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Defining an inflamed tumor immunophenotype in recurrent, metastatic squamous cell carcinoma of the head and neck

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ABSTRACT

Objectives: Immune checkpoint inhibitors have demonstrated clinical benefit in recurrent, metastatic (R/M) squamous cell carcinoma of the head and neck (SSCHN), but lacking are biomarkers that predict response. We sought to define an inflamed tumor immunophenotype in this R/M SCCHN population and correlate immune metrics with clinical parameters and survival.
Methods: Tumor samples were prospectively acquired from 34 patients to perform multiparametric flow cytometry and multidimensional clustering analysis integrated with next-generation sequencing data, clinical parameters and outcomes.
Results: We identified an inflamed subgroup of tumors with prominent CD8+ T cell infiltrates and high PD-1/TIM3 co-expression independent of clinical variables, with improved survival compared with a non-inflamed subgroup (median overall survival 84.0 vs. 13.0 months, p = 0.004). The non-inflamed subgroup demonstrated low CD8+ T cells, low PD-1/TIM3 co-expression, and higher T_{regs}. Overall non-synonymous mutational burden did not correlate with response to PD-1 blockade in a subset of patients.
Conclusion: R/M SCCHN patients with an inflamed tumor immunophenotype demonstrate improved survival. Further prospective studies are needed to validate these findings and explore the use of immunophenotype to guide patient selection for immunotherapeutic approaches.

Introduction

Even with current treatments and in the era of favorable prognosis human papillomavirus (HPV)-associated disease, there remains a significant risk of locoregional recurrence or distant metastases in squamous cell carcinoma of the head and neck (SCCHN) [1,2]. Moreover, 5-year survival in patients with recurrent or metastatic (R/M) SCCHN remains dismal [3]. In recent years, it has become evident that tumor progression is promoted by immune evasion and abrogation of an effective immune response against cancer cells [4]. SCCHN appears closely linked with immunosuppression, and patients often demonstrate impaired immune cell function that correlates with poor outcomes [5,6]. Mechanisms of tumor immune evasion in SCCHN include the development of T cell tolerance, modulation of inflammatory and angiogenic cytokines, downregulation of antigen-processing machinery, and the expression of immune checkpoint ligands or receptors to promote immune evasion [7–9]. These mechanisms are currently serving to define immunotherapy targets for clinical development.

Immune checkpoint receptors inhibit normal T cell activation and costimulation to maintain a homeostatic immune response [10]. Cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and programmed death-1 (PD-1) are expressed on the surface of immune cells and interact with their respective ligands on antigen presenting or tumor cells. High tumor expression of the ligands of PD-1 (PD-L1, L2) and/or PD-1 expression by T lymphocytes can attenuate T cell activation and drive T cell exhaustion favoring tumor immune evasion [11]. Studies have estimated PD-L1 expression in SCCHN at 30–70% [12,13] with HPV+ tumors more frequently harboring infiltrating immune cells that express PD-1, despite its favorable prognosis [14]. Two recently published trials

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have established the efficacy of PD-1 blockade in advanced SCCHN. Anti-PD-1 blockade with pembrolizumab in a heavily pre-treated SCCHN population that was PD-L1+($\geq$ 1% by immunohistochemistry [IHC] in tumor or immune cells) resulted in an overall response rate of 18%, and 25% among those who were HPV+ [15]. The CheckMate-141 trial in platinum-refractory SCCHN using the anti-PD-1 antibody nivolumab demonstrated an improvement in median overall survival (OS) compared with standard chemotherapy – which was greater in those with HPV+ disease and in those with $\geq$ 1% tumoral PD-L1 expression by IHC [16]. These data were the foundation for Food and Drug Administration approval of these agents in 2016. These studies illustrate that response rates to anti-PD-1 monotherapy in SCCHN patients are low. Additionally, there is significant cost and toxicity associated with these agents, together highlighting the critical need to identify predictive biomarkers or immunophenotypes to guide patient selection. The tumor immune microenvironment is built on spatially diverse and complex immune relationships that appear dynamic. Thus alternative immune checkpoint upregulation by T cells represents just one mechanism to explain why capturing PD-1/PD-L1 interactions alone may not predict response to inhibitors [17].

Recent work in other solid tumors has suggested two main tumor immunophenotypes: an inflamed tumor category comprised of a rich T cell infiltrate, a type 1 interferon signature, and a diverse chemokine profile, and a non-inflamed category that lacks these features [18]. These so-called inflamed tumors may respond more favorably to therapies targeting immune checkpoint mechanisms. Here we prospectively characterized the tumor immune microenvironment using multiparametric flow cytometry and a multidimensional clustering algorithm to define an inflamed phenotype in R/M SCCHN and correlated these findings with mutational burden, smoking and HPV status, and survival. Our observations strengthen our understanding of the tumor immune microenvironment specific to SCCHN. Along with advances in genomic biomarkers, immunophenotyping may serve to guide the selection and sequencing of immunotherapies, and identify candidate biomarkers to predict response to checkpoint inhibitors.

Materials and methods

Subjects

Patients with SCCHN undergoing biopsy to confirm metastatic disease and those undergoing surgical resection for locoregional recurrence were identified prospectively. Prior to biopsy, informed consent was obtained for institutional review board-approved protocols. Peripheral blood controls were obtained at the time of biopsy in a subset of patients. Patient demographics and clinical characteristics were recorded.

Multiparametric flow cytometry

At the time of biopsy or surgical resection, fresh tissue samples from each patient were placed in RPMI-1640 with 10% fetal bovine serum (FBS). Tumor was confirmed by hematoxylin and eosin staining. A non-fixed cell suspension was then prepared for staining with fluorescently-conjugated antibody cocktails, as previously described [19]. Single cell suspensions were stained using mouse-anti-human antibodies. Surface antibodies against CD3 (HIT3a; UCHT1), CD8 (RPA-T8), CD14 (M5E2; MphiP9), CD45 (HI30), CD56 (B159), CD279 (EH12.1) and its isotype control (MOPC-21), and HLA-DR (G46-6) were purchased from BD Biosciences. Surface antibodies against CD4 (RPA-T4), CD16 (3G8), CD19 (HIB19), CD33 (WM53), CD66b (G10F5), CD123 (6H6), and TIM-3 (F38-2E2) and its isotype control (MOPC-21) were purchased from BioLegend. The surface antibody against CD45 (2D1) was purchased from eBioscience and the surface antibody against LAG-3 (polyclonal) and its isotype control (polyclonal) were purchased from R&D Systems. The intracellular antibody FOXP3 (236A/E7) was purchased from eBioscience. Cells were analyzed within 72 h of fixation on a BD FACSCanto II HTS or BD Fortessa cell analyzer with FACSDiva software v8.0.1 (BD Biosciences) and analyzed using FlowJo software v10.

Massively parallel genomic sequencing

Targeted next-generation sequencing was performed on DNA extracted from formalin-fixed paraffin-embedded (FFPE) archival tumor samples. Total gDNA concentration was determined using the PicoGreen dsDNA quantification assay (ThermoFisher Scientific, Waltham, MA), and samples with sufficient starting material (> 100 ng) were taken forward to library construction [20]. Briefly, gDNA was fragmented by ultrasonication to 150 base pairs and purified. Size-selected DNA was ligated to specific adapters during library construction (Illumina, Inc., San Diego, CA). Libraries were quantified using qPCR (Kapa Biosystems, Inc., Woburn, MA) and captured using the OncoPanel_v2 bait set using the Agilent SureSelect hybrid capture kit (Agilent Technologies, Santa Clara, CA). Oncopanel_v2 consists of 504 genes and 15 intronic regions with known or potential importance in cancer. The captured libraries then underwent paired end 100 (2 $\times$ 100 nt) sequencing on a Hiseq 2500 (Illumina Inc.).

Read pairs were aligned to the reference sequence b37 edition from the Human Genome Reference Consortium using the Burrows-Wheeler Aligner [21] and de-multiplexed using Picard tools. Mutation analysis for single nucleotide variants and non-synonymous mutational burden was performed using MuTect v1.1.4 in paired mode for tumors with matched germline or single mode for samples without matched germline, and annotated using Oncotator [22]. The alignments were further refined using the GATK tool for localized realignment around indel sites [23]. As part of quality metrics, all samples had to meet a minimum requirement that 80% of targets have a minimum coverage of 30$\times$.

Statistical analysis

Fisher's exact test for categorical variables (Wilcoxon rank sum test for continuous variables) and one-way ANOVA on ranks was used to assess for differences between individual immune subgroups. Spearman's Rho was used to measure the strength of association between variables. All statistical tests used a significance of <0.05 and were two-sided. An unsupervised non-linear dimension reduction method, t-Distributed Stochastic Neighbor Embedding (t-SNE), was used to investigate in reduced dimension space how tumors locate in relation to one another based on multiparametric flow data – using a tree-based algorithm [24]. With n = 20 samples included in t-SNE, the power to differentiate at least 8 markers was 0.8. Overall survival (OS) was determined from the date of diagnosis to death from any cause, otherwise this was censored at date of last follow-up. Time to recurrence (TTR) was determined from the date of completion of initial treatment to recurrence or metastatic disease – and Kaplan-Meier statistics were applied. Data were analyzed using R software package (version 2.15.3) [25].

Results

Clinical characteristics of the cohort

Demographics, clinical characteristics and survival information from 34 patients are summarized in Table 1. With a median age of