Comparison of the Tumor-Immune Microenvironment and Checkpoint Blockade Biomarkers Between Stage III and IV Non-Small Cell Lung Cancer

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INTRODUCTION

Immune checkpoint blockade (ICB) therapies have emerged as an essential treatment modality in non-small cell lung cancers (NSCLC). While ICBs have earned multiple FDA approvals for the first-line treatment of stage IV NSCLC, approvals for locally advanced or stage III NSCLC have been relatively limited.

To characterize the differences in the landscape of the tumor immune microenvironment (TIME) between stage III and stage IV tumors, we combined de-identified RNA-seq, DNA-seq, immunohistochemistry (IHC), and real-world clinical data from the Tempus Oncology Database in a retrospective analysis of pre-treatment tumors from patients with unresectable stage III and IV non-squamous NSCLC.

METHODS

Cohort Selection

(1) Included de-identified records of patients with stage III or stage IV NSCLC with health records and tumor sample data within the Tempus oncology database.

(2) Excluded records of tumors with neuroendocrine or pseudosarcomatous carcinoma histologies.

(3) Included only data from primary tumor samples taken from either the lung or airway and submitted pre-treatment.

(4) Included only data from tumors staged within 30 days of biopsy collection.

PD-L1 Immunohistochemistry

PD-L1 tumor cell percentage was assessed by 22C3 pharmDx IHC assay (Dako North America, Inc.) through Tempus IHC testing or from abstracted clinical notes.

DNA & RNA Sequencing

Tempus|xT targeted panel (648 genes) DNA-seq and full-transcriptome RNA-seq were performed on primary tumor biopsies. Tumor mutational burden (TMB) was calculated using the number of nonsilent mutations/length of coding regions sequenced.

RNA Processing and Analysis

Reads from bulk RNA-seq were pseudo-aligned using kallisto (Bray et al., 2016). Raw counts underwent trimmed means of M-value normalization (TMM) and voom transformation using the R packages *edgeR* (Robinson et al., 2010) and *limma* (Ritchie et al., 2015), respectively.

The relative proportion of immune cells was estimated using a support vector regression (SVR) model (Beaubier et al., 2019).

Differentially expressed (DE) genes were identified using *limma*, with age, sex, histology, tissue source, smoking history, and tumor purity included as covariates in a multivariate linear model.

Gene set enrichment analysis (GSEA) in KEGG was performed using the R package *ClusterProfiler* (Yu et al., 2011) on DE genes (FDR < 0.05).

RESULTS

Table 1: NSCLC Cohort Overview

		Stage III (n=106)	Stage IV (n=285)
Female [n, (%)]		57 (55%)	138 (48%)
Age [median, (IQR)]		69 (62 - 76)	69 (61 - 76)
Any smoking history		85 (80%)	246 (86%)
Histology	*Adenocarcinoma*	96 (91%)	261 (92%)
	Adenosquamous carcinoma	2 (1.9%)	5 (1.8%)
	Non-small cell carcinoma	8 (7.5%)	19 (6.7%)
KRAS mutation		36 (34%)	124 (44%)
EGFR pathogenic mutation		11 (11%)	41 (15%)
TMB		4.0 (2.1-7.4)	3.8 (2.1-6.8)

Figure 1: PD-L1 IHC and *CD274* expression are highly correlated in the TIME of stage III-IV NSCLC

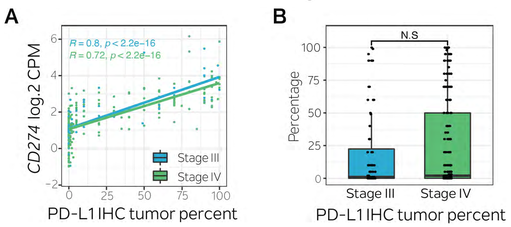


Figure 1. A) Scatter plot of PD-L1 IHC tumor percent and *CD274* RNA expression with regression trendlines. PD-L1 IHC and *CD274* expression in tumor were highly correlated in both stage III (P < 2.2e-16, R= 0.8) and IV (P < 2.2e-16, R = 0.72) tumors. **B)** Boxplot of PD-L1 IHC percentage in stage III and stage IV tumor samples. No significant differences in PD-L1 IHC levels or *CD274* expression by stage were observed.

Figure 2: The relative proportion of CD4+ T cells are increased in stage III compared to stage IV tumors

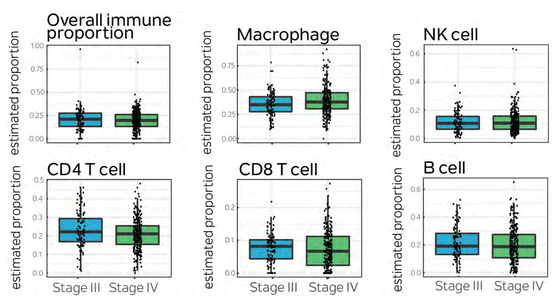


Figure 2. Boxplots of RNA-seq estimated immune cell proportion by stage. Stage IV tumors had a significantly increased proportion of macrophages relative to stage III (P = 0.017). The proportion of CD4+ T cells was significantly higher in stage III tumors compared with stage IV (P = 0.024).

Figure 3: Transcriptome-wide DE analysis between stage III and IV NSCLC tumors

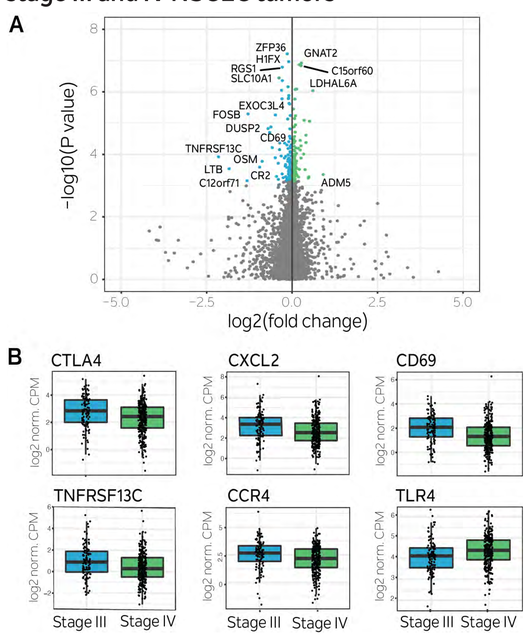


Figure 3. A) Volcano plot of gene expression differences between stage III and IV tumors. Of the 205 genes DE by stage (FDR < 5%), 91 had increased expression in stage III tumors (blue points), and 114 had increased expression in stage IV tumors (green points). **B)** Boxplots of immune-related DE genes. Notably, *CTLA4* expression was significantly increased in stage III tumors, however other immune checkpoint genes were not DE.

Figure 4: GSEA of DE genes revealed significant enrichment of immune pathways in stage III tumors

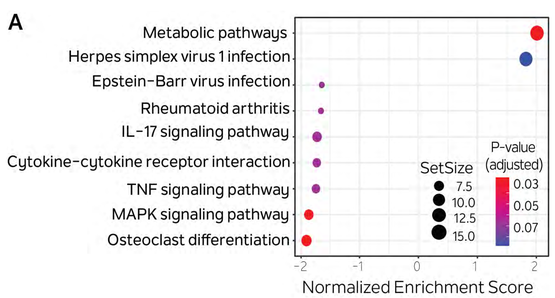
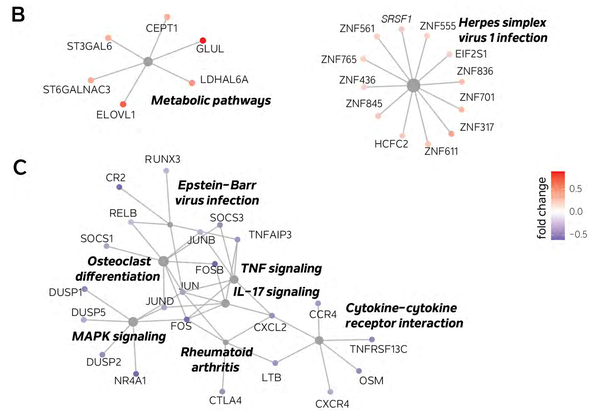


Figure 4A. Dotplot of KEGG pathway enrichment in 9 pathways with adjusted P-values < 0.1. Several immune regulatory pathways containing DE genes were enriched in stage III tumors.



B) Gene networks of enriched pathways enriched in stage IV tumors or **C)** stage III tumors. Immune-related networks in (**C**) were connected through *CXCL2* and Fos-Jun family genes.

CONCLUSIONS

- PD-L1 protein levels (measured by IHC) and *CD274* gene expression (measured by RNA-seq) were concordant in both stage III and IV NSCLC primary tumors, with no significant difference in PD-L1 or *CD274* levels by stage.
- Stage III tumors had a significantly increased relative proportion of estimated CD4+ T cells, and a significantly decreased relative proportion of macrophages compared to stage IV tumors, though overall immune infiltration did not differ by stage.
- Immune activation pathways were enriched in stage III tumors compared to stage IV tumors, suggesting a TIME with greater inflammatory activity and, paradoxically, increased inhibitory signaling due to increased expression of *CTLA4*.
- Taken together, these results suggest the evolution of metastasis in NSCLC tumors is accompanied by consequential changes in the TIME, with implications for ICB use in stage III tumors.

ACKNOWLEDGMENTS

The authors would like to thank the Scientific Communications Team for their assistance in poster design and development as well as the Tempus Medical Affairs team for helpful feedback and suggestions.

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