Abstract

Purpose: Squamous cell carcinoma of the head and neck (SCCHN) is a lethal cancer with a suboptimal 5-year overall survival of approximately 50% with surgery and/or definitive chemoradiotherapy. Novel treatments are thus urgently awaited. Immunotherapy with checkpoint blockade has emerged as a promising option for patients with recurrent/metastatic SCCHN; however, it has not been investigated in the curative-intent setting yet. The purpose of this study was to investigate the T-cell receptor repertoire and the tumor microenvironment in tumor tissues of SCCHN patients with locoregionally advanced disease.

Experimental Design: We performed T-cell receptor sequencing of tumor tissues from 44 patients with locoregionally advanced SCCHN prior to treatment with definitive chemoradiotherapy and correlated the T-cell clonality and the mRNA expression levels of immune-related genes with clinicopathologic parameters.

Results: Clonal expansion of T cells was significantly higher in human papilloma virus (HPV)–negative compared with HPV-positive tumors, signifying more robust antigen presentation in HPV-negative tumors. The latter was supported by the higher percentage of HPV-negative tumors expressing HLA-A protein compared with HPV-positive tumors (P = 0.049). Higher GRZB levels correlated significantly with longer recurrence-free survival (log-rank, P = 0.003) independent of tumor size, nodal stage, and HPV status.

Conclusions: Our findings support clonal expansion of T cells in SCCHN patients with locoregionally advanced disease and imply differences in the antigen presentation capacity between HPV-negative and HPV-positive tumors. Elevated GRZB mRNA levels may also serve as a favorable and independent predictor of outcome in SCCHN patients treated with chemoradiotherapy. These data provide rationale for the introduction of immunotherapeutic approaches in the curative-intent setting.

Clin Cancer Res; 1–11. ©2017 AACR

Introduction

Squamous cell carcinoma of the head and neck (SCCHN) is a lethal cancer that affects approximately 500,000 people/year worldwide and is the sixth most common cancer in global incidence (1). The 5-year overall survival is approximately 50% (2). In the locoregionally advanced stage, chemoradiotherapy and surgery are the mainstay of treatment, but metastatic and locoregional relapses are quite common, underlining the need for more effective treatment strategies to achieve higher cure rates. More recently, immunotherapy has emerged as a promising treatment option with pembrolizumab being expeditiously approved by the FDA for recurrent/metastatic SCCHN in August 2016 (3), its fourth indication after melanoma, non–small cell lung cancer, and renal cell carcinoma. Nivolumab was also shown to increase 1-year overall survival to 36% compared with 16.6% with standard chemotheraphy in a phase III trial. (4) This efficacy highlights the immunogenic nature of SCCHN and supports that immune-based therapies are a rational and promising therapeutic platform for SCCHN.

CD8+ T-cell infiltration has been associated with better overall survival and response to chemoradiotherapy in human papilloma virus (HPV)–positive and HPV-negative patients with locoregionally advanced SCCHN, though this still remains controversial and further studies are needed to confirm these findings (5–9). Previous work has shown that CD8+ T-cell infiltration is essential for the response of tumors to radiotherapy (10–11). In addition, although radiotherapy alone may induce antitumor immune responses, preclinical work has demonstrated that it also augments an immunosuppressive tumor microenvironment through upregulation of PD-L1 and that combination of radiotherapy with anti–PD-L1 blockade...
Saloura et al. evaluated the effects of definitive chemoradiotherapy on the tumor immune microenvironment in locoregionally advanced SCCHN. They reported increased in blood-circulating CD8+ T cells, increases in MDSCs, regulatory T cells (Treg), T-cell receptor (TCR) clonality, and PD-1 positive T cells, indicating that chemoradiotherapy may alter the tumor microenvironment in locoregionally advanced SCCHN. These results suggested that chemoradiotherapy may alter the tumor microenvironment, and thus integration of immunotherapy with chemoradiotherapy in the definitive setting would merit further investigation in SCCHN.

In this study, we pursued a comprehensive characterization of the tumor microenvironment in locoregionally advanced SCCHN by evaluating factors that reflect the inflammatory status of the tumor microenvironment, such as the TCR repertoire and various immune-related markers. More specifically, we sought to investigate the pattern of TCR clonality and the expression of immune-related genes in 44 patients with locoregionally advanced SCCHN prior to treatment with definitive chemoradiotherapy, and to correlate these with clinicopathologic characteristics, such as HPV status, nodal and tumor stage, recurrence-free survival (RFS), and overall survival (OS). Overall, our data give insight into the clonality of the T cells infiltrating HPV-positive and HPV-negative tumors, as well as the association of immune-related genes with survival, and provide rationale for the integration of immunotherapy strategies in the treatment of locoregionally advanced SCCHN.

**Materials and Methods**

**Patient characteristics**

The examined patient cohort consisted of 58 patients with locoregionally advanced SCCHN who were previously treated with an organ-preservation approach with or without platinum-based induction chemotherapy followed by one of the 5-fluorouracil and hydroxyurea (FHX)-based chemoradiotherapy protocols at the University of Chicago, as previously described (16). Of these patients, 14 were excluded from the analysis due to low CD4 and CD8 mRNA levels compared with the rest of the cohort. Of the 44 remaining patients, 39 (89%) had participated in FHX-based chemoradiotherapy clinical protocols at the University of Chicago, ensuring the homogeneity of the treatment received. The remaining 11% of patients received off protocol platinum-based induction chemotherapy followed by FHX-based chemoradiotherapy and did not require significant dose reductions or treatment interruptions. Thirty-six of 44 (82%) of these patients received platinum-based induction chemotherapy followed by FHX-based chemoradiotherapy, whereas 8 of 44 (18%) received FHX-based chemoradiotherapy only. Table 1 shows the clinicopathologic characteristics of this patient cohort.

**Patient samples**

RNA was isolated from fresh-frozen biopsies of 58 patients with locoregionally advanced disease previous to treatment with or without platinum-based induction chemotherapy followed by definitive chemoradiation with one of six FHX-based

**Translational Relevance**

Although immunotherapy was recently approved as a promising treatment option for patients with recurrent/metastatic squamous cell carcinoma of the head and neck (SCCHN), its application in the curative-intent setting is still under investigation. We used pretreatment tumor tissues from 44 patients with locoregionally advanced SCCHN and showed that tumor-infiltrating T cells clonally expand and that patients with higher GRZB levels have significantly longer recurrence-free survival independent of tumor, nodal stage, or human papilloma virus (HPV) status. Furthermore, HPV-positive patients had lower degree of T-cell expansion compared with HPV-negative patients. Our data suggest that this could be secondary to a lower percentage of HPV-positive patients expressing HLA-A, thus making antigen presentation less efficient in these patients. These data underscore the significance of the immune system in SCCHN biology and provide rationale for the introduction of immunotherapy in the curative-intent setting.
chemoradiotherapy protocols at the University of Chicago. Briefly, fresh biopsies were frozen in optimal cutting temperature (OCT) embedding material, a section was cut and stained with hematoxylin and eosin (H&E), and then reviewed by a head and neck pathologist to confirm histology and tumor content. Guided by the H&E slides, the OCT region with the highest tumor burden (≥60%) was cut from the OCT block for further processing. RNA was isolated using the AllPrep RNA/DNA/Protein Mini Kit (Qiagen; #80204) or the RNA/DNA/Protein Purification Kit (Norgen Biotek, #47700) using an Ultrasonicator (Covaris). Samples were obtained from the University of Chicago SCCHN tissue bank (Human Tissue Resource Center). Use of tissues was approved by the University of Chicago Institutional Review Board (IRB#8980).

**Quantitative RT-PCR**

To examine the expression levels of immune-related genes, quantitative RT-PCR from the tumor and tumor bed cDNAs was conducted using TaqMan Gene Expression assays (Thermo Fisher Scientific) and ABI Viia 7 system (Applied Biosystems), according to the manufacturer’s instructions. The TaqMan probes for PD-1, PD-L1, PD-L2, OSC40, OX40, CTLA4, IDO1, B7-H3, GRZB, PRF1, HLA-A, CD4, CD8, CD206, and FoxP3 genes were purchased from Life Technologies. Samples were run in triplicate and a probe for GAPDH was used for data normalization.

**TCRβ sequencing**

The libraries for TCRβ sequencing were prepared using previously described methods (17–21). Briefly, cDNA with 5’-RACE adapter was synthesized. The TCRβ chains were amplified using a reverse primer specific to the constant region and a forward primer for the SMART adapter. Illumina sequence adapters with barcode sequence were then added using the Nextera XT Index kit (Illumina, FC-131-2004). Sequencing was performed by 300-bp paired-end reads on the Illumina MiSeq platform, using a MiSeq Reagent v3 600-cycle kit (Illumina, MS-102-3003).

**TCRβ repertoire analysis**

TCRβ repertoire analysis was performed using Tcrip software. Briefly, sequencing reads were mapped to the human TCRβ reference sequences using Bowtie2 aligner, and then decomposed to the V-D-J components of the CDR3s (17). The inverse Simpson’s diversity index (DI) value was calculated to quantify the clonality of the TCRβ repertoire as previously reported (20, 21). To present the TCRβ repertoire of each sample, we used the Excel program (Microsoft) to generate bar graphs and pie charts.

**IHC in head and neck cancer tissue microarrays**

The expression levels of HLA-A in 57 HPV-negative and 25 HPV-positive SCCHN sections were examined by immunohistochemistry. SCCHN sections were derived from biopsies of patients with local or locoregionally advanced disease previous to treatment with either surgery with or without adjuvant chemoradiation, or definitive chemoradiation. Slides of paraffin-embedded squamous cell carcinoma tumor specimens were deparaffinized, rehydrated, and sections were treated with antigen retrieval buffer (Bond TM Epitope Retrieval 2, AR9640, Leica Microsystems) in a steamer for 20 minutes at 96°C. Anti–HLA-A antibody (Abcam ab52922) was applied on tissue sections for 1-hour incubation at room temperature. Following TBS wash, the antigen–antibody binding was detected with the Bond Refine polymer detection system (DS9800, Leica Biosystems). Tissue sections were briefly immersed in hematoxylin for counterstaining of the nucleus and were covered with cover glasses. An expert head and neck cancer pathologist and an additional reviewer blinded to clinical outcomes performed semiquantitative analysis of HLA-A staining using the following semiquantitative scoring: tissues with moderate or strong staining in >50% of cancer cells were defined as positive, and tissues with no staining, weak staining, or moderate/strong staining in ≤50% of cancer cells were defined as negative. The tissue samples were acquired with written informed consent from all the participating patients following the relevant protocol approval by the University of Chicago Institutional Review Board (IRB 12-2125 and IRB 12-2117). Accordingly, the above experimental procedure involving patient samples was conducted in accordance with the approved guidelines by the University of Chicago Institutional Review Board.

**Statistical analysis**

Gene expression level and TCRβ DI were compared between patient groups using the log-rank test (Kaplan–Meier curves) and between different clinicopathologic characteristics using the Mann–Whitney U test (two-tailed). Univariate and multivariate Cox-regression analysis was also performed. Statistical analysis was carried out using Prism 6 (GraphPad) and R biostatistical software. P < 0.05 was considered statistically significant.

**Results**

**T cells can clonally expand in SCCHN tumors**

To assess the TCR repertoire in the tumors of patients with locoregionally advanced SCCHN, we obtained RNA from the tumors of 58 patients and we first examined CD8 and CD4 mRNA expression levels. Of these samples, 14 were excluded due to low CD8 and CD4 mRNA expression levels compared with the rest of the cohort, with 44 patients remaining for TCRβ and immune-related gene analysis. Subsequently, we performed TCRβ sequencing by amplifying the TCRβ cDNA using a MiSeq Illumina sequencing protocol. An average of 715,539 sequence reads mapped to V, D, J, and C segments were obtained for all tumor samples. From these reads, 69,844 ± 7,184 (SEM) sequence reads mapped to V, D, J, and C segments were obtained for TCRβ for all tumor samples. From these reads, 69,844 ± 7,184 (SEM) were found to be unique TCRβ complementarity determining region 3 (CDR3) clonotypes which are important for the recognition of a unique antigen on an HLA molecule. The frequencies of the CDR3 sequences were then calculated, and clonal expansion for specific TCRβ CDR3 clonotypes was observed in both HPV-positive and HPV-negative tumors. Representative examples of clonal expansion are shown in Fig. 1A.

To assess the distribution of the TCRβ diversity among SCCHN patients, the TCRβ DI was calculated as the inverse Simpson’s diversity index (1/Δs), whereby lower TCRβ DI signifies more clonal expansion. The TCRβ DI among the 44 tumors ranged from 10 to 2,981 with a median value of 220 (Fig. 1B).

**HPV-negative SCCHN tumors exhibit more clonal expansion and express HLA-A more frequently compared with HPV-positive SCCHN tumors**

Given their viral causality, we hypothesized that HPV-positive tumors would be more immunogenic and would thus have more clonal expansion of T cells directed to HPV-related
epitopes, compared with HPV-negative tumors. Contrary to our expectation, the TCRβ DI was significantly lower in HPV-negative tumors compared with HPV-positive tumors (Mann–Whitney U test, \( P = 0.002 \); Fig. 2A). This observation remained statistically significant after removal of heavy smokers (≥10 pack-years) from the HPV-positive group and light smokers (<10 pack-years) from the HPV-negative group (Supplementary Fig. S1; Mann–Whitney U test, \( P = 0.004 \)). The sum of the percentages of the top 10 TCRβ CDR3 clonotypes for each tumor was also calculated and ranged from 7% to 52% (average, 22%±9.5%) in HPV-negative tumors compared with 3% to 37% (average, 14.6%±10.6%) in HPV-positive tumors, and this difference was statistically significant (Supplementary Fig. S2, Mann–Whitney U test, \( P = 0.01 \)).

Figure 2. HPV-negative SCCHN tumors exhibit more clonal expansion and express higher levels of HLA-A compared with HPV-positive tumors. A, TCRβ DI in HPV-negative versus HPV-positive patients (\( N = 44 \), Mann–Whitney U test; *, \( P = 0.002 \)). The y-axis is presented in logarithmic scale. B, Histogram representing the cumulative results of IHC analysis for HLA-A in a separate cohort of patients with locoregionally advanced SCCHN (\( N = 82 \)). The percentage of HPV-negative (\( N = 57 \)) and HPV-positive (\( N = 25 \)) patients that are HLA-A positive or HLA-A negative by IHC are presented. A total of 68% of HPV-negative tumors were HLA-A positive compared with 44% of HPV-positive tumors (Fisher exact test, \( P = 0.049 \)). C, Representative examples of IHC for negative HLA-A staining in HPV-positive (i) versus positive HLA-A staining in HPV-negative patients (ii).
The fact that the TCRβ DI was found to be lower in HPV-negative tumors implies that the T-cell infiltration in these tumors is characterized by greater expansion of T-cell clones compared with HPV-positive tumors. One explanation for this finding could be that the tumor microenvironment of HPV-positive tumors and the chronic nature of the HPV-infection may promote T-cell anergy or exhaustion. However, comparison of the mRNA levels of immune-related genes associated with T-cell anergy and/or exhaustion, such as CTLA4 and PD-1, did not reveal any significant differences between HPV-positive and HPV-negative tumors (Supplementary Fig. S3). In addition, IDO1, PD-L1, PD-L2, CD206, a marker of MDSCs, and FOXP3/CD4, a ratio reflecting the presence of Tregs, were not statistically different between the two groups (Supplementary Fig. S3).

Another possibility that could explain the higher TCRβ DI in HPV-positive SCCHN tumors is that, in these tumors, recognition of neoeptopes by T cells may be less efficient due to defects in HLA expression and/or antigen presentation. Given that the majority of antigens presented by cancer cells that have been reported are mostly associated with HLA-A alleles (22), we chose to concentrate on HLA-A expression defects. To further assess whether HLA-A expression is defective in HPV-positive compared with HPV-negative tumors, the protein levels of HLA-A were compared between HPV-positive (N = 25) and HPV-negative (N = 57) patients using immunohistochemistry in a separate cohort of SCCHN patients with locoregionally advanced disease and available tissue microarrays. Results showed that 44% (11/25) of HPV-positive tumors were HLA-A positive, compared with 68% (39/57) of HPV-negative tumors, a difference that was statistically significant (Fisher exact test, P = 0.049), indicating more frequent HLA-A defects in HPV-positive SCCHN tumors (Fig. 2B). Figure 2C shows representative examples of HLA-A staining in HPV-positive (Fig. 2C (i)) and HPV-negative patients (Fig. 2C (ii)). Correlation of the HLA-A IHC staining with the TCRβ DI was not feasible due to lack of available RNA in this patient cohort.

Association of TCRβ DI with survival outcomes and other clinicopathologic characteristics

Potential correlations of the TCRβ DI with RFS and OS were examined in HPV-positive and HPV-negative patients, but no associations were found (Supplementary Fig. S4). Associations of the TCRβ DI with T stage, N stage, and tumor localization were also examined, but no statistically significant differences were observed (Supplementary Fig. S5).

High pretreatment levels of GRZB are associated with improved RFS in patients with SCCHN treated with definitive chemoradiotherapy

Previous work has shown that CD8+ T-cell infiltration is associated with improved survival and response to chemoradiotherapy of patients with HPV-positive and HPV-negative locoregionally advanced SCCHN (6–9); however, no studies have systematically evaluated the role of the tumor microenvironment in the clinical outcome of these patients. Given several lines of preclinical evidence supporting that an active immune response is critical for a successful radiotherapy response (10), we hypothesized that SCCHN patients with higher levels of intratumoral immune-related markers would exhibit longer disease-specific RFS and/or OS after treatment with chemoradiotherapy.

To investigate this hypothesis, we analyzed the expression levels of CD8, CD4, FOXP3, CD206, PD-1, CTLA4, OX40, IDO1, PD-L1, PD-L2, OX40L, B7-H3, GRZB (granzyme B), PRF1 (perforin), and HLA-A genes with RT-PCR in tumor samples from the previously described 44 patients with locoregionally advanced SCCHN and examined their association with disease-specific RFS and OS of these patients. Univariate and multivariate Cox-regression analysis of each of the immune markers with HPV status, tumor, and nodal stage, which are known to affect RFS and OS in SCCHN, was conducted. Patients with higher GRZB intratumoral levels had significantly longer RFS compared with patients with lower GRZB levels (log-rank test, P = 0.003), and this relationship remained significant after multivariate analysis for HPV status, tumor, and nodal stage (P = 0.032; Fig. 3A). Significantly longer RFS was also observed in patients with higher levels of OX40 (log-rank test, P = 0.008) and PD-L1 (log-rank test, P = 0.027); however, after multivariate analysis, the significance was lost, though a trend was still observed (P = 0.052 for OX40, P = 0.09 for PD-L1; Fig. 3B and C). Higher levels of PD-L2 and HLA-A were also associated with increased RFS with significance or trends to significance observed after multivariate analysis for HPV status, tumor, and nodal stage (P = 0.042 for PD-L2, P = 0.051 for HLA-A; Fig. 3D and E). Interestingly, PD-L1, PD-L2, and HLA-A were also significantly and positively associated with levels of GRZB and PRF1, indicating the presence of an active antitumor immune infiltrate and the generation of adaptive immune resistance (Fig. 4A and B). A positive association with GRZB or PRF1 was also observed with B7-H3, CD206, FOXP3, and CTLA4, further supporting the presence of adaptive immune resistance within SCCHN tumors (Supplementary Fig. S6). Longer RFS was also observed in patients with higher CD8 levels (P = 0.046), PRF1 (P = 0.048), CD4 (P = 0.052), OX40L (P = 0.037), and CTLA4 (P = 0.055), but these associations were lost with multivariate analysis for HPV status, nodal, and tumor stage. Results of multivariate analysis are shown in Table 2. No associations were found between PD-1 (P = 0.16), FOXP3 (P = 0.20), B7-H3 (P = 0.95), IDO1 (P = 0.15), and CD206 (P = 0.53). RFS and OS analysis did not reveal significant associations with any of the examined immune markers after multivariate analysis (Supplementary Table S1).

These results indicate that increased levels of GRZB portend improved response to chemoradiotherapy and maintenance of disease remission in SCCHN patients with locoregionally advanced disease, independent of HPV status, nodal, or tumor stage. Furthermore, expression of PD-L1, PD-L2, and B7-H3, as well as immune-suppressive cell subtypes, such as MDSCs and Tregs, may develop as mechanisms of adaptive immune resistance to an active immune infiltrate.

Association of intratumoral immune markers with other clinicopathologic parameters

We sought to further assess whether any of the aforementioned immune-related genes are associated with other clinicopathologic parameters, such as tumor size, nodal stage, primary tumor location, and HPV status. Interestingly, we found that higher B7-H3 levels correlated significantly with high-risk T stage (Mann–Whitney U test, P = 0.009; Fig. 5A), implicating B7-H3 as a mechanism of immune evasion in
SCCHN. In addition, HPV-positive tumors had significantly higher levels of OX40 (Mann–Whitney U test, \( P = 0.037 \)) and a trend toward increased OX40L levels (Mann–Whitney U test, \( P = 0.07 \); Fig. 5B), implying that the OX40/OX40L pathway may be important in enabling antitumor immune activity in HPV-positive SCCHN. No other markers showed association with T stage and HPV status, and no other significant associations were observed between immune-related markers, nodal stage, and primary location.

Association of intratumoral immune markers with TCRß DI

We performed additional correlations between the mRNA expression levels of the examined immune markers and the TCRß DI in our cohort of 44 patients. Results showed a significantly

---

**Figure 3.**

GRZB predicts recurrence in SCCHN patients treated with definitive chemoradiotherapy. RFS Kaplan-Meier curves analyzed by log-rank test for GRZB (\( P = 0.003 \); A), OX40 (\( P = 0.008 \); B), PD-L1 (\( P = 0.027 \); C), PD-L2 (\( P = 0.175 \); D), and HLA-A (\( P = 0.15 \); E). The median of mRNA values was used to dichotomize patients in high versus low expressing groups. mRNA levels of immune markers were obtained by RT-PCR from pretreatment samples of patients with SCCHN who received definitive chemoradiotherapy. Normalization was performed by GAPDH.
Figure 4.
Correlation of PD-L1, PD-L2, and HLA-A levels with GRZB and PRF1 in pretreatment tumor samples of patients with locoregionally advanced SCCHN. PD-L1, PD-L2, and HLA-A correlate positively with GRZB (A) and PRF1 (B). Pearson correlation coefficient and P value were calculated for each association and shown in each graph separately for each immune marker. mRNA levels were normalized by GAPDH.
positive association between the expression of CD4, CTLA4, and FOXP3 mRNA and the TCRβ DI (CD4: r = 0.44, P = 0.0035; FOXP3: r = 0.49, P = 0.0009; CTLA4: r = 0.43, P = 0.0038; Supplementary Fig. S7), signifying that higher numbers of Tregs may “dampen” the function of cytotoxic T cells and reduce clonal expansion of neoantigen-specific T cells. This finding is in accordance with multiple studies supporting the importance of Tregs in SCCHN biology, with clinical trials actively investigating CTLA4 inhibition not only in recurrent/metastatic SCCHN, but also in the locoregionally advanced, curative-intent setting (23–25). No significant associations were found between GRZB, PRF1, as well as the remaining immune markers, with the TCRβ DI.

Discussion

This study is the first to investigate the TCR repertoire and its associations with various clinicopathologic parameters of prognostic significance in patients with locoregionally advanced SCCHN prior to treatment with chemoradiotherapy. Our data support the pre-existence of clonal expansion of T cells in SCCHN tumors prior to treatment with chemoradiotherapy, signifying the possible presence and recognition of specific neoepitopes by T cells. This finding provides a proof-of-concept for the application of immunotherapeutic strategies that could potentiate and/or unleash the antitumor function of tumor-infiltrating effector T cells that pre-exist and already recognize neoepitopes in patients with locoregionally advanced SCCHN.

Another interesting finding of this study is the significantly higher clonal expansion of T cells in HPV-negative, smoking-related SCCHN tumors compared with HPV-positive SCCHN tumors. A possible reason that could explain this finding is that HPV-positive SCCHN tumor cells may have a defect in their antigen-presenting machinery induced by HPV viral proteins, thus evading recognition by effector T cells. Indeed, our findings support that a significantly lower percentage of HPV-positive SCCHN tumors express HLA-A protein compared with HPV-negative SCCHN tumors, indicating an inherent defect in the expression of HLA-A in HPV-infected SCCHN cancer cells. In accordance to this finding, previous work has also demonstrated

<table>
<thead>
<tr>
<th>Table 2. Univariate and multivariate Cox regression analysis of RFS for immune markers and HPV status, tumor, and nodal stage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>GZRB</td>
</tr>
<tr>
<td>PD-L2</td>
</tr>
<tr>
<td>HLA-A</td>
</tr>
<tr>
<td>OX40</td>
</tr>
<tr>
<td>PD-L1</td>
</tr>
<tr>
<td>CTLA4</td>
</tr>
<tr>
<td>OX40L</td>
</tr>
<tr>
<td>PRF1</td>
</tr>
<tr>
<td>CD4</td>
</tr>
<tr>
<td>CDB</td>
</tr>
</tbody>
</table>

Abbreviations: N, nodal stage; T, tumor stage.

Figure 5.
Associations of clinicopathologic characteristics with immune markers in SCCHN. A, B7-H3 is associated with high-risk tumor stage (Mann-Whitney U test, P = 0.009). RT-PCR for B7-H3 in patients low- (T1-T3) versus high-risk (T4) SCCHN tumor stage. mRNA levels normalized by GAPDH. B, HPV-positive tumors are associated with higher levels of OX40 (Mann-Whitney U test, P = 0.037). RT-PCR for OX40 in patients with HPV-positive versus HPV-negative SCCHN tumors. OX40L also showed a trend for higher expression in HPV-positive tumors (Mann-Whitney U test, P = 0.07). mRNA levels normalized by GAPDH.
that the HPV16 E7 viral oncoprotein interacts with histone deacetylases (HDAC) 1, 2, and 8 at the promoter region of MHC class I genes and transcriptionally downregulates their expression in HPV-infected cervical cancer cells (26). Furthermore, the HPV16 E5 protein has been shown to decrease the expression of MHC class I proteins through their sequestration in the endoplasmic reticulum of W12 cervical intraepithelial cells (27). In addition, Albers and colleagues have demonstrated decreased protein expression of HLA class I antigens in HPV-positive SCCHN compared with normal squamous epithelium (28), whereas Tertipis and colleagues have shown that the expression of the antigen-presenting machinery components, TAP2, LMP2, and LMP7, is decreased in HPV-positive SCCHN cells (29).

Another reason that could have explained the higher T-cell diversity in HPV-positive SCCHN tumors is a more immunosuppressed tumor microenvironment in HPV-positive tumors that could inhibit antigen-specific T-cell expansion, our data though did not reveal any statistically significant differences in the expression of immune-related genes between HPV-positive and HPV-negative SCCHN tumors. Finally, it is possible that the lower TCR β DI in patients with HPV-negative SCCHN is that the neoantigens present in smoking-related SCCHN may be more immunogenic by binding more strongly to the HLA class I and/or the TCR molecules, compared with HPV-related viral antigens. Interestingly, although the mutation load in HPV-positive SCCHN is similar to that of HPV-negative SCCHN (16, 30), it could be speculated that the abundance of HPV-related viral antigens may compete with neoantigens present at potentially lower concentrations within the HPV-infected cancer cells, thus leading to predominance of presentation of viral epitopes. Furthermore, it cannot be excluded that smoking-induced mutations, characterized by transversions of purine nucleotides to other purines and pyrimidine nucleotides to other pyrimidines, may lead to neoepitopes that have different affinity to HLA class I molecules compared with the ones induced by the recently described cytosine deaminase activity of the apolipoprotein B editing enzyme catalytic family of enzymes (APOBEC) which is increased in HPV-positive tumors and is characterized by a predominance of cytosine to thymidine mutations (30). In addition, it can be hypothesized that HPV-infected patients may tend to express specific HLA class I molecules that have inherently lower affinity to HPV-viral epitopes, which could be a mechanism of persistence or immune evasion of the HPV virus. These hypotheses would merit further investigation as potential causes of the difference in the TCR β repertoire diversity between HPV-positive and HPV-negative SCCHN tumors.

The lack of correlation of the TCR β DI with survival outcomes could be explained by a number of reasons. First, only 9 of the 44 patients had recurrent disease, making the survival analysis difficult to derive definite conclusions. Second, it cannot be excluded that the clonally expanded tumor-infiltrating T-cell subtypes include both CD8+ and CD4+ T cells, potentially also Tregs which may “dampen” an antitumor immune response and thus contribute to worse survival outcomes. Third, even in the presence of clonal expansion of CD8+ T cells recognizing robust neoepitopes, the TCR β DI alone does not take into consideration the functional effect of the tumor microenvironment on effector T cells. This is further supported by the lack of association between GRZB and PRF1 with the TCR β DI in our patient cohort, indicating that expansion of effector T cells does not necessarily imply effective antitumor cytotoxicity.

In addition, patients with locoregionally advanced SCCHN with longer RFS expressed higher pretreatment levels of immune markers, compared with patients with shorter RFS. Specifically, higher levels of GRZB and PD-L2 were significantly associated with longer RFS in patients treated with chemoradiotherapy regardless of HPV status, tumor, and nodal stage, suggesting that the pre-existence of an intrinsically inflamed microenvironment enhances chemoradiotherapy effects. Trends to significance were also found for OX-40, PD-L1, and HLA-A. This is in accordance with preclinical data showing that the presence of CD8+ T cells and the induction of an active immune response is necessary for the antitumor effects of radiotherapy (10), as well as with recent data showing significant prolongation of survival by boosting an immune response with PD-1 blockade in patients with metastatic SCCHN (4). The trend of a positive association of HLA-A with longer RFS may also signify more robust antigen presentation, leading to more effective antitumor inflammation. With the above in mind, it would be a rational next step to evaluate the pretreatment levels of immune markers, such as GRZB, PD-L1, PD-L2, OX-40, and HLA-A, as predictors of recurrence in patients with locoregionally advanced SCCHN after chemoradiotherapy in a larger validation cohort and subsequently in a prospective setting.

Expression levels of PD-L1, PD-L2, and B7-H3, which are expressed either by cancer or by stromal cells, as well as markers of immune-suppressive cell subtypes, such as MDSCs (CD1206) and Tregs (CTLA4, FOXP3), were also found to be significantly and positively associated with higher GRZB and PRF1 levels, indicating these pathways as potential mechanisms of adaptive immune resistance in patients with locoregionally advanced SCCHN. These findings suggest that targeting these mechanisms may be a promising approach to potentiate the therapeutic effect of chemoradiotherapy in SCCHN.

Furthermore, our data show that higher levels of B7-H3 are significantly associated with high-risk tumor stage (T4). This implies that B7-H3 may be important in the progression of SCCHN to more aggressive tumor stages by promoting immune evasion and thus warrants further investigation as a modulator of the immunobiology of head and neck cancer. B7-H3, a member of the B7/CD28 superfamily, is a potent inhibitor of CD8+ T-cell activation (31) and has been shown to be overexpressed in multiple cancer types, including SCCHN, while its expression in normal tissues is limited. MGA271 is a humanized monoclonal antibody that is already being investigated in phase I clinical trials, including patients with SCCHN (32). Furthermore, our findings also suggest that HPV-positive SCCHN tumors have significantly higher OX40 levels compared with HPV-negative SCCHN tumors. It is thus possible that HPV-positive patients may benefit more from OX40 agonists which is currently under investigation in clinical trials for patients with SCCHN (33–34).

Limitations of this study are its hypothesis-generating nature and its small sample size, making larger cohorts necessary to validate these results and expand analysis in HPV-positive versus HPV-negative patients. Furthermore, our TCR sequencing methodology did not differentiate between CD4+ and CD8+ tumor-infiltrating T cells, which would require flow cytometry analysis of fresh tumor samples. In addition, due to the limited availability of cancer tissues, we could not correlate the TCR β DI with HLA-A protein expression in our main cohort of 44 patients. Also, although HLA-A mRNA levels in our 44 patient cohort.
showed a trend for lower expression in HPV-positive patients compared with HPV-negative patients, this was not statistically significant (P = 0.06), making any definite conclusion on the association of HLA-A with the TCRβ DI precocious (Supplementary Fig. S8). Finally, it would be important for future studies to confirm the aforementioned immune marker correlations at the protein level.

In conclusion, to our knowledge this is the largest study exploring the TCR repertoire and the immune tumor microenvironment of SCCHN tumors in the locoregionally advanced stage. Our data suggest clonal expansion of T cells infiltrating the tumors of patients with locoregionally advanced SCCHN and provide rationale to introduce immunotherapeutic approaches in the curative-intent setting, and to pursue the identification of relevant neoantigens. Our group is currently pursuing the discovery of neoantigens in SCCHN, which could truly alter the treatment paradigm in the curative-intent setting, either by the introduction of neoadjuvant or adjuvant approaches through neoantigen-based personalized vaccines or personalized adoptive T-cell transfer that could potentially enhance chemoradiotherapy effects or reduce recurrence rates.

In HPV-positive tumors, the T-cell infiltrate seems to be more diverse, and this could be secondary to virally induced defective expression of HLA class I molecules. Approaches that could restore the HLA class I expression would thus merit further investigation as a method to increase response to immunotherapy in HPV-positive SCCHN. Furthermore, locoregionally advanced SCCHN patients with intrinsically inflamed tumors have longer RFS after chemoradiotherapy treatment, compared with patients who have lower levels of intratumoral inflammatory markers, with GRZβ and PD-L2 reaching statistical significance. These immune markers could be further explored as predictive biomarkers to select patients with locoregionally advanced SCCHN who could benefit from immune-boosting treatments prior to chemoradiotherapy or from de-escalation of curative-intent treatment.

Disclosure of Potential Conflicts of Interest
T. Seiwert is a consultant/advisory board member for Amgen, AstraZeneca, Bristol-Myers Squibb, Eisai, Eli Lilly, Innate Pharma, and Merck/MSD. E. Vokes is a consultant/advisory board member for AbbVie, Amgen, AstraZeneca, Boehringer-Ingelheim, Bristol-Myers Squibb, Celgene, Eli Lilly, Genentech, Leidos, Merck, Regeneron, Serono, Takeda, and VentiRx. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: V. Saloura, A. Fatima, T. Seiwert, Y. Nakamura
Development of methodology: V. Saloura, A. Fatima, M. Zewde, Y. Ikeda, T. Seiwert, Y. Nakamura
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): V. Saloura, A. Fatima, M. Zewde, R. Brisson, T. Seiwert, N. Cipriani, M. Lingen, E. Vokes
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): V. Saloura, A. Fatima, M. Zewde, K. Kiyotani, R. Brisson, J.-H. Park, T. Vougiouklakis, R. Bao, T. Seiwert
Writing, review, and/or revision of the manuscript: V. Saloura, A. Fatima, R. Brisson, T. Vougiouklakis, T. Seiwert, N. Cipriani, M. Lingen, E. Vokes, Y. Nakamura
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Zewde, R. Brisson, T. Vougiouklakis, E. Vokes
Study supervision: V. Saloura, Y. Nakamura

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received January 11, 2017; revised March 15, 2017; accepted April 20, 2017; published OnlineFirst April 25, 2017.

References


33. Linch SN, McNamara MJ, Redmond WL. OX40 agonists and combination immunotherapy: putting the pedal to the metal. Front Oncol 2015;5:34.

## Clinical Cancer Research

### Characterization of the T-Cell Receptor Repertoire and Immune Microenvironment in Patients with Locoregionally Advanced Squamous Cell Carcinoma of the Head and Neck

Vassiliki Saloura, Aiman Fatima, Makda Zewde, et al.

*Clin Cancer Res* Published OnlineFirst April 25, 2017.

<table>
<thead>
<tr>
<th>Updated version</th>
<th>Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-17-0103</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplementary Material</td>
<td>Access the most recent supplemental material at: <a href="http://clincancerres.aacrjournals.org/content/suppl/2017/04/25/1078-0432.CCR-17-0103.DC1">http://clincancerres.aacrjournals.org/content/suppl/2017/04/25/1078-0432.CCR-17-0103.DC1</a></td>
</tr>
</tbody>
</table>

- **E-mail alerts** Sign up to receive free email-alerts related to this article or journal.
- **Reprints and Subscriptions** To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
- **Permissions** To request permission to re-use all or part of this article, use this link [http://clincancerres.aacrjournals.org/content/early/2017/06/26/1078-0432.CCR-17-0103](http://clincancerres.aacrjournals.org/content/early/2017/06/26/1078-0432.CCR-17-0103). Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.