# Using Next-Generation Sequencing to Re-Evaluate the Correlation Between Tumor Mutational Burden and The Survival Probability NSCLC Patients

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**Abstract:** Tumor mutational burden （TMB）are considered a prerequisite for anti-tumor immunotherapy. A number of clinical studies have used whole-exome sequencing (WES) to explore the value of TMB in clinical applications. But WES is not currently feasible at the scale of a clinical setting. **Methods:** Following the same analysis done by the original study, the TMB and FGA are examined using the Mann-Whitney U test. For the survival analysis, the standard Kaplan-Meier curves were utilized for the comparison of survival status difference between different factors. Hazard ratios (HRs) for factors were also calculated trough Cox proportional hazards models. Coefficient of determination and related statistical evaluations measures are conducted. Aalen's Additive Regression Model are also tested for the overtime influence of each factor has over the survival object constructed for the survival analysis. **Results:** The majority of the patients progress at between 1 months to 5 months and above. The progression of disease would then slow down and having only few patients that reaches 25 months and above. Given the HRs, the middled aged patient with the MSK-IMPACT profiling on panel 410 are comparatively receiving the lowest possible risks in disease progression. **Conclusions:** In conclusion, while it is verifiable the TMB would heavily impact the NSCLC patient survival, their specific impact relationship are difficult to determine due to the lack of specific data classification, lack of data with solid support, data sensitivity should be improved along with the sample bias problem.

## 1. Introduction

Immune checkpoint inhibitors (ICIs) have dramatically changed the landscape of cancer treatment because of the existence of durable responders among patients with advanced stage including non–small-cell lung cancer (NSCLC). Since immune checkpoint inhibitors (ICIs) are only effective in a subset of patients with advanced non–small-cell lung cancer (NSCLC), there is a compelling need to create clinically effective techniques to identify the subgroup of individuals who are most likely to benefit therapeutically.

Currently, a widely used biomarker of ICIs in NSCLC is the programmed death-ligand 1 (PD-L1) expression approved by the U.S. Food and Drug Administration (FDA). Most NSCLC trials found that tumors with elevated PD-L1 expression had higher response rates, but the response was not consistent. Meanwhile, other studies have found that a higher somatic mutation burden is related to a greater likelihood of immunotherapy response in a variety of tumor types, including NSCLC. Given these studies have proven that tumor mutational burden (TMB) may be related to diverse types of malignancies, TMB as a biomarker of using ICIs treatment has been difficult to apply. TMB is a relative term for each tumor type, and the cutoffs for distinct tumors appeared to be varied. TMB has been quantified in the majority of studies using whole exome sequencing (WES). This method is

neither clinically practicable nor practical at this time. In contrast, consider using targeted next-generation sequencing (NGS) to do genomic study of malignancies is a good technique.

While the original study focused on determine the association of PD-L1 expression with TMB and FGA, with respect to the progression free status of the NSCLC patients classified by the accessibility of the Durable Clinical Benefit (DCB). Although the NGS accurately correlated with the TMB estimates, the sample data this study combined three different gene panels MSK-IMPACT sequencing were tested on. While each of the gene panel tests have different sample size, even when the study states that each of panel tests were standardized on the counts of TMB and FGA, the problem of sample bias is highly possible to exist during the statistical analysis of the original study. Furthermore, based on the provided patient characteristics it is also critical to evaluate how these characteristics affect the patient survival probability statistically.

In this study, we have attempted to re-evaluate the statistical analysis of the original study, with interest to other characteristics during the data collection. Given the possible existing sample bias of the data collected, we would hypothesize that the TMB estimates are limited to the majority gene panel that was subjected to the MSK-IMPACT sequencing, while other characteristics may exist the possibility to affect the survival probability of the NSCLC patients in the study.

## 2. Methodology

### 2.1 Patients

The patients' data from the original study are obtained from the MSKCC reviewed ICI treatment of advanced NSCLC with anti-PD-(L)1 monotherapy or in combination with anti-cytotoxic T-cell lymphocyte-4(anti-CTLA-4) between April 2011 and January 2017, only the patients with tumor molecularly profiled by MSK-IMPACT were included, same as the original study. Among all the patients, cases that were not radiologically evaluable were excluded. Progress-free survival (PFS) was classified as the time period from the patient began immunotherapy to the date of progression. Patients who have not progressed were censored at their last date of scan.

### 2.2 MSK-IMPACT Sequencing

In the original study, MSK-IMPACT assay was performed for the entire 240 patients on the custom gene panel of 341, 410, or 468. The sequencing data are available through the cBioPortal for Cancer Genomics. The somatic nonsynonymous TMB data across the various panels with different sizes were normalized by using the total number of mutations divide by coding region captured in each panel, covering 0.98, 1.06, and 1.22 megabases (Mb) in the 341-, 410-, and 468-gene panels. The fraction of copy number-altered genome (FGA) was standardized as the fraction of genome with log2 copy number gain > 0.2 or less < -0.2 relative to the size of the genome with copy number profiled. Tumor samples used for the assay were collected before immunotherapy treatment in 204 patients, covering 85% of the entire sample size according to the study.

### 2.3 Statistical Analysis

Following the same analysis done by the original study, the TMB and FGA are examined using the Mann-Whitney U test. For the survival analysis, the standard Kaplan-Meier curves were utilized for the comparison of survival status difference between different factors. Hazard ratios (HRs) for factors were also calculated trough Cox proportional hazards models. Following the original study, coefficient of determination and related statistical evaluations measures are conducted. In addition to the statistical analysis, Aalen's Additive Regression Model are also tested for the overtime influence of each factor has over the survival object constructed for the survival analysis. All of the P value reported are two-sided. All of the statistical analysis was conducted through R version 4.1.2 software (www.r-project.org).

## 3. Results

### 3.1 Tumor Mutation Burden associated Patients Characteristics

The original study selected 240 patients with NSCLC been treated with anti-PD-(L)1 therapy alone or combined with anti-CTLA-4 that are both been profiled by MSK-IMPACT and were radiologically evaluable to include into the analysis. The patient demographic (**Table 1**) indicated that the prevailing choice of treatment to be anti-PD-(L)1 therapy alone, with the majority of patient at second line of treatment. Among the cohort, 42 patient's disease (17.5%) eventually did not progress; 69 patients (29%) had durable clinical benefit. The median and mean TMB of the cohort are respectively 7.8 (SNVs/Mb, with a range of 0.9 to 95.6) and 10.3 (SNVs/Mb).

Before conducting the survival analysis with the Kaplan-Meier Curves, Levene's test was conducted for the FGA and TMB, such indicating the two factors are non-parametric. The patients where been further categorized in age groups based on their age distribution (**Figure 1**).

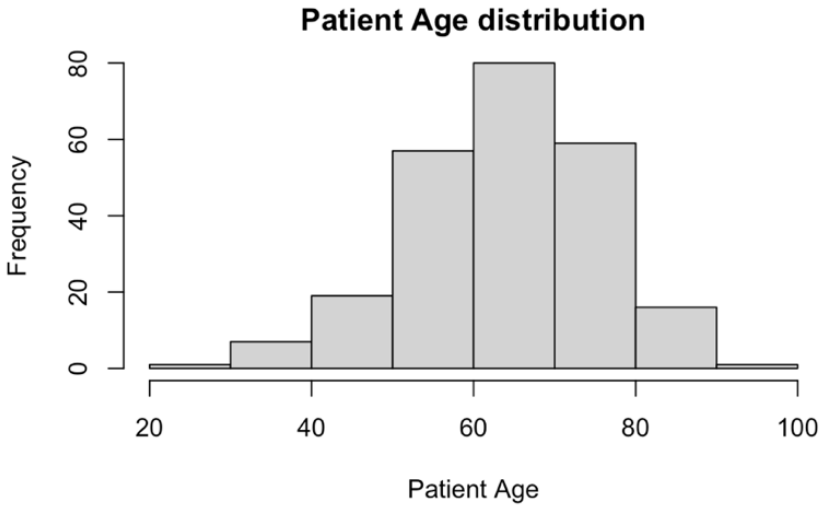



Figure 1. Patient Age distribution

Table 1. Patient Characteristics

| Characteristics | No. (%) |
|---|---|
| No. of Patients | 240 |
| Median age, (Range) | 66 (22-92) |
| Sex | |
| Male | 118 (49) |
| Female | 122 (51) |
| Gene Panels | |
| 341 | 56 (23.3) |
| 410 | 164 (68.3) |
| 468 | 20 (8.3) |
| Smoking status | |
| Ever | 193 (80) |
| Never | 47 (20) |
| Progression Free Status | |
| Progressed | 198 (82.5) |
| Not progressed | 42 (17.5) |
| Treatment line | |
| First | 51 (21) |
| Second | 127 (53) |
| Third or more | 62 (26) |
| Treatment Type | |
| PD-(L)1, monotherapy | 206 (86) |
| PD-(L)1+CTLA-4, combination | 34 (14) |
| Clinical Benefit | |
| DCB | 69 (29) |
| NDB | 158 (66) |
| Not evaluate (< 6-month follow-up) | 13 (5) |
| Abbreviations: DCB, durable clinical benefit, NDB, no durable benefit; PD-(L)1, programmed cell death-1 or programmed death-ligand 1. | |

### 3.2 Kaplan-Meier Curves of Survival Analysis

The overall KM curve was first illustrated with 95% confidence interval without categorification by any factors (**Figure 2**). With a labeled summary statistic based on the PFM time (**Table 2**).

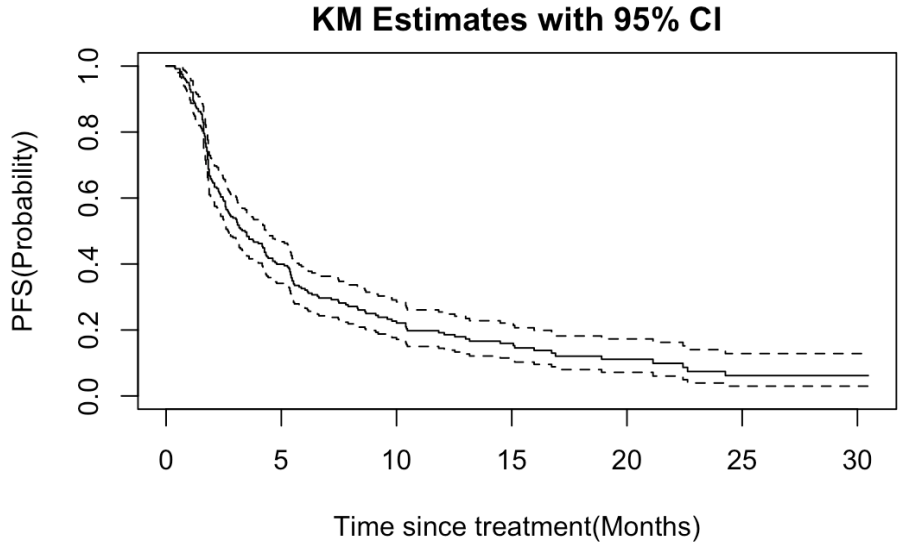


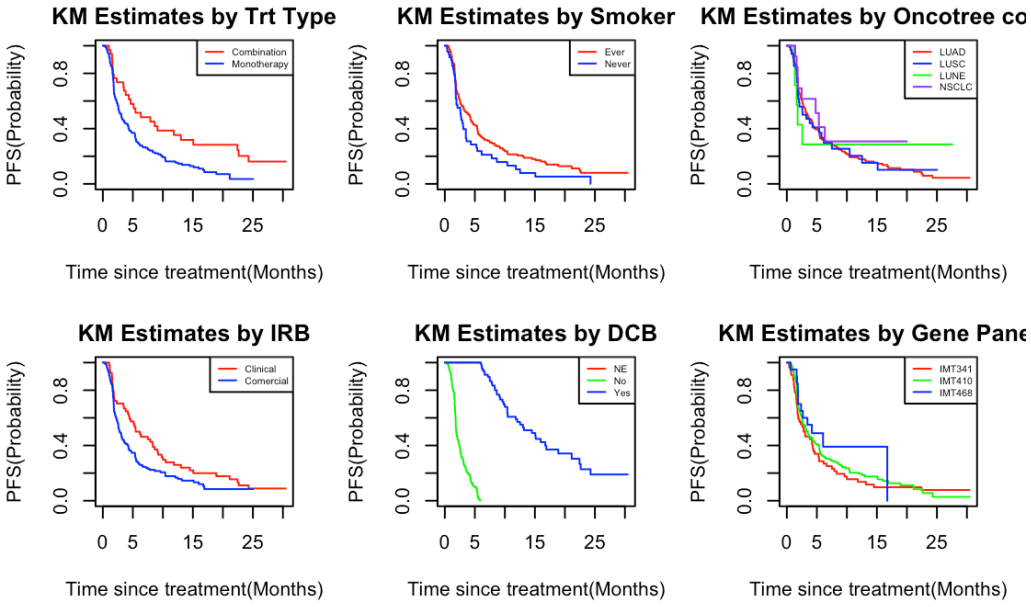

Figure 2. Overall Kaplan-Meier Curve with 95% CI

Table 2. Summary statistics of the Overall KM curve

| time n. | risk n. | event | survival | std.err | lower 95% CI | upper 95 % CI |
|---|---|---|---|---|---|---|
| 1 | 228 | 14 | 0.9417 | 0.0151 | 0.9125 | 0.972 |
| 5 | 87 | 128 | 0.3994 | 0.0321 | 0.3413 | 0.467 |
| 10 | 39 | 36 | 0.2211 | 0.0287 | 0.1715 | 0.285 |
| 15 | 24 | 10 | 0.1595 | 0.0266 | 0.1151 | 0.221 |
| 20 | 11 | 6 | 0.1113 | 0.025 | 0.0717 | 0.173 |
| 25 | 5 | 4 | 0.0618 | 0.0231 | 0.0298 | 0.128 |
| 30 | 2 | 0 | 0.0618 | 0.0231 | 0.0298 | 0.128 |

The KM curve and its summary statistics indicated a discrepancy in the survival probability on the PFM measures. It is shown that the majority of the patients progress at between 1 months to 5 months and above, the progression of disease would then slow down and having only few patients that reaches 25 months and above.

The KM curve can then be categorized by all of the factors involved in the survival analysis, allowing the graphically illustration of the effect of each factor have over the KM curve estimation (**Figure 3**).



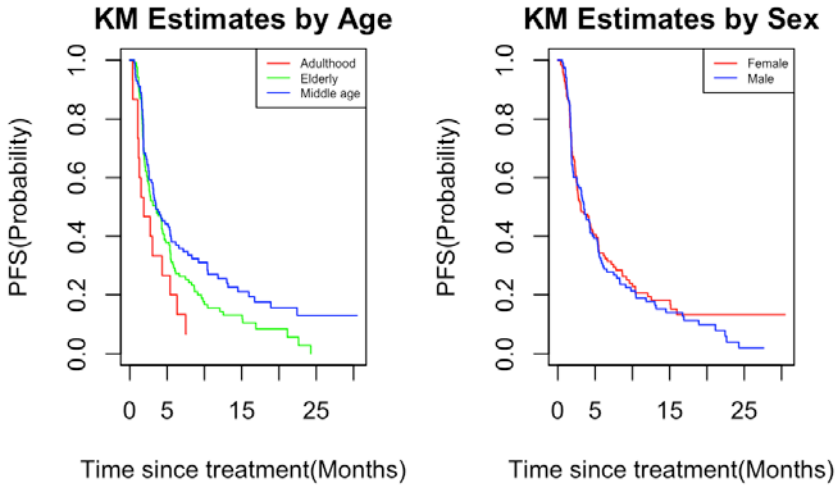



Figure 3. Kaplan-Meier Curve by various factors with 95% CI

Based on the multiple KM curve estimates, it is possible to visually observe the effects of these variables has over the survival probability and the survival time. Based on the visual KM estimates, for the patients to have the best possible survival probability, the patients with lung adenocarcinoma, taking a combination treatment, been a smoker, treatment been approved by clinical Institutional Review Board, have access to durable clinical benefits, with MSK-IMPACT profiling on gene panel IMPACT410.

Table 3. Summary statistics of Age and DCB KM curves

| Group | Time | Risk | Event | Survival | Std.err | Lower 95% Cl | Upper 95% Cl |
|---|---|---|---|---|---|---|---|
| Age (Adulthood) | 1 | 13 | 2 | 0.867 | 0.0878 | 0.7111 | 1.000 |
| | 5 | 4 | 9 | 0.267 | 0.1142 | 0.115 | 0.617 |
| Age (Elderly) | 1 | 121 | 5 | 0.690 | 0.0177 | 0.9257 | 0.995 |
| | 5 | 40 | 70 | 0.379 | 0.0448 | 0.3004 | 0.477 |
| | 10 | 16 | 21 | 0.166 | 0.0369 | 0.1076 | 0.257 |
| | 15 | 10 | 3 | 0.131 | 0.0342 | 0.0788 | 0.219 |
| | 20 | 4 | 3 | 0.084 | 0.0318 | 0.0400 | 0.176 |
| Age (Middle Age) | 1 | 94 | 7 | 0.931 | 0.0253 | 0.8825 | 0.982 |
| | 5 | 43 | 49 | 0.442 | 0.0497 | 0.3545 | 0.551 |
| | 10 | 23 | 12 | 0.311 | 0.0474 | 0.2303 | 0.419 |
| | 15 | 14 | 7 | 0.211 | 0.0449 | 0.1388 | 0.320 |
| | 20 | 7 | 3 | 0.156 | 0.0430 | 0.0910 | 0.268 |
| | 25 | 5 | 1 | 0.130 | 0.0430 | 0.0681 | 0.249 |
| | 30 | 2 | 0 | 0.130 | 0.0430 | 0.0681 | 0.249 |
| DCB (No) | 1 | 146 | 14 | 0.911 | 0.0226 | 0.8681 | 0.957 |
| | 5 | 16 | 128 | 0.101 | 0.0240 | 0.0636 | 0.161 |
| DCB (Yes) | 1 | 69 | 0 | 1.000 | 0.0000 | 1.0000 | 1.000 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | 5 | 69 | 0 | 1.000 | 0.0000 | 1.0000 | 1.000 |
| | 10 | 39 | 20 | 0.679 | 0.0598 | 0.5714 | 0.807 |
| | 15 | 24 | 10 | 0.490 | 0.0669 | 0.3749 | 0.640 |
| | 20 | 11 | 6 | 0.342 | 0.0695 | 0.2295 | 0.509 |
| | 25 | 5 | 4 | 0.190 | 0.0685 | 0.0936 | 0.385 |
| | 30 | 2 | 0 | 0.190 | 0.0685 | 0.0936 | 0.385 |

Given the original study focuses heavily on the effect of durable clinical benefit, therefore the summary statistics of KM curves grouped by durable clinical benefit are analyzed. The age groups are analyze along given its indicative graphical illustration (**Table 3**).

Based on the summary statistics, it is indicative that the patients without access to the durable clinical benefit faces a significantly higher risk of disease progression. For the age group classification, the elderly group faces the highest risk of disease progression as expected.

### 3.3 Hazard ratios & Log rank tests

To take a more systematic approach in understanding the effect of these factors have over the survival analysis, Hazard ratios (HRs) based on the Cox proportional hazards models where been illustrated in **Table 4**. Given the HRs, the middled aged patient with the MSK-IMPACT profiling on panel 410 are comparatively receiving the lowest possible risks in disease progression.

Tbale 4. Hazard ratios collected from forest graph

| | | | | | |
|---|---|---|---|---|---|
| Sex | Female | N = 122 | reference | | |
| | Male | N = 118 | 1.15 | (0.86-1.5) | 0.339 |
| Age_group | Adulthood | N = 15 | reference | | |
| | Elderly | N = 124 | 0.76 | (0.42-1.4) | 0.37 |
| | Middle Age | N = 104 | 0.64 | (0.35-1.2) | 0.16 |
| Gene.Panel | MPACT341 | N = 56 | reference | | |
| | MPACT410 | N = 164 | 0.75 | (0.52-1.1) | 0.137 |
| | MPACT468 | N = 20 | 0.63 | (0.33-1.2) | 0.178 |
| FGA | | N = 230 | 1.21 | (0.56-2.6) | 0.627 |
| TMB | | N = 240 | 0.98 | (0.97-1.0) | 0.052 |
| Trt_lines | 1 | N = 51 | reference | | |
| | 2 | N = 127 | 1.38 | (0.91-2.1) | 0.131 |
| | 3 | N = 39 | 1.36 | (0.82-2.2) | 0.227 |
| | 4 | N = 8 | 1.18 | (0.51-2.7) | 0.701 |
| | 5 | N = 8 | 1 | (0.42-2.4) | 0.998 |
| | 6 | N = 5 | 1.24 | (0.43-3.6) | 0.695 |
| | 7 | N = 2 | 1.53 | (0.35-6.8) | 0.574 |
| Smoker | Ever | N = 193 | reference | | |
| | Never | N = 47 | 1.39 | (0.94-2.1) | 0.101 |
| Treatment. Type | Combination | N = 34 | reference | | |
| | Monotherapy | N = 206 | 1.9 | (1.18-3.1) | 0.009** |

### 3.4 Aalen's Additive Regression Model Analysis

While the HRs were able to present the proportional risk associated with the factors, it is also important to consider that the effect of the hazards are possibility additive, the effect changes over the

survival probability over time. Therefore, based on the Cox proportional hazard model used to construct the HRs, Aalen' additive regression models were conducted (**Figure 5**). Based on the result the steep slope indicated for a negative effect on the survival probability over time, while the positive slope indicated for a relatively positive effect on the survival probability over time. Among the factors, it is visible that each of the treatment lines except treatment line 4, would always experience an certain level of steep sloping down, then proceeds to an upward slope. Of all treatment lines, treatment line 4 and 5 can be seen as a turning point of treatment effectiveness. While on other hand, the patient cohort that identified as "never a smoker" appears to be subjected to heavy negative impact on the survival probability overtime.

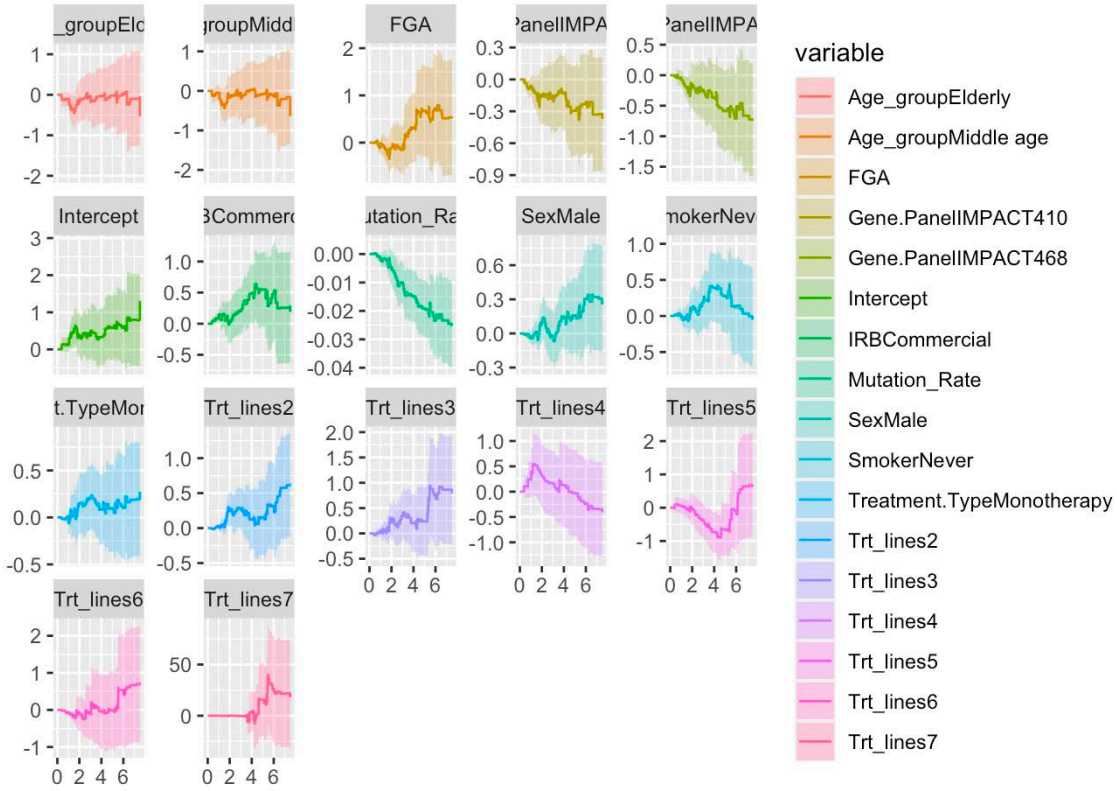



Figure 5. Aalen's regression model

### 3.5 Statistical Analysis Evaluation

The Cox proportional hazard model were then be evaluated for $R^2$ tests for statistical significance in predicting the dataset that was studied (**Table 4**). Coefficient of determination (COD, $R^2$), Measure of explained randomness (MER), and Measure of explained variance (MEV) were measured. Goodness of fit are also evaluated for the Cox model.

Table 4. $R^2$ Statistical test

| COD | MER | MEV |
|---|---|---|
| 0.130464 | 0.1558703 | 0.1009256 |
| Abbreviations: COD, Coefficient of determination; MER, Measure of explained randomness; MEV, Measure of explained variance. | | |

Given the $R^2$ test, based on the statistical point of view, the $R^2$ value fails to state the prediction power of the Cox model, while the MEV suggested that the model could only explain approximately 10% of the dataset used. While the goodness of fit test reports a Chi-squared value of 12.277 on 4 degrees of freedom, with an P value of 0.0154, helped explained the model meets the expected prediction power to a certain extent.

## 4. Discussion

While researching the original paper, the original study provided the conclusion that TMB has not correlated to the PD-L1 expression. Based on the study, it is both due to the lack of eligible samples went through MSK-IMPACT sequencing and the possibility that there are other candidates that can better correlates to the immunotherapy response. Pan et al has taken such direction and have listed out 52 candidate genes in predicting the clinical benefit for ICB therapy in the NSCLC patient. In their study, they investigated 52 independent agnostic gene panels selected based on significant mutation frequencies vs. deceased patient ratio, compared the overall survival rate with no mutations, single mutation, compound mutation (two or more) on the study cohort of 350 patients. Among the 350 patients, 230 were determined to have mutation signature, 145 having compound signature, all of the patients have varied PD-L1 expression level, stating that the prediction made with mutation signature are independent of PD-L1 expression level. This conclusion overshadows on the original study, questioning whether or not the prediction made with TMB faces the similar problem despite the lack of eligible PD-L1 expression level.

In another prospective study [7], ctDNA testing was found to be a good fit for NSCLC patient molecular monitoring. Thompson et al have detected 275 mutations in 45 genes, and 86 of 102 patients (84 percent) had at least one ctDNA mutation, with EGFR variations being the most prevalent. For 52 of the 102 patients, a tissue sample with appropriate quality and quantity of DNA for NGS was unavailable or unavailable (51%). The amount of tDNA was insufficient for 24 of 52 (46.2%) individuals, similar to prior results for tissue NGS [8]. As a result, a liquid biopsy was the only method of molecular monitoring for more than half of our patients. While circulating tumour cells (CTCs) constitute a type of liquid biopsy, CTCs from NSCLC patients are not always identifiable [9, 10]. Furthermore, ctDNA is easily detectable in the blood of lung cancer patients [11, 12], and the sensitivity of variation identification in ctDNA has recently been proven to be greater than in CTCs [13]. As a result, ctDNA testing is well-suited for molecular surveillance of NSCLC patients.

## 5. Conclusion

The original study indicated the importance over the inclusion of TMB and PD-(L)1 expression to be significant contributors in elevating the prediction power of the analysis model. However, based on the statistical analysis, although TMB would certainly provide statistical significance in the prediction power for the survival of the patients, it is difficult to confirm such statement based on the presented dataset given the lack of critical PD-(L)1 expression data. The data coverage for other perspective of the patient data are also a problem that should be resolved. In conclusion, while it is verifiable the TMB would heavily impact the NSCLC patient survival, their specific impact relationship is difficult to determine due to the lack of specific data classification, lack of data with solid support, data sensitivity should be improved along with the sample bias problem.

## References

[1] Mark Yarchoan, Alexander Hopkins, Elizabeth M. Tumor mutational burden and response rate to PD-1 inhibition. N Engl J Med. 2017 Dec 21; 377(25): 2500–2501.

[2] Naiyer A. Rizvi, Matthew D. Hellmann, Alexandra Snyder, et al. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. Science, 2015, 348(6230): 124-128.

[3] Cheng DT, Mitchell TN, Zehir A, et al. Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT): A hybridization capture-based next-generation sequencing clinical assay for solid tumor molecular oncology. J Mol Diagn,2015,17:251-264.

[4] Zehir A, Benayed R, Shah RH, et al. Mutational landscape of metastatic cancer revealed from prospective clinical sequencing of 10,000 patients. Nat Med, 2017, 23:703-713.

[5] Hira Rizvi, Francisco Sanchez-Vega, et al. Molecular Determinants of Response to Anti–Programmed Cell Death (PD)-1 and Anti–Programmed Death-Ligand 1 (PD-L1) Blockade in Patients with Non–Small-Cell Lung Cancer Profiled with Targeted Next-Generation Sequencing. J Clin Oncol. 2018 Mar 1; 36(7): 633–641.

[6] Pan, D., Hu, A. Y., Antonia, S. J., & Li, C.-Y. (2021). A gene mutation signature predicting immunotherapy benefits in patients with NSCLC. Journal of Thoracic Oncology, 16(3), 419–427.

[7] Thompson JC; Yee SS; Troxel AB; Savitch SL; Fan R; Balli D; Lieberman DB; Morrissette JD; Evans TL; Bauml J; Aggarwal C; Kosteva JA; Alley E; Ciunci C; Cohen RB; Bagley S; Stonehouse-Lee S; Sherry VE; Gilbert E; Langer C; Vachani A; Carpenter EL; (n.d.). Detection of therapeutically targetable driver and resistance mutations in lung cancer patients by next-generation sequencing of cell-free circulating tumor DNA. Clinical cancer research: an official journal of the American Association for Cancer Research.

[8] Zill OA, Greene C, Sebisanovic D, Siew LM, Leng J, Vu M, et al. Cell-Free DNA Next-Generation Sequencing in Pancreatobiliary Carcinomas. Cancer Discov. 2015; 5:1040–8.

[9] Allard WJ, Matera J, Miller MC, Repollet M, Connelly MC, Rao C, et al. Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. Clin Cancer Res. 2004; 10:6897–904.

[10] Krebs MG, Hou JM, Sloane R, Lancashire L, Priest L, Nonaka D, et al. Analysis of circulating tumor cells in patients with non-small cell lung cancer using epithelial marker-dependent and -independent approaches. J Thorac Oncol. 2012; 7:306–15.

[11] Lanman RB, Mortimer SA, Zill OA, Sebisanovic D, Lopez R, Blau S, et al. Analytical and Clinical Validation of a Digital Sequencing Panel for Quantitative, Highly Accurate Evaluation of Cell-Free Circulating Tumor DNA. PLoS One. 2015;10: e0140712.

[12] Schwaederle M, Husain H, Fanta PT, Piccioni DE, Kesari S, Schwab RB, et al. Detection rate of actionable mutations in diverse cancers using a biopsy-free (blood) circulating tumor cell DNA assay. Oncotarget. 2016; 7:9707–17.

[13] Punnoose EA, Atwal S, Liu W, Raja R, Fine BM, Hughes BG, et al. Evaluation of circulating tumor cells and circulating tumor DNA in non-small cell lung cancer: association with clinical endpoints in a phase II clinical trial of pertuzumab and erlotinib. Clin Cancer Res. 2012; 18:2391–401.