection	41 (82.0)
Ipsilateral	13 (31.7)
Bilateral	28 (68.3)
Re-irradiation only	1 (2.0)
Re-irradiation with CT (postoperative VOKES protocol)	4 (8.0)

Data are expressed as frequency and percentage [n (%)] or median [interquartile range]. CT, chemotherapy; Gy, Gray; IMRT, intensity-modulated radiation therapy; Q3w, every three weeks; RT, radiotherapy; RTCT, chemoradiotherapy; T/N Tumor/Node. [a]Comprising tumor progression. [b]Comprising cetuximab. [c]Computed on the 43 patients with radiotherapy.

definitive chemoradiation, 5 (10.0%) by radiotherapy only, and 7 (14.0%) by induction chemotherapy and chemoradiation. Nineteen (44.2%) patients in the irradiated area cohort had received a mean irradiation dose at the site of tumor recurrence of ≥66 Gy (tumor and/or node). Table SI describes the treatments of the initial tumors of the irradiated area cohort.

Immune microenvironment. Table III details the immune microenvironment (CD3+ and CD8+ TILs, PD-L1) expression on tumor and immune cells) in the intratumoral and stromal regions.

CD3+ TIL numbers. The median number of CD3+ TILs in the intratumoral regions was significantly lower in the irradiated area cohort [median, 20.5; interquartile range (9.0-70.0) vs. 58.0 (27.0-101.0); P=0.003].

A total of 34% of tumors in the irradiated area (n=17) showed a high CD3+ TIL count compared to 66% (n=33) in the *de novo* group (P=0.001). Similar results were found for CD3+ TIL count in the stromal regions (P=0.016). 36% (n=18) of irradiated tumors had a low number of CD3+ TILs in intratumoral and stromal regions compared to 14% (n=7) in the *de novo* group (P=0.001). Results were similar with the IPTW method.

CD8+ TIL numbers. CD8+ TIL counts did not differ between the two cohorts in either intratumoral or stromal regions. The median number was 21.5 [3.0-64.0] in the irradiated area cohort vs. 30.0 [13.0-87.0] in the *de novo* cohort in intratumoral compartments (P=0.273) and 71.0 {24.0-131.0} vs. 97.5 [48.0-158.0] in the stromal compartments (P=0.129).

Expression of PD-L1 by tumor and immune cells. The percentage of tumors with PD-L1+ tumor cells (TPS ≥1%) was significantly lower in the irradiated area cohort than the *de novo* cohort (56.0% vs. 86.0%, P<0.001) (Table III). The percentage of tumors with PD-L1+ immune cells was significantly lower in the intratumoral regions (48.0% vs. 72.0%, P=0.014) and also lower in the stromal regions (62.0% vs. 78.0%, P=0.081) in the irradiated area cohort compared to the *de novo* cohort. One-third of irradiated tumors had a negative TPS and a low expression of PD-L1 by immune cells (ICs) (Table III) compared to 10% in the *de novo* cohort (P=0.004). Results were similar using the IPTW method.

Immune phenotype. Fig. 2 presents the proportion of microenvironment phenotypes I to IV in each cohort. We observed type I phenotype, adaptive immune resistance, in 63% of tumors in the *de novo* cohort vs. 52% in the irradiated area cohort, whereas types II and IV were found more frequently in the irradiated area cohort (P=0.032 in bivariate analyses and P<0.001 after the IPTW method).

Median follow-up time was 18 months (interquartile range 10-27). The tumor microenvironment (CD3+ and CD8+ TILs, PD-L1+ tumor cells, and PD-L1+ immune cells) was not significantly associated with PFS in the *de novo* cohort (Table IV) or the irradiated area cohort (Table SII).

Discussion

The present study aimed to assess antitumor immune response biomarkers in head and neck squamous cell carcinoma (HNSCC) occurring in irradiated areas: Few data are currently available. We observed a significantly lower infiltration of CD3+ tumor-infiltrating lymphocytes (TILs) in tumors in irradiated areas compared to *de novo* tumors, in both intratumoral and stromal regions, a significantly lower expression of programmed death-ligand 1 (PD-L1) on tumor cells and immune cells, and no difference in CD8+ TIL infiltration, except for the high number of intratumoral CD8+ TILs, which was considered to be a statistical artifact.

As there are no randomized clinical trials stratified according to whether patients had previously received radiation or not, we

Table III. Description of the expression of immune response biomarkers in irradiated area compared to *de novo* tumors.

Biomarkers	*De novo* (n=50)	Irradiated area (n=50)	P-value	Adjusted P-value[a]
$CD3^+$ TILs				
Intratumoral				
Number	58.0 (27.0-101.0)	20.5 [9.0-70.0]	0.003[b]	0.088
High	33 (66)	17 (34)	0.001[c]	<0.001
Stromal				
Number	185.5 (107.7-361.0)	139.0 (63.0-215.0)	0.020[b]	0.046
High	30 (60)	18 (36)	0.016[c]	0.008
Intratumoral low and stromal low $CD8^+$ TILs	7 (14)	18 (36)	0.011[c]	0.001
Intratumoral				
Number	30.0 (13.0-87.0)	21.5 (3.0-64.0)	0.273[b]	0.261
High	25 (50)	21 (42)	0.422[c]	0.005
Stromal				
Number	97.5 (48.0-158.0)	71.0 (24.0-131.0)	0.129[b]	0.121
High	28 (56)	24 (48)	0.423	0.577
Intratumoral low and stromal low PD-L1	15 (30)	16 (32)	0.829	0.098
TPS ≥1%	43 (86)	28 (56)	<0.001[c]	<0.001
Immune cells (ICs)				
Intratumoral=High	36 (72)	24 (48)	0.014[c]	<0.001
Stromal=High	39 (78)	31 (62)	0.081[c]	0.058
TPS <1% and PD-L1 IC intratumoral=0	5 (10)	17 (34)	0.004[c]	<0.001

Data are expressed as frequency and percentage [n (%)] or median [interquartile range]. IC, immune cells; TILs, tumor-infiltrating lymphocytes; PD-L1, programmed death-ligand 1; TC, tumor cells; TPS, tumor proportion score. For $CD3^+$ and $CD8^+$ TIL counts, values were dichotomized (low/high) according to the median cell number calculated from all samples and considered high if >40 $CD3^+$ cells and >30 $CD8^+$ cells were observed in intratumoral regions and if >160 $CD3^+$ cells and >90 $CD8^+$ cells were observed in stromal regions. Expression of PD-L1 by tumor cells was considered positive if TPS ≥1%. The expression of PD-L1 by immune cells was considered high with a score of 1-2 in intratumoral components and 2-5 in stromal components. [a]Analyses were adjusted by the inverse probability of treatment weighting (IPTW) method using the propensity score. [b]Mann Whitney U test. [c]Chi-square test.

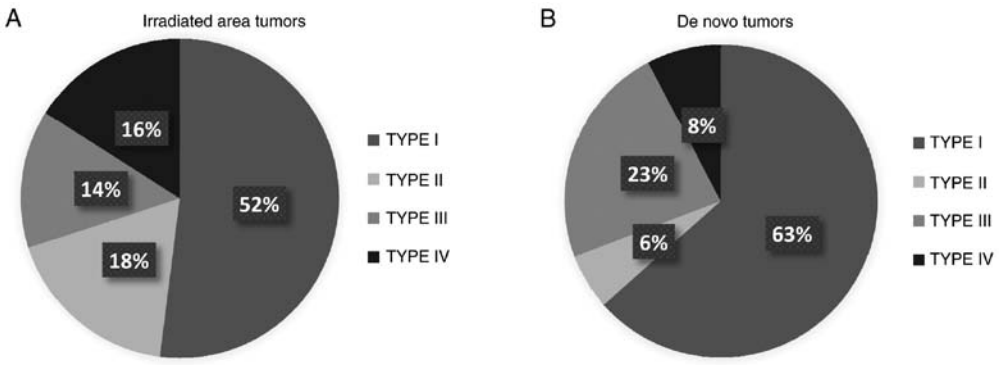

Figure 2. Distribution of the four types of immune phenotype among irradiated area tumors (A) and *de novo* tumors (B) TYPE I, adaptive immune resistance ($TPS^+/CD8^+$ TIL high count); TYPE II, immunological ignorance ($TPS^-/CD8^+$ TIL low count); TYPE III, intrinsic induction ($TPS^+/CD8^+$ TIL low count); TYPE IV, immune tolerance ($TPS^-/CD8^+$ TIL high count). TPS, tumor proportion score; TIL, tumor-infiltrating lymphocyte.

had no comparative data; lower PD-L1 expression by tumor and immune cells or lower numbers of TILs, whether in intratumoral or stromal compartments, might have been expected, as immune checkpoint inhibitors (ICIs) seem to be less effective for locoregional relapses than for metastatic lesions (3). Our results confirm the hypothesis that antitumor treatments modify the microenvironment of recurrent tumors, especially for radiotherapy. This is why trials are being conducted with concomitant

Table IV. Prognostic factors of PFS for the *de novo* cohort in univariate and multivariate analyses.

	Univariate		Multivariate	
Characteristics	HR (95% CI)	P-value	HR (95% CI)	P-value
Age (years)		0.139		
≤60	1			
>60	2.29 (0.76-6.87)			
Sex		0.334		
Male	0.60 (0.21-1.69)			
Female	1			
Smoking		0.603		
Current smoker	1			
Former smoker	1.59 (0.60-4.19)			
Never smoker	0.99 (0.20-4.77)			
Alcohol		0.458		
Yes	1.42 (0.56-3.62)			
No	1			
Tumor location		0.003		
Other	1			
Hypopharynx	4.08 (1.59-10.45)			
Oropharynx location and p16		0.167		
Oropharynx $p16^+$	1			
Oropharynx $p16^-$	4.57 (0.53-39.26)			
Differentiation		0.331		
Moderately/poorly/undifferentiated	1			
Well-differentiated	1.73 (0.57-5.22)			
pT stage		0.921		
Tx-T1-T2	1			
T3-T4	0.95 (0.37-2.48)			
pN stage		0.014		
Nx-N1-N2	1			
N3-N4	3.34 (1.28-8.72)			
AJCC disease stage (8th edition)		0.009		
I/II	1			
III	0.62 (0.12-3.21)			
IV	3.62 (1.29-10.18)			
Smoking history (pack*year)		0.422		
<40	1			
>40	1.44 (0.59-3.49)			
Former drinker		0.996		
Yes	1.00 (0.30-3.32)			
No	1			
Resection margins		0.044		0.015
Positive (R1 or R2)	1		1	
Negative (RO or limit)	0.38 (0.15-0.97)		4.10 (1.31-12.79)	
Lymphovascular invasion		0.007		
Yes	4.36 (1.49-12.76)			
No	1			
Perineural invasion		0.019		0.021
Yes	4.61 (1.29-16.45)		5.16 (1.28-20.80)	
No	1		1	
Number of lymphadenopathies		0.001		0.037
1	1		1	
>1	5.29 (1.92-14.62)		3.90 (1.09-14.02)	

Table IV. Continued.

	Univariate		Multivariate	
Characteristics	HR (95% CI)	P-value	HR (95% CI)	P-value
Number of nodes removed		0.068		
<30	1			
≤30	2.36 (0.94-5.91)			
Extranodal spread		0.503		
Yes	1			
No	1.40 (0.52-3.76)			
P16		0.116		
Positive (≥70%)	1			
Negative (<70%)	5.08 (0.67-38.49)			
TPS		0.092		
<1	2.42 (0.86-6.80)			
≥1	1			
PD-L1 IC intratumoral expression		0.159		
Low	1.91 (0.78-4.71)			
High	1			
Combined score		0.075		
TPS <1% AND PD-L1 IC intratumoral=0	2.74 (0.90-8.31)			
TPS ≥ 1% OR PD-L1 IC intratumoral ≥1	1			
PD-L1 IC stromal expression		0.052		
Low	2.50 (0.99-6.31)			
High	1			
CD3 intratumoral counts		0.935		
Low	0.96 (0.38-2.41)			
High	1			
CD3 stromal counts		0.208		
Low	1.76 (0.73-4.25)			
High	1			
CD3 intratumoral + stromal counts		0.953		
CD3 intratumoral low AND CD3 stromal low	0.96 (0.28-3.29)			
CD3 intratumoral high OR CD3 stromal high	1			
CD8 intratumoral counts		0.864		
Low	1.08 (0.45-2.61)			
High	1			
CD8 stromal counts		0.185		
Low	1.83 (0.75-4.46)			
High	1			
CD8 intratumoral + stromal counts		0.194		
CD8 intratumoral low AND CD8 stromal low	1.81 (0.74-4.44)			
CD8 intratumoral high OR CD8 stromal high	1			

HR, hazard ratio; 95% CI, 95% confidence interval; AJCC, American Joint Committee on Cancer; IC, immune cells; TILs, tumor-infiltrating lymphocytes; PD-L1, programmed death-ligand 1; PFS, progression-free survival; TPS, tumor proportion score; WHO, World Health Organization. For $CD3^+$ and $CD8^+$ TIL counts, values were dichotomized (low/high) according to the median cell number calculated from all samples and considered high if >40 $CD3^+$ cells and >30 $CD8^+$ cells were observed in intratumoral regions and if >160 $CD3^+$ cells and >90 $CD8^+$ cells were observed in stromal regions. Expression of PD-L1 by tumor cells was considered positive if TPS ≥1%. The expression of PD-L1 by immune cells was considered high with a score of 1-2 in intratumoral components and 2-5 in stromal components.

strategies such as PembroRad (Clinical Trial NCT02707588), KEYNOTE-412 (Clinical Trial NCT03040999) and REACH trial (Clinical Trial NCT02999087).

In the irradiated area cohort, we observed a lower $CD3^+$ TIL count, which may suggest that pretreated tumors belong to immunologically unresponsive group of tumors, as Yuan *et al* described (23). However, there was a persistent infiltration of $CD8^+$ TILs in tumors from irradiated areas. $CD8^+$ TILs are cytotoxic T lymphocytes and therefore play a major role in antigen-specific antitumor immune responses (24). Studies of several malignant tumors have revealed that the frequency of $CD8^+$ and $CD3^+$ TILs has a prognostic value (25-28). Although we observed slightly lower densities of $CD8^+$ TILs in the irradiated area cohort than in the *de novo* cohort, previous radiation therapy or chemoradiation did not significantly affect their presence.

This therefore highlights that irradiated tissues retain the ability for antitumor action, as the main driver leading to immune response is the preexistence of antitumor cytotoxic T cells that were specifically hampered by immune checkpoints (8). It may corroborate previous observations that radiotherapy has the capacity to recruit $CD8^+$ TILs that are specific to tumor antigens and to increase the expression of PD-L1 (29).

Moreover, it constitutes a strong rationale for the use of ICI treatment for tumors occurring in previously treated areas, in order to activate cytotoxic antitumor immune responses. This is being assessed in the ADJORL1 trial (Clinical Trial NCT03406247), which is investigating the use of immunotherapy after salvage surgery for previously treated tumors.

$CD3^+$ TILs are important, as CD3 is a T cell co-receptor that aids activation of both cytotoxic cells and T helper cells. The persistent rate of cytotoxic $CD8^+$ TILs and the reduction in $CD3^+$ TILs could reflect a decrease in other classes of T cells, such as helper T cells or regulatory T cells.

Our results do not explain why locoregional recurrences can be the center of hyperprogression when using ICIs and may suggest that other mechanisms are likely to be involved. To date, we do not yet understand why the rate of hyperprogression, which can range from 9 to 29%, is greater in locoregional recurrences compared to distant metastases; prospective trials should investigate this further (15,16,30).

Significantly fewer tumors in the irradiated area cohort expressed PD-L1 on tumor cells than in the *de novo* cohort, but more than half (56%) were positive according to our cut-off of ≥1%. This reduction compared to *de novo* tumors may provide first indicators of why these tumors are less responsive to immunotherapy and lend weight to the argument of introducing ICIs earlier in therapeutic strategies for HNSCC, associated or not with cytotoxic agents or radiation. This was the rationale behind the KEYNOTE-048 phase III randomized trial, which showed a significantly longer overall survival in the frontline setting with anti-PD-1 pembrolizumab monotherapy vs. the EXTREME regimen [12.3 months vs. 10.3 months for the PD-L1 combined positive score (CPS) ≥1 population and 14.9 months vs. 10.7 months for the PD-L1 CPS ≥20 population] (14). The pembrolizumab with chemotherapy arm also showed a significantly longer overall survival in the frontline setting vs. the EXTREME regimen (13.6 months vs. 10.4 months for the PD-L1 CPS ≥1 population and 14.7 months vs. 11.0 months for the PD-L1 CPS ≥20 population) (14).

The PembroRad phase II randomized trial (Clinical Trial NCT02707588) is evaluating pembrolizumab combined with radiotherapy vs. cetuximab combined with radiotherapy in locally advanced HNSCC (LA HNSCC), and preliminary data seem to indicate a good tolerance (31). Other trials are assessing combination strategies with ICIs, such as the REACH trial, assessing the anti-PD-L1 antibody avelumab combined with cetuximab and radiotherapy (Clinical Trial NCT02999087), the KEYNOTE-412 trial, assessing pembrolizumab combined with chemoradiation (Clinical Trial NCT03040999) in LA HNSCC, or the JAVELIN trial, assessing avelumab in combination with chemoradiation in LA HNSCC (Clinical Trial NCT02952586).

Interestingly, our study indicates that patients with tumors in previously irradiated areas could be the best candidates to receive the addition of anti-CTLA4 to anti-PD-1/PD-L1 agents, as we found a significantly lower expression of PD-L1 on tumor cells in these tumors. Indeed, anti-CTLA4 agents seem to recruit $CD8^+$ TILs and their combination with anti-PD-1/anti-PD-L1 agents has a strong rationale for the induction of an antitumor immune response, especially when expression of PD-L1 is low. Trials assessing the combination of anti-CTLA4 and anti-PD-1/PD-L1 agents are ongoing, including the KESTREL phase III randomized trial (Clinical Trial NCT02551159) and the CheckMate 651 phase III randomized trial (Clinical Trial NCT02741570). However, CheckMate 714 phase II trial results were negative, suggesting that a combination of chemotherapy and immunotherapy could be of more interest than combining immunotherapies (Clinical Trial NCT02823574).

Various tumor proportion score (TPS) thresholds are used in different studies of HNSCC, with the cut-off ranging from 1 to 50%, illustrating that debate exists around choosing a threshold (3,7,28,32). We analyzed data from both intratumoral and stromal regions; there is currently no clear consensus on whether PD-L1 expression on all cells or specific cell populations should be analyzed, and which emergent scores should be used (6). CPS is one of these emergent scores, which takes into account the ratio of PD-L1 expressing cells to the total number of tumor cells and was able to predict response to pembrolizumab (7,14). An elevated pretreatment neutrophil-to-lymphocyte ratio can also predict poor prognosis with local invasion and distant metastases (33).

Immune phenotype. The predominant tumor microenvironment type in the irradiated area cohort was adaptive immune resistance (type I). Yang *et al* noted that HNSCC had typically diverse profiles of TILs, dividing tumors into two main categories: Inflamed or non-inflamed (24) Lei *et al* suggested that an immunoscore based on TIL phenotype may be helpful in better classifying patients (34). This study shows that tumors in irradiated areas can be still inflamed and links to ideas from Teng *et al*, who suggested that anti-PD-1/PD-L1 agents may be most effective in tumors with type I/inflamed microenvironments, because of preexisting intratumoral T cells (21). The persistent infiltration of $CD8^+$ TILs shown in this study is encouraging for this entity of tumors occurring in patients who have previously received radiation, since $CD8^+$ TILs have recently been presented as a promising favorable prognostic biomarker in HNSCC after adjuvant chemoradiation (28). However, given the known poor prognosis of patients with these tumors, $CD8^+$ TILs may be dysfunctional T cells,

as Psyrri *et al* suggested when studying the mechanisms of resistance to nivolumab (35).

Prognostic factors in the *de novo* cohort were advanced stage and site of disease; our results are consistent with previous findings that tumors in the hypopharynx have a poor prognosis (36,37). Expression of PD-L1 on immune cells tended to be a PFS prognostic factor. In the irradiated area cohort, no relevant prognostic factor was highlighted, which could be explained by the initial poor prognosis. Indeed, we found the same tumor characteristics that have previously been shown to confer a poor prognosis for patients with locoregional relapses: Poor differentiation, high pN stage, positive resection margins, and lymphovascular or perineural invasion (Table I).

Yang *et al* performed a meta-analysis of studies that assessed the prognostic role of PD-L1 expression in HNSCC (24). They concluded that PD-L1 could not be recommended as a prognostic factor in HNSCC, nor could it be used to stratify the risk in HPV-related HNSCC.

While PD-L1 has mainly been described as a prognostic factor when expressed on tumor cells (PD-L1$^+$ tumor cells), the importance of immune cells (PD-L1$^+$ immune cells) is beginning to be revealed. Indeed, Kim *et al* depicted PD-L1$^+$ immune cells as a favorable prognostic factor in HNSCC with a positivity cut-off of 5% (38). The two-year follow-up data from CheckMate 141 described an OS benefit with nivolumab irrespective of PD-L1 expression (39), suggesting that PD-L1-negative patients may benefit from ICI treatment because of PD-L1 expression by stromal cells. Kim *et al* also found that PD-L1 expressed by immune cells was a favorable prognostic factor in HNSCC (38) and some trials have started to use PD-L1 expression as a stratification factor, such as the IMpassion130 trial in triple-negative breast cancer (40).

The limitations of this study include the bias typical of retrospective studies and the fact that patients had not received ICI treatment, as clinical trials using immunotherapy were ongoing.

In conclusion, our results show the persistence of cytotoxic cells but lower expression of PD-L1 and fewer CD3$^+$ TILs in tumors in irradiated areas. This study provides first potential explanations of the fact that these lesions are less responsive to immunotherapy, although they may still retain antitumor capacities. The assessment of immune response biomarkers in patients treated with immunotherapy in randomized trials is required to decipher the molecular mechanisms involved in acquired resistance to treatments.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

All authors contributed equally to this research study. CP, JS, XSG and LG conceived and designed the experiments. CP, JT, JCF and GD made substantial contributions to acquisition of the data. XSG performed the histological examination. CP, JS, XSG and LG analyzed and interpreted the patient data. CB was involved in revising the manuscript critically. All authors were involved in the writing of the manuscript and approved the final manuscript.

Ethics approval and consent to participate

All patients were managed in the Lorraine Institute of Oncology according to the standards of good clinical practice. This retrospective study was approved by the local institutional review board and has been declared to Commission for information technology and civil liberties ('CNIL'), registered as a standard declaration by CNIL correspondent of the Institute. All patients were informed of the research performed with tumor tissue specimens and gave their consent. Ethical approval was not necessary for this retrospective study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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