Th2 cells are associated with recurrence following radiation in human papillomavirus negative head and neck cancer.

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Short Report

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Abstract

Background

Human papillomavirus (HPV)-negative head and neck squamous cell carcinoma (HNSCC) leads to the death of over 360,000 patients annually worldwide. Curative therapy for this disease commonly consists of surgery followed by radiation therapy. Previously, we linked the expression of PD-L1 with clinical radioresistance in HNSCC; however, the relationship between outcome following radiation and immune function is unclear.

Methods

We used two well-annotated cohorts to examine clinical outcomes. The discovery cohort included 94 patients diagnosed with HPV-negative HNSCC who were treated uniformly with surgery and adjuvant radiation. The validation cohort consisted of 97 patients with similar treatment. Immune infiltrates in tumors were derived from RNAseq gene expression in tumor tissues using xCell. The association between each immune cell type and clinical outcomes was tested using Cox proportional hazards models. Immune cell types significantly associated with overall survival (OS), distant metastasis (DM), or locoregional recurrence (LRR) in the discovery cohort were examined in the independent validation cohort. Genes that carry nonsynonymous somatic mutations or have mRNA expression that is associated with the abundance of CD4 + T helper 2 (Th2) cell infiltrate in tumors were also identified in the full TCGA HPV-negative HNSCC cohort as well as nonoverlapping cohorts from the International Cancer Genome Consortium (ICGC) and the National Cancer Institute's Clinical Proteomic Tumor Analysis Consortium (CPTAC).

Results

In the discovery cohort, we identified intratumoral cell types significantly associated with OS (monocytes, CD4 + memory T cells & Th2 cells), DM (CD4 + memory T cells, sebocytes and epithelial cells) or LRR (common lymphoid progenitor (CLP) cells, CD4 + memory T cells, Th2 cells, and immature dendritic cells (iDCs). However, only a positive association between high levels of Th2 cells and LRR was identified in the validation cohort. We also identified *CREBBP/EP300* and *CASP8* among the genes that carry somatic mutations significantly associated with the presence of Th2 cells. Additionally, pathway analysis of genes correlated with Th2 cells revealed potential repression of the antitumor immune response and activation of BRCA1-associated DNA damage repair in multiple cohorts.

Conclusions

Th2 infiltrate is enriched in HPV-negative HNSCC tumors and is associated with LRR in two independent cohorts of patients following surgery and radiation. Th2 infiltrate is associated with mutations in *CASP8* and *CREBBP/EP300* and pathways previously shown to impact the response to radiation. Further investigation is warranted to investigate the mechanisms underlying the role of Th2 cells in mediating radioresistance in HPV-negative HNSCC.

Introduction

Head and neck squamous cell carcinoma (HNSCC) leads to the death of over 360,000 patients annually worldwide[1]. Generally, curative therapy for this disease consists of surgery, radiation or a combination of the two, with the most aggressive tumors additionally receiving cytotoxic chemotherapy as a radiation sensitizer. Outcomes following therapy are driven by tumor characteristics as well as human papillomavirus (HPV) status, with HPV-positive tumors exhibiting far more robust responses to therapy and better outcomes than HPV-negative tumors[2].

HNSCC is unique compared to most other cancers in that locoregional recurrence (LRR) following local therapy, and not distant metastasis (DM), is the most common cause of death due to disease[3]. Thus, resistance to radiation is a direct cause of death in HNSCC and is particularly prominent in HPV-negative disease. However, in HPV-negative tumors, there are no clinically utilized biomarkers of therapeutic resistance or any biologically driven sensitizers currently in standard use. In these tumors, mutations in genes such as *TP53*, *CREBBP*, *EP300*, and *CASP8* predict radioresistance and are associated with worse disease-free survival[4–7].

In addition to its direct cytotoxic effects, the response to radiation therapy also relies on immune activation to exert maximal antitumor effects[8]. This is via multiple pathways, including, but not limited to, the release of tumor antigens and proinflammatory cytokines/chemokines as well as the activation of tumor-infiltrating lymphocytes (TILs) and dendritic cells[9,10]. Furthermore, overexpression of PD-L1 has been associated with worse local control following radiation[11]. These observations, coupled with the activity of anti-PD1 in treatment-refractory HNSCC, led to the exploration of concurrent immunotherapy and chemoradiation in this disease[12,13]. While several trials exploring the combination of radiation and immunotherapy in this setting are ongoing, a large trial combining anti-PD1 with curative intent chemoradiation in an unselected patient population was closed early due to futility[13]. This finding highlights the lack of understanding of the interactions between the antitumor immune response and radiation, particularly radiation delivered in conventional doses and fractionation schedules (1.8–2.2 Gy per day over 30–35 days).

In the current study, we utilized two populations of HPV-negative HNSCC treated with surgical resection followed by adjuvant conventionally fractionated radiation to examine tumor-infiltrating lymphocytes associated with LRR. Pre-treatment tumors from both cohorts were digitally dissected for the abundance of TILs using an integration of gene set enrichment analysis and deconvolution approaches (xCell).

Univariable and multivariable analyses for the association between immune cell type and clinical outcome were performed in the discovery cohort. Significant cell types from this analysis were then examined in the independent validation cohort. High Th2 infiltrate was identified to be significantly associated with locoregional recurrence in both cohorts. Analysis of somatic mutations associated with Th2 infiltrate from whole-exome sequencing data of the entire head and neck cancer genome atlas (TCGA) (opposed to our subset analysis of uniformly treated patients above) identified genes directly associated with immune response (HLA-A) and those previously identified as associated with radiation resistance in HNSCC (*EP300*, *CASP8*)[7,14]. Gene expression data from the same cohort identified DNA damage repair and regulation of the cell cycle as key pathways associated with Th2 infiltrate in HNSCCs.

Methods

Clinical outcome datasets

All studies were approved by the institutional review board (IRB) where applicable. The discovery cohort consists of a total of 94 patients from the HN TCGA cohort with HPV-negative HNSCC treated with surgery and postoperative radiation, for which detailed treatment and clinical outcomes are known and published previously[14]. The validation cohort consisted of 97 patients with HPV-negative HNSCC treated with surgery and postoperative radiation for which expression data necessary for TIL prediction were available, with tumor characteristics and gene expression data published previously[11].

Genomic and proteomic datasets

RNA sequencing data available for the discovery cohort as well as the full Head and Neck TCGA cohort have been previously described and downloaded from cBioPortal[15], while Illumina mRNA array data from the LRR validation cohort have been published previously[16]. Unique (eg. nonoverlapping with TCGA) RNA sequencing data from the International Cancer Genome Consortium (ICGC) data portal[17,18] (accessed 10/1/2021) were used to validate the pathway analysis. A separate pathway validation was performed using proteomic data from the National Cancer Institute's Clinical Proteomic Tumor Analysis Consortium (CPTAC) Head and Neck cohort (Accessed 11/1/2021)[19].

Digital dissection of the tumor microenvironment for TIL abundance

RNAseq data from both the discovery and validation cohorts were used for the prediction of TIL abundance in tumors using xCell[20]. xCell quantifies 64 immune, stromal, and tumor cell abundances in a heterogeneous tumor microenvironment from tissue RNA-seq data[20]. Analysis was performed using the web server xCell: https://xcell.ucsf.edu/ (analyzed 9/10/2021). Immune cell types present in at least 15 tumors and significantly associated with clinical outcome in the discovery cohort were selected for further validation using the validation cohort.

Identification of somatic mutations associated with Th2 cell infiltrates

Somatic mutations significant in the TCGA HPV-negative HNSCC cohort by MutSig[21] and identified as cancer genes in OncoKB[22] were determined. Average derived Th2 infiltrate was compared between mutant versus wild type for each mutation using one-way ANOVA and post hoc comparison (SPSS v27). Unadjusted p-values are shown for survival analysis. For pathway analysis, the multiple comparisons effect was adjusted using the Benjamini-Hochberg (BH) method[23].

Identification of genes positively or negatively correlated with Th2 cell infiltrates by expression. Ingenuity pathway analysis (IPA) (Qiagen) was performed to evaluate genes with at least a 1.5-fold difference between the upper and lower tertiles of derived Th2 infiltrate at an FDR of 0.05 in the HPV-negative TCGA cohort (n=409). Pathway activation scores for the “BRCA1 in DNA damage response” and the “Tumor immune microenvironment” pathways were then generated using the following method. Th2 scores were initially divided into tertiles. Then, if the pathway activation z-score was > 0 (activated in the highest Th2 tertile), all expression values with log Fold change (FC) > 0 comparing Th2 level highest tertile vs. lowest tertile were unconverted, while all expression values with log FC < 0 in Th2 level using this comparison were multiplied by -1. Conversely, if the pathway activation z-score was < 0 (suppressed in the highest Th2 tertile), all expression values with log FC < 0 using the same Th2 tertile comparison were unconverted, while expression values with log FC > 0 using this comparison were multiplied by -1. Following these conversions, the mean expression for all genes or proteins in a pathway was calculated to give the pathway score.

Survival analysis

Overall survival (OS) was defined as the time of diagnosis until death or the last follow-up. Time to locoregional recurrence (LRR) or distant metastasis (DM) was defined as the time from diagnosis until either an event or the last follow-up. Clinical variables included site (oral cavity, oropharynx and larynx/hypopharynx), nodal stage (0-1 versus ≥2a) and tumor stage (1-2 versus 3-4). xCell z-scores were transformed by $\log_{10}$ (score * 100 + 1) for the survival analysis. A univariable Cox proportional hazards (PH) model was used to test the associations between the abundance of each immune infiltrate and OS, DM or LRR. Variables significant on univariable analysis were included in multivariable analysis where indicated. Kaplan-Meier analysis (SPSS (v27)) was performed using a log-rank test comparing the lower versus upper two tertiles of derived Th2 cells in both the TCGA and institutional cohorts.

Results

Our discovery cohort consisted of 94 patients from the HN TCGA cohort treated with surgery and postoperative radiation for whom detailed treatment and outcomes, including patterns of failure, are known, which is not available for the full TCGA cohort. The tumor characteristics of this subset are shown in Table 1. All patients had HPV/p16-negative tumors and were treated with ~ 60 Gy following surgical resection. Most patients had tumors of the oral cavity (63.8%) or larynx/hypopharynx (31.9%), with a small number of oropharyngeal tumors (4.3%), reflecting the higher proportion of HPV-positivity of these tumors as well as the usual nonoperative management of these tumors. The majority of tumors

were staged as either T3 (24.5%) or T4 (59.6%), with close to half (48.9%) found to have nodal involvement following resection.

Table 1
Tumor characteristics of the discovery cohort.

Nodal stage	n	Percent
0	44	46.8
1	20	21.3
2x	1	1.1
2a	2	2.1
2b	13	13.8
2c	8	8.5
3	2	2.1
x	4	4.3
Tumor stage		
1	1	1.1
2	12	12.8
3	23	24.5
4	56	59.6
x	2	2.1
Site		
Oral cavity	60	63.8
OPX	4	4.3
Larynx/hypopharynx	30	31.9

Th2 cell type is significantly associated with clinical outcome in two similarly treated HNSCC cohorts.

To identify an association between outcome and pretreatment TILs and other cell types, we initially examined the discovery cohort and investigated the association of clinical variables (tumor stage, nodal stage and site) and cell types (as a continuous variable) with overall survival (OS). We found that nodal stage was significantly associated with OS ($p = 0.03$). The presence of monocytes (HR 2.1, 95% CI 1.008–4.34, $p = 0.05$), Th2 cells (HR 6.1, 95% CI 1.7–21.7, $p = 0.005$) and CD4 + memory T cells (HR 5.9, 95% CI 1.7–20.6, $p = 0.006$) was associated with worse OS in this cohort (Fig. 1a). On multivariable Cox

regression analysis including both the immune cell types as well as nodal stage, nodal stage (p = 0.008), monocytes (p = 0.02), and Th2 cells (p = 0.008) remained significant.

We next evaluated the association between intratumoral cell populations and distant metastasis (DM) or locoregional recurrence (LRR). Time to DM was significantly associated with higher nodal stage (p = 6.4e-4). Additionally, we found that CD4 + memory T cells (HR 11.63, 95% CI 1.8–75.9, p = 0.01), sebocytes (HR 0.068, 95% CI 0.01–0.48, p = 0.007) and epithelial cells (HR 8.7e-3, 95% CI 3.56e-4–0.21, p = 0.01) were significantly associated with DM on univariable analysis (Fig. 1b). After adjusting for nodal status in a multivariable model, the presence of CD4 + memory cells remained significantly associated with DM (p = 0.012). We next examined LRR and found no significant association with any clinical variables evaluated. However, LRR was significantly associated with specific immune infiltrates, including common lymphoid progenitor cells (CLPs) (HR 9.5 95% CI 1.21–74.9, p = 0.03), Th2 cells (HR 9.0 1.4–57.0, p = 0.02), CD4 + memory T cells (HR 7.351843, 95% CI 1.3–41.3, p = 0.024) and immature dendritic cells (IDCs) (HR 0.34, 95% CI 0.17–0.67, p = 0.002) (Fig. 1c).

To validate the associations seen between intratumoral cell types and clinical outcome, we used gene expression data from an independent patient population treated in a similar fashion (e.g., surgery and postoperative radiation)[11]. We repeated the same analysis using xCell to predict cell type abundance in this validation cohort and focused only on the cell types identified to be significant in the discovery cohort. Regarding OS and DM, none of the cell types identified in the discovery cohort were found to be significant in the validation cohort. However, we found that the presence of Th2 cells was associated with significantly worse LRR both in the discovery and validation cohorts when the same cutoff value was used (lower tertile vs. others) (p = 0.019) (Fig. 1d).

Somatic mutations and gene expression associated with Th2 cell infiltration

To explore the potential relationship between tumor signaling and derived Th2 infiltrate, we initially examined differences in the presence of Th2 based upon tumor mutation in the entirety of the HPV-negative Head and Neck TCGA cohort for which both mutational and gene expression data were available (n = 406). Mutations in several genes, specifically *CASP8*, HRAS, and HLA-A, were associated with an increased presence of derived Th2 infiltrate (Fig. 2a). Although neither *EP300* nor *CREBBP* mutation was individually associated with increased Th2 infiltrate, if taken together, mutations in these functionally similar histone (or lysine) aceytltransferases were also associated with increased Th2 infiltrate (p = 0.042, Fig. 2a).

We next examined the relationship between Th2 infiltrate and gene expression using RNA-seq data in the HPV-negative Head and Neck TCGA cohort, for which only gene expression data were required (n = 409). These data were analyzed using IPA, as described in the Methods, to identify pathways associated with derived Th2 infiltrate. This analysis identified several cancer- and immune-related pathways (hits related to cancer or immune response shown in Fig. 2b**).** For instance, the “BRCA1 in DRR” pathway positively

and the “Tumor microenvironment (TME)” pathway negatively associated with Th2 infiltrate. Signaling nodes and expression patterns for both pathways are shown in Fig. 2c and Fig. 2d, respectively, with nodes significantly positively and negatively correlated with derived Th2 cells.

To confirm the relationship between these two pathways and derived Th2 infiltrate, pathway activation scores were generated as described in the Materials & Methods. As expected, a high degree of positive correlation was observed between derived Th2 infiltrate and BRCA1 in the DDR pathway ($r = 0.73$, $p < 2.2e-16$), while a significant negative correlation was observed with the TME pathway ($r=-0.45$, $p < 2.2e-16$) (Fig. 3a). We next generated pathway activation scores using gene expression data from a separate nonoverlapping HPV-negative HNSCC cohort and observed similar associations with derived Th2 infiltrate (Fig. 3b). Specifically, derived Th2 infiltrate was correlated with activation of BRCA1 in the DDR pathway ($r = 0.77$, $p = 5.6e-9$) and repression of the TME pathway ($r=-0.44$, $p = 0.007$). We also analyzed proteomic data from the separate HPV-negative Head and Neck CPTAC database and again found the same pathway associations, with derived Th2 infiltrate associated with activation of BRCA1 in DDR ($r = 0.59$, $p < 2.2e-16$) and repression of the TME pathway ($r=-0.48$, $p = 1.6e-7$) (Fig. 3c).

Discussion

Patients with HNSCC are currently stratified by HPV/p16 status and smoking history, with HPV/p16-negative disease and ≥ 10 pack years of smoking history having worse prognosis and disease control. However, there is a large variance in response to therapy within these groups, and there is a significant unmet clinical need for further development of molecular, histological, and gene expression prognostic markers to guide therapy for patients. Previously, several attempts have been made to characterize HNSCC tumors based on immune infiltrate and potentially link these factors to outcome following standard therapy. While interesting, these efforts have been hampered by small numbers, the heterogeneity of HNSCC itself, and a lack of uniformly treated patients[24–26].

A prior study published by our group demonstrated that high PD-L1 expression was associated with increased local recurrence after treatment with surgery and postoperative radiation in patients with HPV-negative HNSCC tumors[11]. In contrast, in this same population, patients with high CD8 TIL infiltrate and low PD-L1 expression had no local failures or death due to disease[11], suggesting that pretreatment immune infiltrates in the TME have a strong influence on outcomes. In the current study, we build upon this previous work to link an in-depth investigation of the TME in uniformly treated patients to elucidate additional prognostic biomarkers to predict treatment response in HNSCC.

While the role of TILs in solid tumors is well known, the focus of these investigations has primarily been on CD8 + or Th1 TILs as effector immune cells or regulatory T cells (Tregs) as suppressive immune cells. Conversely, Th2 cells and their signature cytokines (IL-4, IL-5, and IL-13) are largely characterized by their role in helminth infection or allergic responses and their promotion of IgE-mediated eosinophilic responses[27]. In the context of cancer, Th2 cells are generally considered to be protumorigenic[28]. However, there are reports of the tumoricidal properties of Th2 cells, including a preclinical study that demonstrated

that adoptive transfer of Th2 cells elicited a potent antitumor response by inducing a type II inflammatory response[29]. Reported potential protumorigenic actions of Th2 cells include recruitment and repolarization of macrophages to suppressive (M2-like) lineages[30], stimulation of the production of vascular endothelial growth factor (VEGF)[31], and production of immunosuppressive cytokines[28]. The variability of the Th2 response in different preclinical tumor models may be in part due to plasticity between Th1 versus Th2 polarization, with factors such as VEGF having been shown to repolarize T cells from a Th1 to a Th2 phenotype[32,] while PD1 blockade has been shown to revert the Th2 to Th1 phenotype[33]. The role of Th2 cells in the tumor microenvironment of clinical tumor samples is also unclear, with Th2 cells having been associated with aggressive tumor histologies, including HNSCC[34,] as well as histologies with better prognosis, such as Hodgkin's lymphoma[35]. Analogous to our findings in this report, a recent study by Rui et al demonstrated that high Th2 infiltrate in prostate cancer samples was associated with increased recurrence after prostatectomy[36].

In addition to validating the Th2 gene signature as a prognostic biomarker for local recurrence in HPV-negative HNSCC, we also examined genetic mutations associated with Th2 infiltrate. We found that mutations in HLA-A, *CASP8*, and HRAS correlated with the Th2 gene signature. Additionally, when taken together, *CREBBP* and *EP300* mutations were also associated with increased Th2 infiltrate. *CASP8* and *CREBBP/EP300* mutations were of particular interest since we had previously shown that both mutations lead to increased resistance to radiation in HNSCC[6,7]. However, caspase 8 has been shown to be critical in activating the nuclear factor-κβ (NF-κβ) pathway and causing the IL-1 secretion needed for the Th2 response[37]. This discordance between the dependence of Th2 function on caspase 8 and an association between *CASP8* mutation and Th2 infiltrate may be at least partially explained by cancer-associated missense mutations in *CASP8* associated with epithelial cancers that inhibit its apoptotic function but chronically activate NF-κβ signaling[38]. In patients whose tumors exhibit a Th2 gene signature, we also found increased expression of genes related to the cell cycle and DNA repair, which can confer radiation resistance. These effects may in part be through the effects of the Th2 cytokine IL-4, which has been shown to increase the expression of nonhomologous end joining DNA repair proteins and induce the proliferation of keratinocytes through c-myc-driven G_0/G_1 to S phase cell cycle progression[39,40].

While our findings have revealed a novel association between outcome and derived Th2 infiltrate in HPV-negative HNSCC, there are several limitations to our approach. Foremost, both our discovery and validation cohort analyses were performed in a retrospective fashion using prediction models for infiltrates based on mRNA expression data. Due to this limitation, further analyses of the TME in these samples, including direct TIL visualization, functional analysis of TIL infiltrates or measurement of cytokine levels/activity, were not possible and should be considered in the future via a prospective validation study. Despite these limitations, our study did reveal a potential new prognostic biomarker for HPV-negative HNSCC that could be utilized to inform future clinical trial design and possibly intensify treatment for patients with HPV-negative HNSCC with high Th2 infiltrate or high PD-L1 expression to improve survival.

Conclusion

Analysis of tumors from patients with HPV-negative HNSCC demonstrates the importance of pretreatment tumor immune infiltrate on treatment outcomes and locoregional recurrence. Increased Th2 immune infiltrate was associated with local recurrence in a discovery cohort and then confirmed in a separate validation cohort. These findings point to potential immunotherapeutic approaches to augment the response to radiation and decrease LRR in HPV-negative HNSCC patients.

Declarations

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Figures

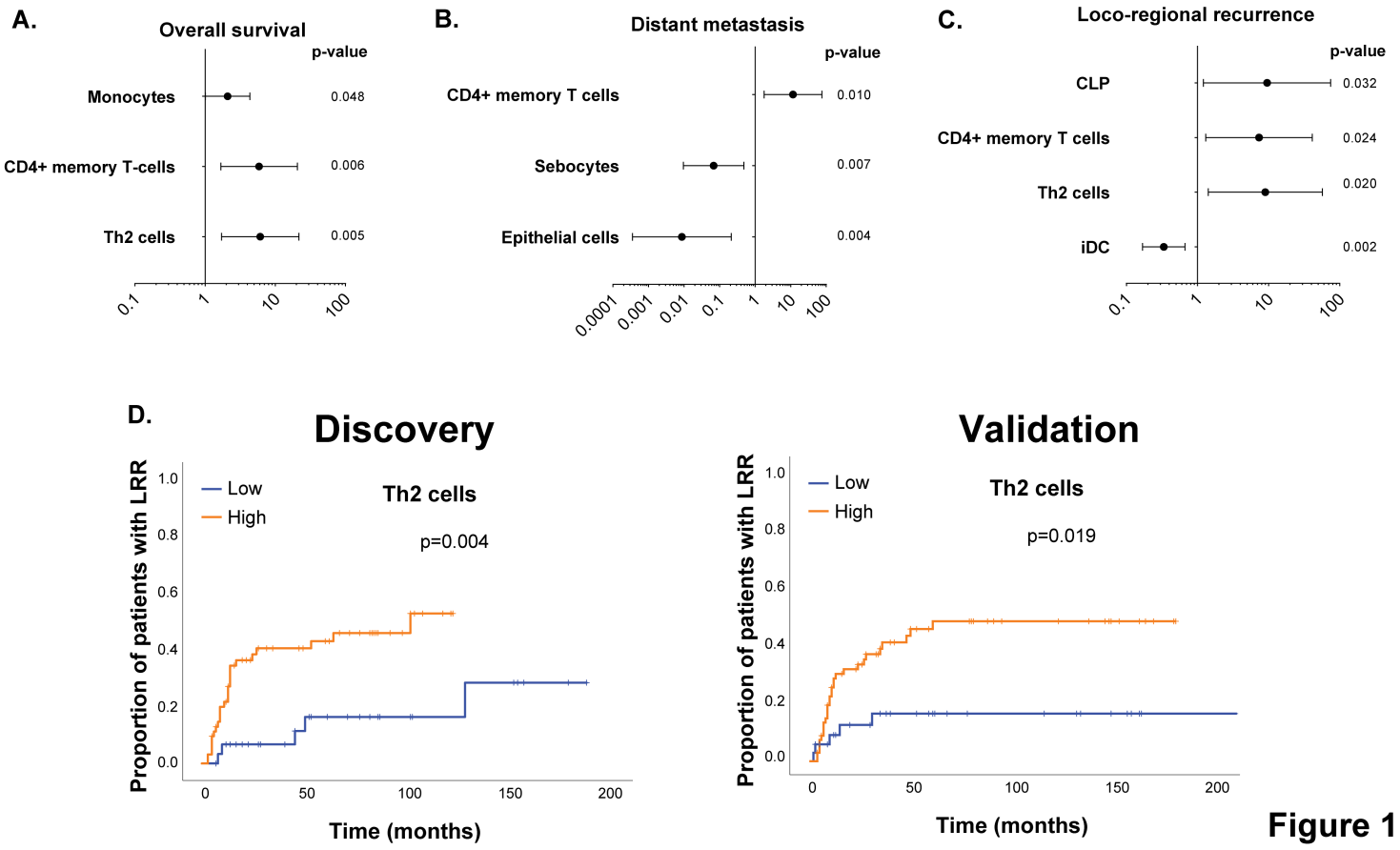

Figure 1

Figure 1

Association between immune subtype and outcome in HPV-negative HNSCC treated with radiation. A-C) Forrest plot of Univariable analysis for overall survival (**A**), distant metastasis (**B**) and locoregional recurrence (**C**) following surgery and adjuvant radiation. **D**) Kaplan-Meyer curve showing loco-regional recurrence as a function of high (upper two tertiles) versus low (lower tertile) Th2 infiltrate in the discovery and validation cohorts. Cox PH model was used in **A-C**. Log-rank test was used in **D**.

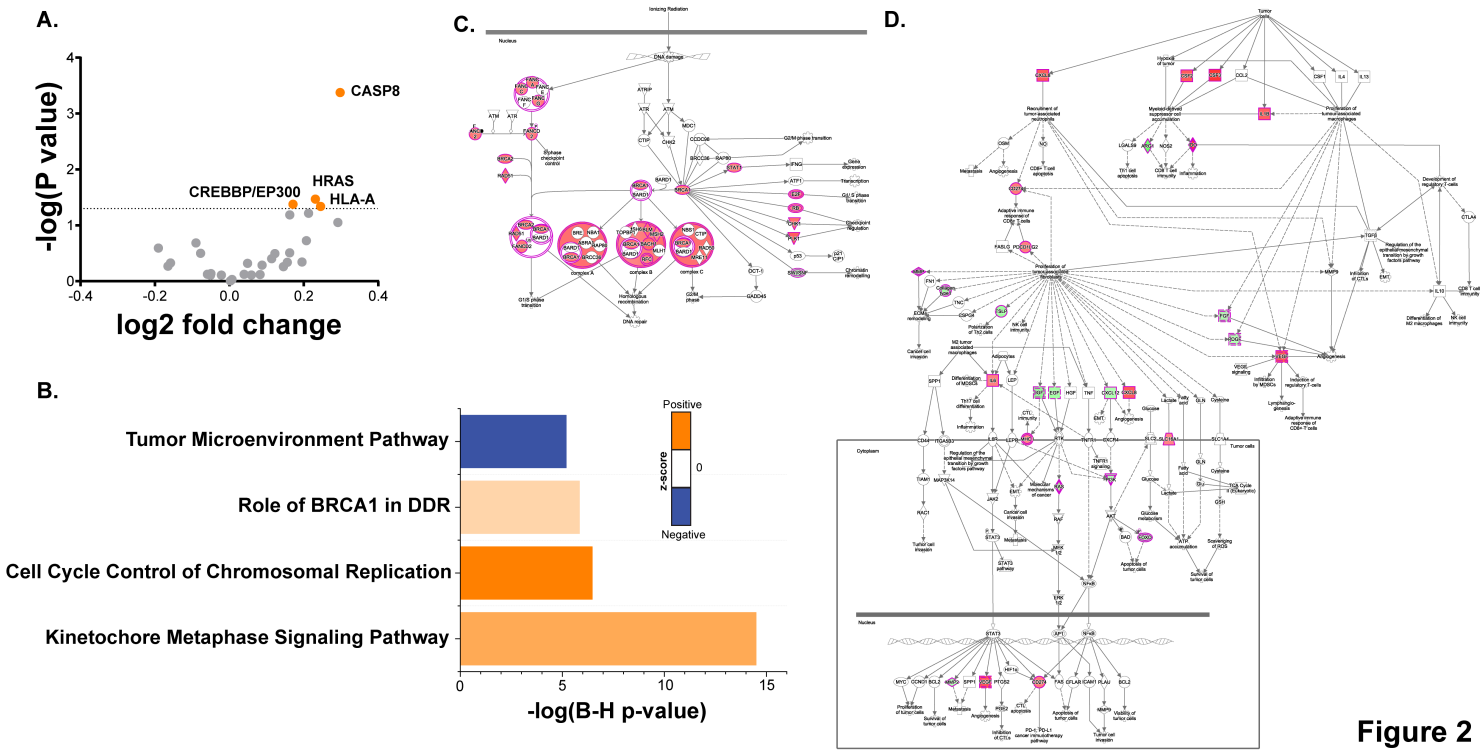

Figure 2

Figure 2

Tumor alterations associated with derived Th2 infiltrate. A) Volcano plot of mutations in HNSCC and Th2 infiltrate from the full HPV-negative TCGA HNSCC cohort with mutational and genes expression data (n=406). Mutations exhibiting significantly altered Th2 infiltrate (unadjusted p-value and fold change (mean mutant/mean wild type) shown, with orange indicating the mutation is associated with increased infiltrate. **B**) Ingenuity pathway analysis of genes associated with Th2 from the full TCGA Head and Neck cohort (n=409) including z-scores and B-H adjusted p-values. **C**) “Role of BRCA1 in DDR” and “Tumor Microenvironment Pathway” from IPA shown. Targets in red and green are significantly (FDR <0.05, fold change >1.5) positively and negatively correlated with derived Th2 infiltrate.

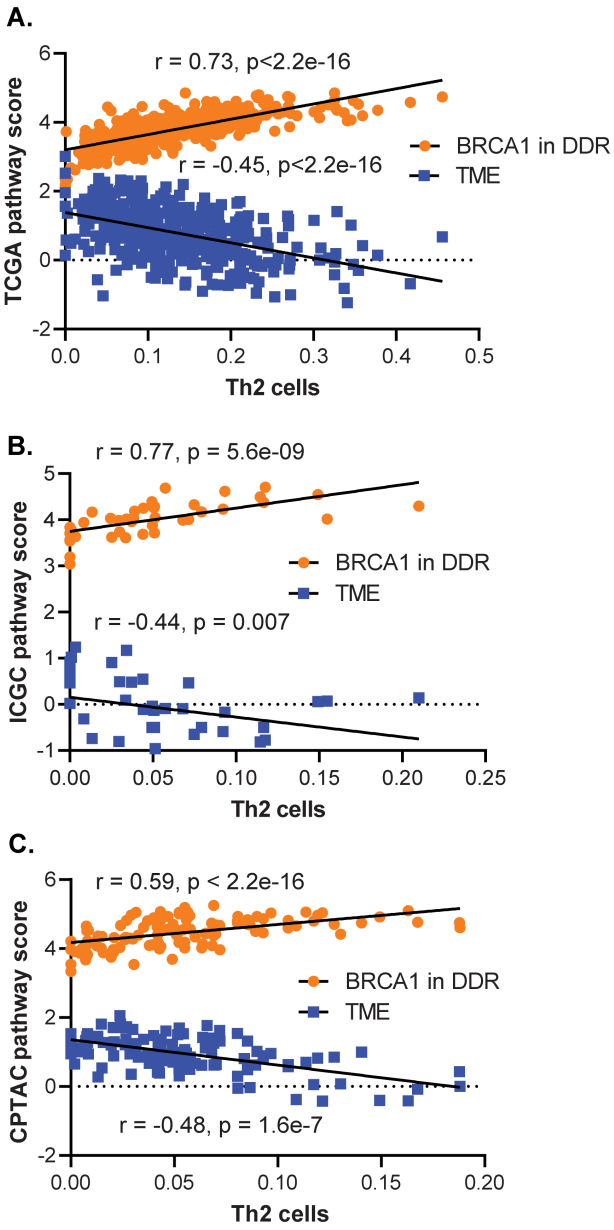

Figure 3

Figure 3

DDR and TME score are significantly associated with derived Th2 infiltrate in multiple clinical cohorts. BRCA1 in DDR and TME scores (as calculated in the Materials and Methods section) and derived Th2 infiltrate in the full HPV-negative TCGA cohort with gene expression data available (n=409) (**A**), non-overlapping HPV negative HNSCC patients from the International Cancer Genome Consortium (ICGC) data (n=40) (**B**) and the National Cancer Institute's Clinical Proteomic Tumor Analysis Consortium (CPTAC) Head and Neck cohort (n=110)(**C**).