Original Article

Differential impact of intratumor heterogeneity (ITH) on survival outcomes in early-stage lung squamous and adenocarcinoma based on tumor mutational burden (TMB)

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Background: Molecular biomarkers are reshaping patient stratification and treatment decisions, yet their precise use and best implementation remain uncertain. Intratumor heterogeneity (ITH), an area of increasing research interest with prognostic value across various conditions, lacks defined clinical relevance in certain non-small cell lung cancer (NSCLC) subtypes. Exploring the relationship between ITH and tumor mutational burden (TMB) is crucial, as their interplay might reveal distinct patient subgroups. This study evaluates how the ITH-TMB dynamic affects prognosis across the two main histological subtypes of NSCLC, squamous cell and adenocarcinoma, with a specific focus on early-stage cases to address their highly unmet clinical needs.

Methods: We stratify a cohort of 741 early-stage NSCLC patients from The Cancer Genome Atlas (TCGA) based on ITH and TMB and evaluate differences in clinical outcomes. Additionally, we compare driver mutations and the tumor microenvironment (TME) between high and low ITH groups.

Results: In lung squamous cell carcinoma (LUSC), high ITH predicts an extended progression-free survival (PFS) (median: 21 *vs.* 14 months, P=0.01), while in lung adenocarcinoma (LUAD), high ITH predicts a reduced PFS (median: 15 *vs.* 20 months, P=0.04). This relationship is driven by the low TMB subset of patients. Additionally, we found that CD8 T cells were enriched in better-performing subgroups, regardless of histologic subtype or ITH status.

Conclusions: There are significant differences in clinical outcomes, driver mutations, and the TME between high and low ITH groups among early-stage NSCLC patients. These differences may have treatment implications, necessitating further validation in other NSCLC datasets.

Keywords: Non-small cell lung cancer (NSCLC); intratumor heterogeneity (ITH); tumor mutational burden (TMB); molecular biomarkers

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Introduction

Background

Tumor mutational burden (TMB), a measure of non-inherited mutations per megabase of DNA, has become an actively used clinical biomarker for response to immuno-oncology (IO) therapies. Among patients receiving IO therapy, a higher somatic TMB is significantly associated with better overall survival (OS) (1). Several trials have evaluated the relationship between TMB and response to IO therapy. Checkmate 026 phase III, POPLAR phase II, and Checkmate 012 phase I all showed a statistically significant relationship between high TMB and better outcomes in patients receiving IO therapy (2-4). In 2020, pembrolizumab was granted accelerated approval for use in resistant solid tumors in adults and children with TMB ≥10 (5).

Another biomarker currently under clinical consideration is intratumor heterogeneity (ITH), a term used to describe the genetic and epigenetic diversity within a single tumor (6). This diversity can arise from various factors, including spatial distribution, temporal evolution, and the influence of the tumor microenvironment (TME) (6). ITH has demonstrated a significant association with survival; the more subclones within a tumor, the shorter the recurrence-free survival and OS (7,8). It has also been implicated as a significant driver of T-cell Receptor (TCR) diversity, an important factor in post-surgical recurrence risk in localized lung adenocarcinoma (LUAD) (9). However, in pan cancer studies, LUAD and lung squamous cell carcinoma (LUSC) were among the three indications where ITH had no association with outcome (8). When applying more conservative ITH estimation methods, the relationship between ITH and outcome becomes less consistent and varies significantly among indications (10). The relationship between ITH, the TME, and outcome warrants further investigation.

Various methods have been developed over time to estimate ITH (11). Some, like MATH (12) and AFH (13), quantify heterogeneity directly through the distribution of variant allele frequencies, while others, such as PyClone (14) and SciClone (15), use allele read counts and copy number variation to estimate a clonal architecture. PyClone and SciClone require more computational power and higher sequencing depth, while MATH and AFH are simpler but less sensitive and specific.

Rationale and knowledge gap

TMB and ITH have been instrumental in distinguishing subgroups within clinical and research settings. Although TMB is an effective predictive biomarker in non-small cell lung cancer (NSCLC), this is only true for patients with high TMB receiving IO therapy. ITH has identified subgroups that, due to higher recurrence rates, could benefit from more frequent post-surgical screening (9).

TMB and ITH have been jointly utilized to investigate outcomes in melanoma patients, shedding light on the relationship between neoantigen load and its distribution among clones and subclones (13). It has been observed that immune surveillance and lymphocyte infiltration of tumors are primarily driven by clonal mutations, while those showing poor responses to immunotherapy often have a higher proportion of subclonal mutations (14). Despite both TMB and ITH being derived from similar tumor DNA sequencing techniques, they offer unique insights into tumor characteristics. By integrating these two molecular biomarkers, researchers can identify patient subgroups that are likely to benefit from existing and forthcoming IO therapies.

The relationship between TMB, ITH, and outcomes in LUSC and LUAD currently represents a knowledge gap that, if filled, could benefit a substantial population given the prevalence and mortality rate of NSCLC.

Highlight box

Key findings

- In early-stage lung squamous cell carcinoma (LUSC), high intratumor heterogeneity (ITH) is predictive of a longer progression-free survival (PFS), while in lung adenocarcinoma (LUAD) high ITH predicts a shorter PFS.
- This relationship is driven by the subset of patients with low tumor mutational burden (TMB).

What is known and what is new?

- Prior work has shown no statistically significant association between ITH and clinical outcomes in specific subtypes of non-small cell lung cancer.
- The relationship between ITH and clinical outcomes is dependent on histology and disease stage and is driven by the low TMB subset of the cohort.

What is the implication, and what should change now?

- In the context of molecular biomarkers, LUSC and LUAD each have a unique relationship with TMB and ITH. Furthermore, the disease stage has an impact on the predictive value of these biomarkers.
- Additional studies are warranted to explain why the predictive value of ITH is histology-dependent and driven by the low TMB.

Table 1 Characteristics of patients with early-stage NSCLC

Indication	LUSC (n=362)	LUAD (n=379)	P value
Age, years	68±9	65±10	0.002
Sex			<0.001
Female	101 [28]	203 [54]	
Male	261 [72]	176 [46]	
Race category			0.20
White	266 [73]	294 [78]	
Non-White	96 [27]	85 [22]	
Stage			0.01
I	219 [60]	263 [69]	
II	143 [40]	116 [31]	
PFS, months	19.3 (9.5–37.5)	18.6 (11–32.1)	0.60
OS, months	22.5 (11.7–43.9)	22.7 (14.5–39.1)	0.60
Tissue TMB	5.8 (4.2–8.5)	5.26 (2.2–10)	0.01
ITH	2.0 (1.1–2.8)	1.8 (1.0–2.7)	0.20

Data are presented as median (interquartile range), mean ± SD or number [percentage]. Patient demographics obtained from TCGA. TMB was calculated by dividing total mutation counts by 38 Mb and is reported in mut/Mb. ITH values were generated by PyClone (see Methods). P values for sex, race category, and stage are based on Chi-squared test; age is based on two-sided *t*-test; all others are based on two sided Mann-Whitney test. SD, standard deviation; PFS, progression-free survival; OS, overall survival; TMB, tissue mutational burden; mut/Mb, mutations per Megabase of DNA; ITH, intratumor heterogeneity; TCGA, The Cancer Genome Atlas; NSCLC, non-small cell lung cancer; LUSC, lung squamous cell carcinoma; LUAD, lung adenocarcinoma.

Objective

We aimed to evaluate the predictive value of a combined TMB and ITH patient stratification approach. We centered our analysis on early-stage NSCLC patients who have had successful primary tumor resections, a demographic characterized by high recurrence rates (16). Our combined approach is designed to help identify patient subgroups that could benefit from additional therapy, thereby improving long-term clinical outcomes. There has been no prior effort to utilize both ITH and TMB to stratify NSCLC patients. We selected The Cancer Genome Atlas (TCGA) dataset for this work due to its wide acceptance and high number of patients who have undergone a successful primary tumor resection and rigorous follow-up. The TCGA database has been used across large collection of studies to evaluate the relationship between tumor and patient characteristics and clinical outcomes. Here we cite several studies where LUSC and LUAD are specifically evaluated (17-22). We believe this to be an ideal cohort to study the relationship between recurrence risk and molecular biomarkers. Lastly, we included driver gene mutation prediction and TME immune cell enrichment into our analysis. We present this article in accordance with the REMARK reporting checklist (available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-24-226/rc).

Methods

Patient data

All patient data was sourced from TCGA and cBioportal. For all indications studied, TCGA single nucleotide variants (SNV), copy number variant (CNV), neoantigen load, and RNASeq patient data were obtained from GDCPortal (23). Clinical patient data was obtained from cBioPortal (24,25). Data for 927 NSCLC patients was available, with 362 LUSC and 379 LUAD patients remaining after excluding stage 3 and stage 4 patients and any patients without complete survival and molecular data. Age, sex, and full early-stage breakdown can be found in *Table 1*. All patients underwent primary tumor resection. Additional details on the methods of TCGA sample and data selection, collection, storage, and processing can be found on the GDCPortal (23). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Study design

Cases were selected based on the availability of clinical and molecular data through TCGA and associated databases. Cases were all retrospective and stratified based on histology (LUSC *vs.* LUAD) and molecular biomarkers (TMB and ITH). Time period, end of follow up period, and median follow up time can be found on the GDCPortal (23). Clinical endpoints were PFS and OS; definitions and calculation methods were defined by TCGA authors and can be found on the GDCPortal and the cBioPortal. Variables considered were disease histology, stage, TMB, and ITH. Sample size was not chosen specifically but rather was based on the total number of available cases.

TMB calculation

TMB was calculated using mutation counts obtained

from the PanCancer Atlas project, dividing the total number of mutations by 38 megabase. This approach to TMB calculation, specifically the choice of 38 Mb, was based on the methods used in several publications cited here (17,18,20-22).

ITH calculation

To estimate ITH, we used Pyclone (RRID:SCR_016873) (26), a Bayesian statistical model that estimates a clonal architecture and outputs a clone count. For SNV data, we used VarScan 2 variant aggregation and masking maf files from TCGA. For CNV data, we used ASCAT2 allele-specific copy number files from TCGA. PyClone begins its clustering with a random seed, resulting in a slightly variable output of clone counts. For each sample, we performed PyClone three times per day for 3 days, yielding a more consistent clone count. Only clones that had greater than one mutation were counted. In addition to ITH based on PyClone we also generated a Mutant Allele Tumor Heterogeneity (MATH) score, a method used in other indications to estimate tumor heterogeneity. This method solely relies on the width of the allele frequency distribution and does not take copy number variation into account (12).

Cutoff selection

Maximally selected rank statistics were used to select the optimum cutoff value for TMB and ITH. This approach has been previously used in several cancer biomarker studies (27-31). The standard log-rank statistic was calculated for a range of cutoff values. The value at which survival outcomes between groups had the best separation and the most balanced samples sizes were selected as cutoffs. P values were adjusted using the false discovery rate to minimize the effect of multiple testing bias. Group comparisons were performed using the two tailed *t*-test and the Mann-Whitney test for continuous variables and the chi-square test was used for discrete variables. For Kaplan-Meier survival analysis, the log-rank test was used. All statistical analyses were performed using Python packages statistics, KaplanMeier, and lifelines. Maximally selected rank statistics were calculated with the R package maxStat.

Driver gene detection

Driver genes were detected using OncoDriveClust (32) via the R package maftools (33), using its default settings. OncDriveClust selects genes that are under selective pressure based on the number of nonsynonymous and synonymous mutations present within a gene. We only considered genes with P values less than or equal to 0.05. P values were calculated using the Z test.

Immune composition via single sample gene set enrichment analysis (ssGSEA)

For RNASeq, we used UQ-FPKM STAR gene counts. We performed single cell gene ssGSEA via the GenePattern (RRID:SCR_003201) (34) platform using the 29 immune cell types derived by Faruki *et al.* (35). We ran modules ssGSEA version 10.1.0 and ssGSEA.ROC version 1 to generate plots, normalizing expression data with log.rank as a parameter for ssGSEA. P values were calculated using the two-sided Wilcoxon test.

Results

Demographics

The TCGA cohort included 927 NSCLC patients, with 362 LUSC and 379 LUAD patients remaining after excluding stage patients. LUSC patients were older at diagnosis than LUAD, with a mean age of 68 versus 65 years (P=0.002); however, this difference is likely not clinically significant. LUSC patients had an approximately 2:1 male predominance as compared to LUAD, which had an approximate 1:1 male-to-female ratio (P<0.001). Additionally, LUAD was diagnosed at earlier stages 9% more often than LUSC (percent of LUAD patients diagnosed at stage I minus percent of LUSC patients diagnosed at stage I) (P=0.01). Although the difference in distributions of TMB between LUSC and LUAD was statistically significant, the 0.54 difference in the median TMB is likely not clinically significant. The difference in the distributions of ITH for LUSC and LUAD was not statistically significant. However, LUSC did have a narrower TMB range with a higher mode (*Figure 1*). A complete list of patient dataset characteristics can be found in *Table 1*.

TMB and ITH

Uni- and multivariate hazard regression analysis was performed. Uni- and multivariate hazard ratios for TMB and ITH in all LUSC early-stage patients were 0.98 (95%

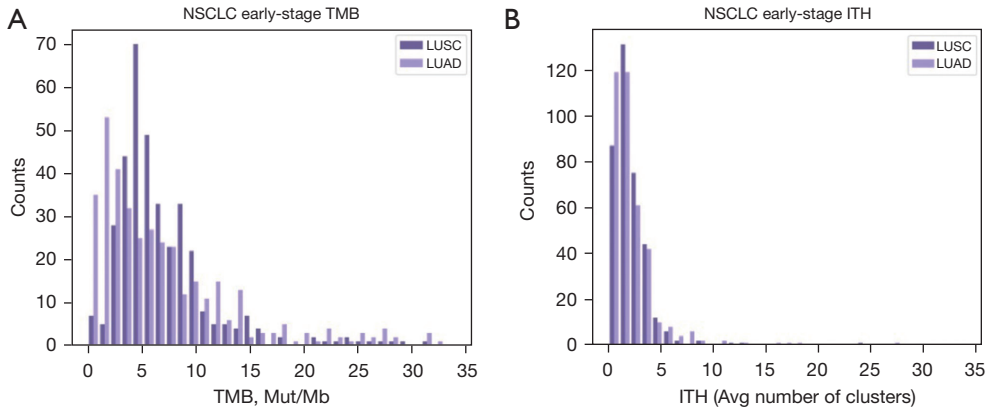

Figure 1 TMB and ITH distributions for patients with early-stage NSCLC. The vertical axis and horizontal axes represent the sample count with a given TMB or ITH value. LUSC and LUAD are plotted next to each other. (A) TMB sample distribution; (B) ITH sample distribution. NSCLC, non-small cell lung cancer; TMB, tumor mutational burden; ITH, intratumor heterogeneity; mut/Mb, mutations per Megabase of DNA; LUSC, lung squamous cell carcinoma; LUAD, lung adenocarcinoma.

CI: 0.96–1.00, P=0.10), 0.95 (95% CI: 0.88–1.02, P=0.20), and 0.98 (95% CI: 0.96–1.01, P=0.10), respectively. Uni- and multivariate hazard ratios for TMB and ITH in all LUAD early-stage patients were 1.00 (95% CI: 1.00–1.02, P=0.50), 1.02 (95% CI: 0.88–1.02, P=0.20), and 1.00 (95% CI: 0.99–1.02, P=0.80), and 1.02 (95% CI: 0.98–1.06; P=0.30), respectively.

At the clinical TMB cutoff of 10 mut/MB in LUSC and LUAD trends were not statistically significant (Figure S1A-S1D). Cutoffs were selected as described in the methods section with P values adjusted for multiple comparison testing. At a TMB cutoff of 4, LUSC had a statistically significant difference in outcomes, with High TMB patients experiencing a 4 month longer median PFS (20 *vs.* 16 months, $P_{adjusted}$ =0.02, Figure S1E). OS differences can be found in Figure S1F.

For LUAD, none of the evaluated TMB cutoffs yielded any statistically significant differences in survival (Figure S1G). OS differences can be found in Figure S1H.

At an ITH cutoff of 1.4, there was a statistically significant difference in outcomes, with high ITH patients experiencing a 7-month longer median PFS than their low ITH counterparts (21 *vs.* 14 months, $P_{adjusted}$=0.009, *Figure 2A*). OS differences can be found in *Figure 2B*. Unlike LUSC, the selected ITH cutoff of 2.7 in LUAD showed that high ITH individuals had a 2-month shorter median PFS than their low ITH counterparts (17 *vs.* 19 months, $P_{adjusted}$=0.043, *Figure 2C*). OS differences can be found in *Figure 2D*.

We stratified with both TMB and ITH cutoffs to create 4 groups: low TMB patients with high/low ITH abbreviated as LTHI/LTLI where LT refers to low TMB and HI/LI refer to high/low ITH and high TMB patients with high/low ITH abbreviated as HTHI/HTLI where HT refers to high TMB and HI/LI refer to high/low ITH. We selected cutoffs according to the selection approach outlined in the methods section and adjusted P-values for multiple comparison testing. For LUSC, we selected 9 and 1.4 for TMB and ITH, respectively. Univariate hazard regression analysis focused on ITH across high and low TMB LUSC groups based on the selected TMB cutoff yielded the following hazard ratios: 1.01 (95% CI: 0.93–1.09, P=0.90) and 0.89 (95% CI: 0.81–0.99, P=0.03). LTHI had a 7-month longer median PFS than LTLI (21 *vs.* 14 months, $P_{adjusted}$=0.02, *Figure 2E*). OS differences can be found in *Figure 2F*. For LUAD, we selected 6 and 2.7 and found that, in contrast to LUSC, LTHI had a 5-month shorter median PFS than LTLI (15 *vs.* 20 months, $P_{adjusted}$=0.04, *Figure 2G*). OS differences can be found in *Figure 2H*. Univariate hazard regression analysis focused on ITH across high and low TMB LUAD groups based on the selected TMB cutoff yielded the following hazard ratios: 1.01 (95% CI: 0.98–1.05, P=0.50) and 1.05 (95% CI: 0.91–1.22, P=0.50), respectively.

At these same cutoffs, the high TMB patients for both the LUSC and LUAD groups showed trends between high and low ITH that matched those observed in the low TMB groups. These relationships were not statistically significant

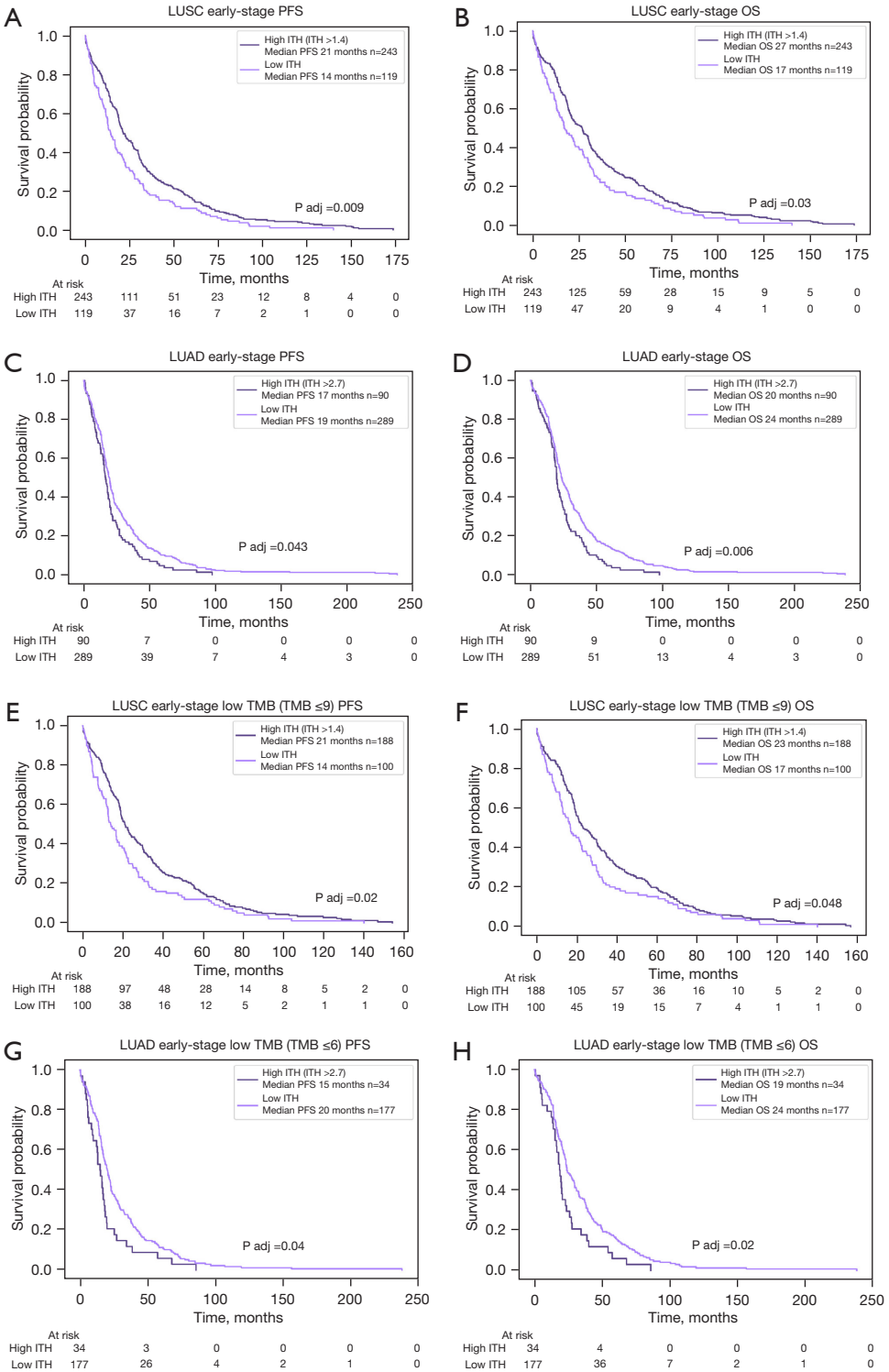

Figure 2 Survival outcomes in patients with early-stage NSCLC stratified by ITH alone and with low TMB. Kaplan-Meier survival plots of early-stage NSCLC patients stratified by ITH and low TMB at selected cutoffs based on output from the maxStat R package. Below each Kaplan-Meier plot is a risk table showing the number of at risk individuals in each group over time. P values were adjusted when multiple comparisons were performed using the false discovery control rate via SciPy Stats python package and are labeled P_{adj} on the plot. (A) Early-stage LUSC: PFS stratified by ITH >1.4. (B) Early-stage LUSC: OS stratified by ITH >1.4. (C) Early-stage LUAD: PFS stratified by ITH

>2.7. (D) Early-stage LUAD OS: stratified by ITH >2.7. (E) Early-stage LUSC with low TMB (TMB ≤9): PFS stratified by ITH >1.4. (F) Early-stage LUSC with low TMB (TMB ≤9): OS stratified by ITH >1.4. (G) Early-stage LUAD with low TMB (TMB ≤6): PFS stratified by ITH >2.7. (H) Early-stage LUAD with low TMB (TMB ≤6): OS stratified by ITH >2.7. P values based on log-rank test and adjusted with false discovery rate control. LUSC, lung squamous cell carcinoma; LUAD, lung adenocarcinoma; PFS, progression-free survival; OS, overall survival; TMB, tumor mutational burden; ITH, intratumor heterogeneity; NSCLC, non-small cell lung cancer.

(Figure S2A-S2D). Of note, neoantigen load across all comparisons showed no statistically significant differences but was correlated with TMB.

The relationship between ITH and outcome was primarily driven by low TMB in both LUSC and LUAD. Therefore, our subsequent analyses focused on this subset.

ITH estimation concordance

We generated MATH scores, another method used to estimate tumor heterogeneity, for LUSC and LUAD. For both LUSC and LUAD MATH scores were higher in the high ITH subgroups. However, only LUAD had a weakly positive statistically significant correlation between ITH and MATH scores (Spearman's coefficient =0.13, P=0.01).

Driver genes

A full list of predicted driver genes for both LUSC and LUAD can be found in *Table 2*. In the LUSC LTHI group, five driver genes were predicted, while four were predicted in the LTLI group. Among the predicted drivers for LUSC LTHI, *CHD3*, *NFE2L2*, and *PIK3CA* have been previously described. *CHD3* has been found to be associated with increased CD8 T cell infiltration, *NFE2L2* with increased programmed cell death ligand 1 (PD-L1) expression, and *PIK3CA* with longer survival. Mutations in *NFE2L2* and *PIK3CA* were present in 20% and 10% of the samples, respectively, while other mutations occurred less frequently (below 5%). In the LUSC LTLI group, two predicted driver genes, *NFE2L2* and *TRPS1*, have been previously described. *TRPS1* has been found to be associated with multidrug resistance. Mutations in *NFE2L2* and *LRRCC1* were present in 15% and 41% of the samples, respectively, while other mutations had frequencies less than 5%. Interestingly, *NFE2L2* was the only mutation present in both LUSC groups at relatively similar frequencies.

In the LUAD LTHI group, driver gene prediction yielded two candidates, *KRAS* and *EGFR*, both are well-studied with documented associations with survival outcomes. Mutation frequencies were 21% and 35%, respectively. In the LUAD LTLI group, five predicted driver genes included *KRAS*, *BRAF*, and *EGFR*. *KRAS* mutations were present in 27% of the samples, like LUAD LTHI, but the *EGFR* mutation frequency was significantly lower at 14%. *BRAF* mutations were present in only 6% of the samples.

To test if the presence of driver genes was an important factor for the relationship between ITH, TMB, and clinical outcome, we performed a survival analysis after removing the EGFR-positive patients. LTLI had a 6 month longer median PFS than LTHI (20 *vs.* 14 months, P=0.03, *Figure 3A*). OS differences can be found in *Figure 3B*. Removing EGFR-positive patients from the analysis led to a one-month improvement in outcomes. This suggests that, at least for the LTHI group, EGFR might offer a benefit in terms of longevity.

Immune cell enrichment

Single sample GSEA yielded several statistically significant enriched and depleted cell types in both LUSC and LUAD. In LUSC LTHI, CD8 T cells and type 2 helper T cells were enriched, while CTLA4 and PDCD1 expression was reduced. Conversely, in LUAD LTHI, CD8 T cells and type 1 helper T cells were depleted, while B cells were enriched. Enrichment score plots along with P values can be found in *Figure 4* for both LUSC and LUAD.

Discussion

Key findings

We present novel findings demonstrating the differential prognostic value of ITH in various histological subtypes of early-stage NSCLC. Prior work has not shown an association between ITH and clinical outcome in LUSC and LUAD (8,43). However, in early-stage patients who received successful primary tumor resection, we observed a statistically significant association between ITH and clinical outcomes. In LUSC, high ITH is prognostic of a better outcome, while in LUAD, high ITH is prognostic

Table 2 Predicted driver genes for early-stage LUSC and LUAD with low TMB, stratified by selected ITH cutoffs

Group	Predicted driver genes	Significance	P value	Mutation frequency	Reference
LUSC LTHI: TMB ≤9, ITH >1.4	*CHD3*	Associated with CD8 T cell infiltration in LUSC	0.001	0.027	Lv and Lin, 2022 (36)
	OR2T34	Unknown	0.006	0.032	
	NFE2L2	Associated with higher TMB and PD-L1 expression	0.006	0.197	Xu *et al.*, 2020 (37)
	ZNF236	Unknown	0.01	0.032	
	PIK3CA	Associated with longer PFS and OS	0.03	0.096	McGowan *et al.*, 2017 (38)
LUSC LTLI	*INPPL1*	Unknown	0.001	0.04	
	NFE2L2	Associated with higher TMB and PD-L1 expression	0.003	0.15	Xu *et al.*, 2020 (37)
	TRPS1	Associated with multidrug resistance	0.007	0.05	Liu *et al.*, 2018 (39)
	LRRCC1	Unknown	0.045	0.41	
LUAD LTHI: TMB ≤6, ITH >2.7	*KRAS*	Associated with mutation specific outcomes	<0.001	0.21	Jones *et al.*, 2021 (40)
	EGFR	Associated with better OS	0.01	0.35	Yang *et al.*, 2022 (41)
LUAD LTLI	*KRAS*	Associated with mutation specific outcomes	<0.001	0.27	Jones *et al.*, 2021 (40)
	BRAF	Associated with mutation specific outcomes	<0.001	0.056	Di Federico *et al.*, 2022 (42)
	ERBB2	Unknown	<0.001	0.034	
	USP29	Unknown	0.001	0.023	
	EGFR	Associated with better OS	0.001	0.14	Yang *et al.*, 2022 (41)

The selected cutoffs for LUSC were 9 and 1.4 for TMB and ITH, respectively. The selected cutoffs for LUAD were 6 and 2.7 for TMB and ITH, respectively. Driver genes were identified using OncoDriverClust. Mutation frequencies were calculated by dividing the number of mutated samples by total samples. The clinical significance of the mutated genes was determined based on the publications present in the source column. Genes without published literature were deemed to lack known clinical significance in LUSC or LUAD. P values based on Z test. LUSC, lung squamous cell carcinoma; LUAD, lung adenocarcinoma; PFS, progression-free survival; OS, overall survival; TMB, tumor mutational burden; ITH, intratumor heterogeneity; PD-L1, programmed cell death ligand 1; LTHI, low TMB high ITH; LTLI, low TMB low ITH.

of a worse outcome, particularly in patients with low TMB. Interestingly, in LUAD patients ITH and MATH, another method used to estimate tumor heterogeneity, had a positive statistically significant correlation, while in LUSC patients they did not. Overall our findings are also clinically significant according to the American Society of Clinical Oncology definition with a 7-month difference for LUSC and a 5-month difference for LUAD (44).

Strengths and limitations

The primary strength of our study lies in the comprehensive approach of incorporating multiple parameters to examine the association between molecular biomarkers and clinical outcomes across various histological subgroups. By integrating driver gene prediction and assessing relative immune cell enrichment, we gain valuable insights into the factors potentially influencing the relationship between ITH and prognosis. Secondly, in contrast to prior work, we have examined LUSC and LUAD as distinct entities, each with a unique relationship among ITH, TME, and outcome. Lastly, the subgroups studied had a relatively large sample size from TCGA. The main limitation of our work is that there is no gold standard or clinically validated method for ITH estimation. The method we chose represents one of the most popular and well-studied approaches. We also included MATH, another tumor heterogeneity method, and evaluated its correlation with PyClone derived ITH. Additionally, our work focuses on patients with early-stage disease and does not suggest specific treatment implications; it is primarily intended to generate hypotheses. Although the statistical method used, maximally selected rank

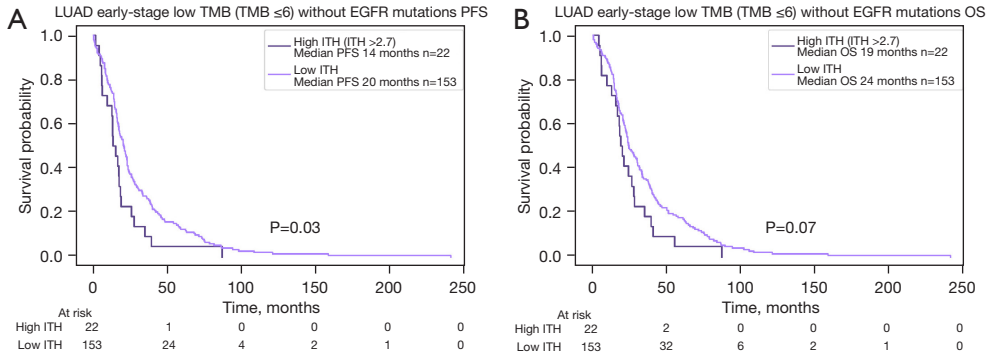

Figure 3 Survival outcomes in patients with early-stage LUAD, low TMB, and without EGFR driver mutations, stratified by ITH. Kaplan-Meier survival plots of early-stage LUAD patients without EGFR mutations stratified by ITH and low TMB at selected cutoffs based on output from the maxStat R package. Below each Kaplan-Meier plot is a risk table showing the number of at risk individuals in each group over time. Since only one comparison was made, P values were not adjusted. (A) Early-stage LUAD with low TMB (TMB ≤6): PFS stratified by ITH >2.7. (B) Early-stage LUAD with low TMB (TMB ≤6): OS stratified by ITH >2.7. P values based on log-rank test. LUAD, lung adenocarcinoma; PFS, progression-free survival; OS, overall survival; TMB, tumor mutational burden; ITH, intratumor heterogeneity.

statistics, have been utilized across several other biomarker studies they are vulnerable to multiple testing bias for which we adjusted out P values, nonetheless our findings require further validation.

Explanations of findings & comparison with similar research

To explain these findings, we first focused our analysis on the molecular differences between LUSC and LUAD. Certain driver genes play an important role in the aggressiveness of tumors. Recent work has identified NFE2L2 as a possible biomarker for response to immune checkpoint inhibitors and has shown that tumors with NFE2L2 mutations exhibit a poor prognosis (37). Additionally, a recent phase 2 clinical trial has shown significant efficacy of TORC1/2 inhibitors in NFE2L2-mutated LUSC tumors (45). Although NFE2L2 was identified as a driver gene in the LUSC cohort, it was present at very similar mutation frequencies in both LTHI and LTLI groups and likely does not explain the difference in clinical outcomes that we observed. PIK3CA, on the other hand, was identified as a driver gene only in the LTHI group and was present in 10% of the patients. Mutations in PIK3CA have been associated with longer survival and time to relapse in LUSC patients (38). Evaluating outcomes with PIK3CA carriers removed would be informative. LRRCC1 was identified as a driver gene only in the LTLI group and was present in 41% of patients. This protein maintains the structural integrity of the centrosome and plays a key role in mitotic spindle formation (46). Mutations in LRRCC1 may imply genomic instability and a faster cell replication cycle. Mitotic spindle disruption has also been studied as a possible explanation for the efficacy of electromagnetic therapies in *in vitro* and *in vivo* studies (47).

In contrast to LUSC, LUAD has several well-studied driver genes. Some of them were predicted by our approach. In LUAD LTHI, KRAS and EGFR mutations were present in a significant percentage of patients, and their possible co-occurrence may explain the shorter disease recurrence time in this group. However, it is clear that EGFR on its own does not explain the relationship that we observed. Although these mutations were also present in the LTLI group, EGFR specifically had a much lower mutation frequency. The LTLI group, as a whole, had a greater number and diversity of predicted driver genes, possibly as a consequence from a more selective TME. In recent years, the idea of cell competition within tumors has gained significant attention and has been hypothesized to act as an additional selective dimension (48). In tumors with developed treatment resistance, clonal competition has been shown to modulate the proliferation of aggressive and highly fit clones (49). Additionally, differences in tumor suppressor mutation profiles, especially in LUSC, have been shown to have a negative impact on immune surveillance and infiltration of tumors (50). However, in this setting increased ITH may rescue these types of tumors from their immunologically "cold" state. In LUAD, on the other hand, the observed increased rate of metastasis may be accelerated in tumors with high ITH (51). Overall, it is still

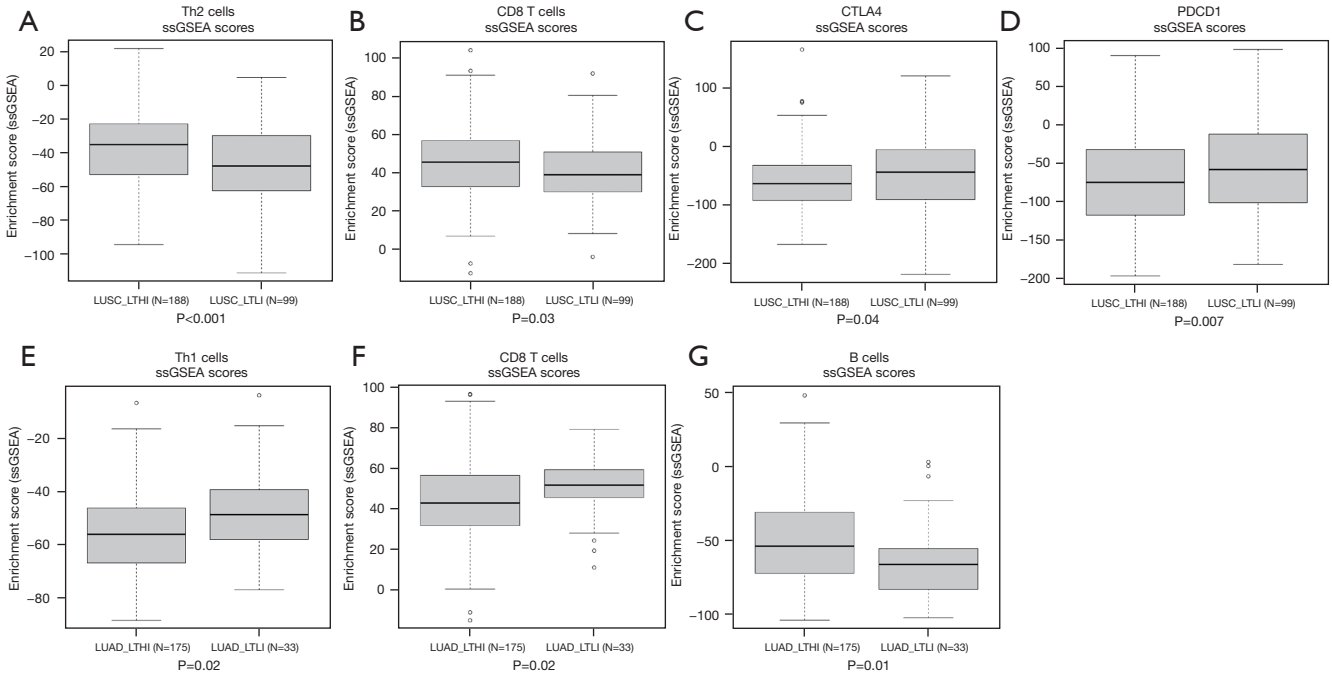

Figure 4 Immune cell enrichment in patients with early-stage NSCLC with low TMB, stratified by selected ITH cutoffs. For LUSC, the selected cutoffs were 9 and 1.4, respectively. For LUAD, the cutoffs were 6 and 2.7 for TMB and ITH, respectively. Plots were generated by the ssGSEA. ROC module within the Genepattern tool kit. The vertical axis represents the enrichment scores, and the horizontal axis displays the two groups of interest: LTHI and LTLI. The line within each box represents the median of the distribution. The top and bottom of each box represent the 3rd and 1st quartile of the distribution, respectively. The vertical lines extending to horizontal lines represent 1.5 the interquartile range. Empty circles beyond the horizontal lines are considered represent distributional outliers. The Wilcox P value is located below the horizontal axis labels. A total of 1 LUSC LTLI, 2 LUAD LTHI, and 1 LUAD LTLI patients were excluded from the analysis due to insufficient RNASeq data. (A) Type 2 helper T cells LUSC LTHI *vs.* LTLI enrichment plot. (B) CD8 T cells LUSC LTHI *vs.* LTLI enrichment plot. (C) CTLA4 LUSC LTHI *vs.* LTLI enrichment plot. (D) PDCD1 LUSC LTHI *vs.* LTLI enrichment plot. (E) Type 1 helper T cells LUAD LTHI *vs.* LTLI enrichment plot. (F) CD8 T cells LUAD LTHI *vs.* LTLI enrichment plot. (G) B cells LUAD LTHI *vs.* LTLI enrichment plot. P values were calculated using the two-sided Wilcoxon test. NSCLC, non-small cell lung cancer; LUSC, lung squamous cell carcinoma; LUAD, lung adenocarcinoma; ssGSEA, single sample gene set enrichment analysis; ITH, intratumor heterogeneity; TMB, tumor mutational burden; LTHI, low TMB high ITH; LTLI, low TMB low ITH; ROC, receiver operating characteristic.

unclear why ITH has differential impact based on histology. Nevertheless it is apparent that the TME plays a key role in integrating the complex qualities of each tumor.

Both LUSC and LUAD subgroups that displayed better performance were enriched for CD8 T cells. Traditionally, this T cell subtype is considered the main driver of antitumor immunity; it can also undergo differentiation into numerous other subtypes and states, notably into an exhausted state, especially in the presence of immune checkpoints, Th2 cells, and regulatory T cells (52). Interestingly, Th2 cells were enriched in the better-performing LUSC LTHI group, suggesting that their immune-suppressive effect may have been modulated. Overall, markers of immune suppression were generally reduced in the TME, LUSC LTHI had a depletion of CTLA4 and PDCD1 (53). In LUAD, there was no statistically significant difference in immune checkpoint expression. Th1 cells, considered protective against cancer, were enriched in the better-performing LUAD LTLI group. Contrary to expectations and despite previous studies associating B cell depletion with worsened clinical outcomes, the better-performing LUAD LTLI group experienced a reduction in B cells (44). The observed variations in immune cell populations across both LUSC and LUAD highlight a complex relationship between pro- and anti-tumor factors. Although other work has explored

the relationship between ITH and the TME, a direct relationship between specific clonal architectures and TME changes has not yet been established (54).

Implications and future studies

Although our work does not provide a clear explanation for why high ITH confers a survival benefit in LUSC and a disadvantage in LUAD, it does illustrate that the relationship between outcome and clonal heterogeneity is complex, especially in the context of TMB. This relationship does not appear to be tied to specific driver genes, regardless of whether they are well-studied in the case of LUAD or bioinformatically-derived in the case of LUSC. Additionally, the TME within the high ITH groups does not appear to show any unique differences. The enrichment for CD8 T cells in both of the better performing groups, LTHI in LUSC and LTLI in LUAD, is likely a downstream effect of a combination of factors that lead to their recruitment and proliferation. As prior work in melanoma has shown (6), there may be a relationship between clonal versus subclonal neoantigens and T cell recruitment; however, this has yet to be explored in NSCLC. The next step involves evaluating how neoantigens are distributed across different types of clonal architectures (i.e., 1 large and 2 small clones *vs.* 3 equally sized clones) and TME composition across histologic subtypes. To answer these questions, a comprehensive analysis of the TME is necessary. Future work needs to make use of thorough H&E whole slide image analysis to directly characterize the interactions between stroma, immune, and neoplastic cells.

Conclusions

In summary, we are the first to demonstrate a differential relationship between ITH and histologic subtype in patients with early-stage NSCLC by TMB. Although the role of ITH in clinical care remains unclear, there is a significant association between the histologic subtype, ITH, and notable differences in the TME. Further validation is warranted, with a focus on other NSCLC datasets and other indications that exhibit coexisting squamous and adenocarcinoma histologies, such as esophageal, cervical, and head & neck.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was

conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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