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Tumor Immune Microenvironment Alterations using Induction Cetuximab in a Phase II Trial of Deintensified Therapy for p16-Positive Oropharynx Cancer

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Abstract

Background

We sought to characterize early changes in $CD8^+$ tumor-infiltrating lymphocytes and tumor transcriptomes after induction cetuximab in a cohort with p16-positive oropharyngeal cancer on a phase II clinical de-escalation trial.

Methods

Tumor biopsies were obtained before and one week after a single cetuximab loading dose in eight patients enrolled in a Phase II trial of cetuximab and radiotherapy. Changes in $CD8^+$ tumor-infiltrating lymphocytes and transcriptomes were assessed.

Results

One week after cetuximab, five patients (62.5 %) had an increase in $CD8^+$ cell infiltration with a median (range) fold change of + 5.8 (2.5 – 15.8). Three (37.5 %) had unchanged $CD8^+$ cells (median [range] fold change of – 0.85 [0.8 – 1.1]). In two patients with evaluable RNA, cetuximab induced rapid tumor transcriptome changes in cellular Type 1 Interferon signaling and keratinization pathways.

Conclusions

Within one week, cetuximab induced measurable changes in pro-cytotoxic T-cell signaling and immune content.

Introduction

The anti-EGFR antibody cetuximab remains a component of multi-agent treatment paradigms for primary, recurrent, and metastatic head and neck squamous cell carcinoma (HNSCC), albeit with overall modest response rates and survival benefit.[1-3] Cetuximab promotes anti-tumor immunologic activity through antibody-dependent cellular cytotoxicity (ADCC), priming NK cells and EGFR-specific cytotoxic T-lymphocytes to target malignant cells.[4] The immunologic activity of cetuximab has particular importance in a rapidly-evolving era of immunotherapy for HNSCC.[5] Novel combinatorial trials of cetuximab with anti-PD-1 inhibitors have begun to emerge, suggesting potential synergy and improvement in patient outcomes in unresectable recurrent or metastatic (R/M) HNSCC.[6,7] As these new therapies are evaluated, there is a need to co-develop predictive biomarkers in order to facilitate patient selection onto the most effective therapy.

To date, most biomarker strategies in this space have leveraged pre-treatment features and little attention has been paid to using mid-treatment biomarker approaches. Without midtreatment biopsies, the temporal dynamics of how cetuximab treatment alters the tumor immune-microenvironment (TIME), and how these changes may influence activity of the PD-1/PD-L1 and CTLA-4 axes in HNSCC remain poorly understood. In two separate Phase II trials of patients treated with cetuximab monotherapy, Jie et al showed a dynamic increase in PD-1^{+}TIM-3^{+} cytotoxic tumor-infiltrating lymphocytes (TILs) and FOXP3^{+}CTLA4^{+} regulatory TILs in the HNSCC TIME four weeks after cetuximab infusion.[8,9] These TIL populations attenuated cetuximab-mediated ADCC *in vitro* and correlated with poor clinical response and outcomes. However, these TIME alterations were not uniform; other critical immune pathways altered by cetuximab likely exist and require further characterization. These pathways may mediate differential response to subsequent immunotherapies in patients with R/M HNSCC previously treated with cetuximab.[10,11]

A better understanding of TIME changes influenced by cetuximab could lead to the identification of immunotherapy combinations specifically tailored to the aberrations in a patient's unique TIME. Toward this goal, our objective in the present study was to evaluate change in $CD8^+$ TIL content and TIME transcriptomes after a single cycle induction dose of cetuximab nested in a Phase II trial cohort of patients with p16-positive oropharynx cancer being treated with cetuximab and definitive radiotherapy.

Materials and Methods

This study was approved by the Michigan Medicine Institutional Review Board with a waiver of informed consent (HUM 00080561).

Patient Cohort and Specimens

Included patients were prospectively enrolled in a multicenter phase II trial of cetuximab and definitive radiotherapy for low-risk p16-associated oropharynx cancer, of which the clinical characteristics, treatment plan, and results have previously been reported.[12] Tumor p16 positivity (≥ 70% tumor cells with strong and diffuse nuclear and cytoplasmic staining on immunohistochemistry [IHC]) was used as a surrogate marker for HPV status. Patients received a cetuximab induction loading dose (400 mg/m^2) one week prior to start of radiotherapy with concurrent weekly cetuximab (250 mg/m^2). Each patient had biopsies of the primary tumor obtained prior to the loading dose and approximately one week later. All serial biopsies were obtained prior to the start of definitive radiotherapy. Tumor tissue obtained from surgical biopsy of the primary tumor was formalin-fixed and paraffin-embedded (FFPE) and banked for the studies described below.[13]

The trial enrolled a total of 42 patients; our cohort included eight of these patients. Reasons for exclusion of remaining patients from our analysis included lack of matched pre- and post-cetuximab biopsy specimens, insufficient tumor tissue on pre- and post-cetuximab biopsy specimens, and exhaustion of

available tumor tissue from pre- and post-cetuximab biopsy specimens prior to $CD8^+$ TIL quantification and transcriptomic analysis.

Immunohistochemistry for $CD8^+$ TILs

IHC for $CD8^+$ TILs was performed according to our lab's established heat-induced epitope retrieval protocol (CD8 antibody at 1:40 dilution [Novocastra VP-C320]).[13,14] Following hematoxylin and eosin (H&E) staining of sections from each FFPE block, our head and neck pathologist (J.B.M.) identified partial TMA cores, those with extensive tumor necrosis, and those consisting of < 50 % parenchymal tumor, to exclude from $CD8^+$ TIL scoring and subsequent DNA/RNA isolation.[13,14] Intratumoral $CD8^+$ TIL subsets were manually counted by the first author (JDS) at 200x magnification (20x objective lens). Mean TIL counts per triplicate tumor cores were calculated and averaged as previously reported.[13,14] GraphPad Prism (San Diego, Ca) software was used for summary descriptive statistics and to compare pre- and post-cetuximab induction dose TIL counts with student's t-test ($\alpha = 0.05$).

Transcriptome Sequencing and Quantification

In total, we identified two tumor-normal tissue pairs with sufficient material from pre- and post-cetuximab induction dose biopsy specimens for molecular analysis. Regions of sections with >60% tumor content were identified from the corresponding H&E by pathologist J.B.M. and regions were collected for RNA isolation using the Qiagen AllPrep Kit, as described.[15] RNA was subsequently advanced for next-generation sequencing (NGS) if samples met our previously defined quality standards by Qubit and Bioanalyzer analysis.[16,17] Total RNA was then submitted to the University of Michigan Advanced Genomics Core for library preparation and sequencing. Briefly, we used 500 ng of RNA for library preparations or as much RNA as was available with the Illumina TruSeq Stranded Total RNA library prep kit (Cat #: RS-122-2201/2). The protocol was followed exactly according to the manufacturer's recommendations, with a single modification that we used 14 PCR cycles to amplify the library prior to the final bead purification. The samples were then pooled and loaded on an Illumina HiSEQ4000 and

paired end sequenced to 75 nucleotide length in each direction. A summary of sequencing quality statistics including total unique mapped reads for each sample is provided in **Supplementary Table 1.** FPKM and TPM were quantified from the data as previously described. [18,19]

CIBERSORT and Gene Set Enrichment Analysis

Immune cell infiltrate content from bulk RNAseq data was assessed using the CIBERSORT algorithm.[20] Gene set enrichment analysis (GSEA) was performed on all genes using the software GSEA v4.03 from the Broad Institute (http://software.broadinstitute.org/gsea/index.jsp). Pre-ranked gene lists were prepared based on the log2-fold change in FPKM and the gene sets used were selected from the Molecular Signatures Database v7.0, including hallmark gene sets, motif gene sets, gene ontology (GO) gene sets and oncogenic signatures gene sets.

Results

Our focused cohort consisted of eight patients, all of whom were male with minimal comorbidities, tobacco and alcohol use history (**Table 1**).[12] All patients completed protocol treatment without interruptions or delays. With a median (range) follow-up duration of 7.2 (4.9 – 11.1) years, all remain alive with no evidence of disease recurrence.

The mean (SD) $CD8^+$ TIL count among all patients was 101.3 (66.6) before and 270.1 (236) after cetuximab induction dose ($p = 0.07$) (**Figure 1**). Five of eight patients (62.5 %) showed an increase in $CD8^+$ TILs after cetuximab loading dose, with a median (range) fold change of + 5.8 (2.5 – 15.8). The remaining three patients (37.5 %) had largely unchanged $CD8^+$ TILs after cetuximab induction dose, with a median (range) fold change of – 0.85 (0.8 – 1.1) (**Figure 1**).

Transcriptome sequencing and GSEA was performed on pre- and post-cetuximab loading dose biopsy specimens for patients one and two, the only two patients with sufficient tumor material remaining for

transcriptome analysis. The tumors of these two patients showed a + 15.7 and + 6.5-fold change in $CD8^+$ TIL count after cetuximab loading dose, respectively (**Figure 1**). Patient one's tumor showed a significant upregulation of transcripts involved in the innate immune response to IFN-α signaling, including interferon-induced protein 44 (*IFI44,* $\log_2$ fold change 3.14) and lymphocyte antigen 6 family member E (*LY6E*, $\log_2$ fold change 3.07). **(Figure 2)**. Conversely, patient one's tumor showed a concomitant downregulation of transcripts involved in epithelial cell keratinization, including small proline rich protein 2E (*SPRR2E,* $\log_2$ fold change -4.17) and (*LCE3D,* $\log_2$ fold change -3.32)**.** Patient two's tumor also showed downregulation of epithelial cell keratinization transcripts, including transglutaminase 3 (*TGM3*, $\log_2$ fold change -6.89) and cornifelin (*CNFN,* $\log_2$ fold change -6.20) but also transcripts essential for aerobic metabolism via the mitochondrial electron transport chain, including peptidylpropyl isomerase F (*PPIF*, $\log_2$ fold change -2.04). Transcripts essential to generation of adaptive immune responses to antigenic stimuli were significantly upregulated in the tumor of patient two, including immunoglobulin heavy constant gamma 1 (*IGHG1*, $\log_2$ fold change 4.55) and Bruton tyrosine kinase (*BTK*, $\log_2$ fold change 2.23) **(Figure 2)**. For a complete list of mapped transcripts and changes with cetuximab induction dose, see **Supplementary Table 2** and **3**.

Discussion

Our objective in the present study was to characterize the early changes in the TIME of patients with p16-positive oropharyngeal cancer after a single cetuximab infusion. We showed that cetuximab influenced rapid changes in $CD8^+$ TIL content and transcriptomic profiles in primary tumors that were measurable one week after initial infusion. Critically, different magnitudes of changing $CD8^+$ TIL content were observed in different patients. In three patients cetuximab did not alter $CD8^+$ TIL content, while increases were observed in the other five patients. As such, our findings are novel in that they suggest that TIME alterations that can be used to sub-divide populations can be identified in a clinically relevant timeframe that would enable timely patient selection for a personalized combinatorial immunotherapy paradigm. For example, the tumors that had enhanced $CD8^+$ TIL content may benefit most from combined

immunotherapy, while those with static or decreased content may better respond to other forms of intervention.

Jie et al have previously shown that cetuximab induced reliable and reproducible changes in TIL populations approximately 4 weeks after cetuximab infusion that correlated with treatment response in patients with HNSCC.[8,9] After a single neoadjuvant cetuximab dose, patients whose tumors failed to respond to treatment showed a significant increase in $CD4^+FOXP3^+$ regulatory TILs that co-expressed the immunoinhibitory CTLA-4 and TGF-β proteins.[9] Concomitantly, poorly-responsive tumors were characterized by a significant increase in $CD8^+$ TILs expressing PD-1 and TIM-3.[8] These TIME alterations were measured approximately one month after initial cetuximab infusion. The authors posited that the immunoinhibitory environment promoted by cetuximab in certain patients may be targeted by combinatorial blockade of PD-1, CTLA-4, and/or TIM-3 to break acquired resistance. Our data extends their discovery by suggesting that TIME alterations occur even earlier after initial cetuximab infusion.

An early biomarker timepoint of response to cetuximab would be a valuable tool when developing combinatorial regimens in HNSCC. In a single arm Phase II trial, the combination of cetuximab and pembrolizumab had an impressive response rate compared to either agent alone, suggesting synergy in R/M HNSCC.[6] While an encouraging result, the TIME characteristics that predict poor rates and durability of response to this combination are unknown. $CD8^+$ TIL content does correlate with response to immune checkpoint inhibition in various HNSCC populations,[21] but few studies have been able to assess how early dynamic changes in TIL content correlate with outcome.

Due to our small cohort size, we were not able to perform analysis to correlate change in $CD8^+$ TILs with response to cetuximab, recurrence, and survival. Further, our study utilized limited primary tumor biopsy specimens and our results thus do not account for the vast intra- and intertumor heterogeneity in HNSCC TIME immune cell content, spatial distribution and transcriptomic programs.[27] Tumor HPV positivity was

determined solely by p16 IHC. While confirmatory HPV DNA ISH is not routinely indicated per expert guidelines, there is a small subset of p16 IHC positive tumors that lack HPV DNA.[28,29] We did not have sufficient tumor specimen to profile early alterations in markers of T-cell exhaustion in the TIME. Our transcriptomic data suggests that cetuximab induces rapid changes in transcriptional programs for diverse cellular processes related to epithelial cell differentiation and keratinization, oxidative phosphorylation, and innate and adaptive immune responses. However, we lacked sufficient tumor material for downstream validation of these findings, which could be a focus of future research in this area. Nevertheless, these preliminary findings provide support for further in-depth characterization of rapid changes induced by cetuximab and their potential utility as early predictive biomarkers. Indeed, with the recent technological advances in spatial transcriptomic analysis of FFPE specimens, early-treatment biomarkers have great potential in the near future.[30] Further, while our field is still in the early phases of advancing mid-treatment liquid (plasma, saliva, urine, etc.) biomarkers for cancer monitoring,[31,32] our data suggests that as trials for liquid biopsy based biomarkers are advancing, mid-treatment tumor biopsies may also be a useful tool for characterizing the biologic response to systemic therapy and assist with thoughtful development of improved, more precise immunomodulatory therapies.

Conclusions

Herein, we showed that cetuximab induces rapid alterations in the TIME of primary HNSCC just one week after a single induction dose. In a rapidly evolving era of combinatorial immunotherapies for primary, recurrent, and metastatic HNSCC, we hypothesize that this early dynamic response in $CD8^+$ TIL content coupled with more in-depth characterization of tumor transcriptomic changes may serve as a useful, early biomarker for adaptive treatment selection paradigms.

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Table and Figure Captions

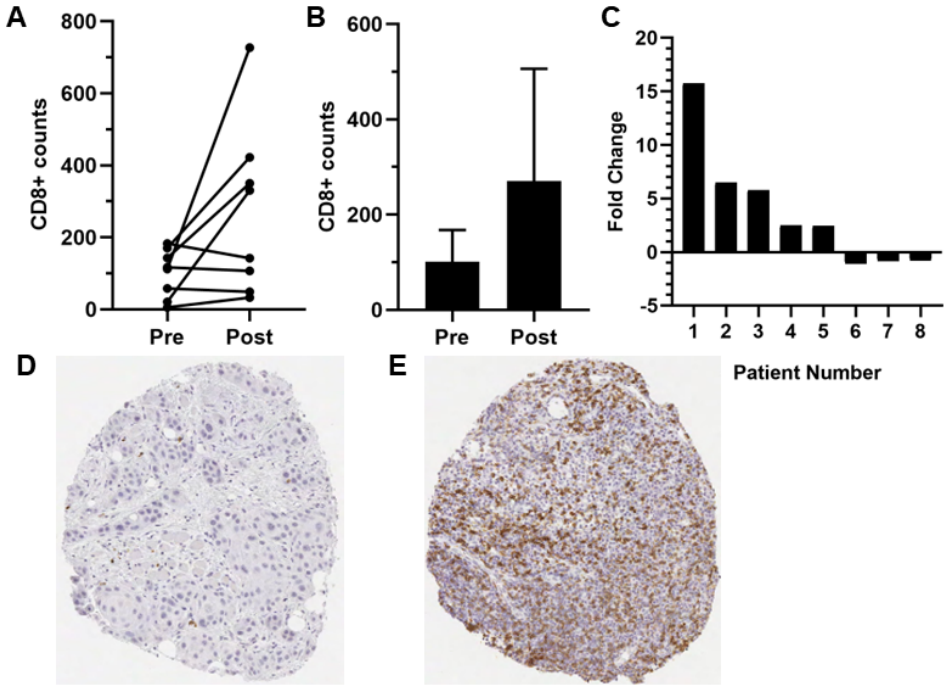
TABLE 1. Patient characteristics (n = 8). All had p16-positive squamous cell carcinoma of the oropharynx. Patient 4 was a former tobacco user of < 10 pack-years, the remaining patients were never-smokers. Patients were staged according to the American Joint Committee on Cancer (AJCC) 7th edition staging manual.

FIGURE 1. CD8$^+$ TIL content before (pre) and after (post) cetuximab induction dose, per patient (**A**). Mean (SD) CD8$^+$ TIL content before (pre) and after (post) cetuximab induction dose (**B**). Fold change in CD8$^+$ TIL content after cetuximab treatment (**C**). Representative tumor cores showing marked change in CD8$^+$ TILs before (**D**) and after (**E**) cetuximab treatment.

FIGURE 2. GSEA plots showing transcriptomic programs significantly upregulated (A) or downregulated (B) after cetuximab loading dose in patients one and two. ES: enrichment score; FDR: false discovery rate.

SUPPLEMENTARY MATERIAL.

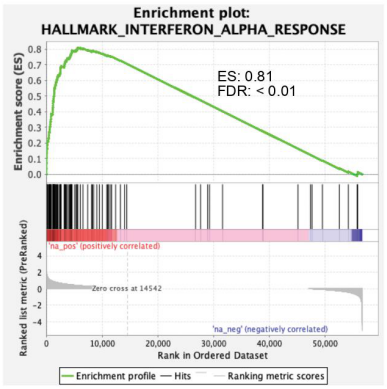
Transcriptome sequencing quality statistics (Supplementary Table 1). Comparative summary of mapped transcripts, patient one (Supplementary Table 2) and patient two (Supplementary Table 3). CIBERSORT summary (Supplementary Table 4). GSEA summary (Supplementary Table 5).



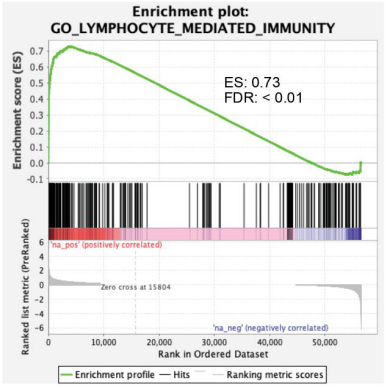
HED_27344_UMCC2009078 Manuscript Figure 1.png

A

Patient 1 (Post/Pre_Cetuximab)

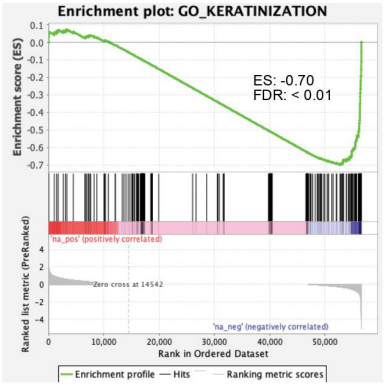


Patient 2 (Post/Pre_Cetuximab)

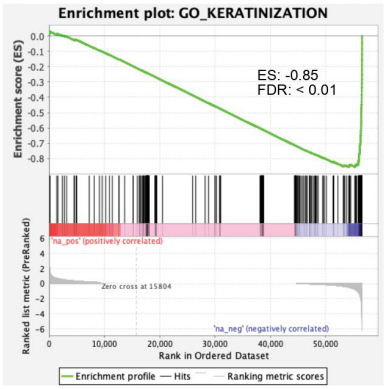


B

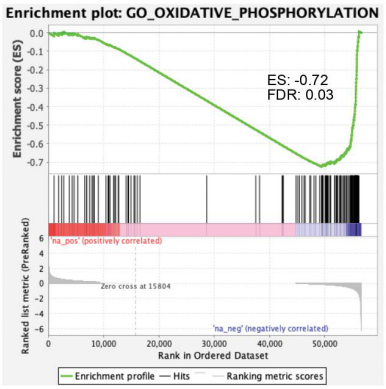
Patient 1 (Post/Pre_Cetuximab)



Patient 2 (Post/Pre_Cetuximab)



Patient 2 (Post/Pre_Cetuximab)



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Patient No.	Sex	Age, years	Charlson comorbidity index (CCI)	Primary Site	T Stage	N Stage	M Stage	Overall Stage
1	Male	76	2	Tongue base	1	2b	0	3
2	Male	71	2	Tonsil	3	2b	0	4
3	Male	57	2	Tongue base	2	1	0	3
4	Male	55	2	Tongue base	2	2b	0	4
5	Male	61	2	Tongue base	2	2b	0	4
6	Male	60	3	Tonsil	2	2b	0	4
7	Male	53	2	Tonsil	2	2a	0	4
8	Male	68	3	Tongue Base	2	2c	0	4