

S. Yeo^{1,2}, W. Figgitt³, K. Karapanos³, M.M. Yeung^{2,3}, D. Huang³, N. Vukelic^{1,4}, F. Atashrazm^{1,5}, P. Wang^{2,6}, A.T. Papenfuss^{2,6}, N. Smallwood^{7,8}, K. Sutherland^{2,6}, V. Naranbhai^{7,8}, D. Steinfert^{2,9}, S. Parakh^{1,4}, T. Leong^{1,2,5}, **S.Q. Wong^{2,3,*}**

1. Olivia Newton-John Cancer Research Institute, Melbourne, Australia , 2. University of Melbourne, Melbourne, Australia, 3. Peter MacCallum Cancer Centre, Melbourne, Australia, 4. La Trobe University, Melbourne, Australia , 5. Austin Health, Melbourne, Australia , 6. Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia , 7. Monash University, Melbourne, Australia , 8. Alfred Hospital, Melbourne, Australia , 9. Royal Melbourne Hospital, Melbourne, Australia

Presenting Author and Correspondence: stephen.wong@petermac.org

INTRODUCTION

Checkpoint inhibition has significantly improved outcomes in metastatic non-small cell lung cancer (NSCLC); however, primary and acquired resistance remain common, and robust predictive biomarkers are lacking. While PD-L1 expression is routinely used and tumor mutational burden remains investigational, both demonstrate limited predictive accuracy. Circulating tumour DNA (ctDNA) methylation profiling offers a minimally invasive approach to capture tumor-derived epigenetic alterations and identify novel biomarkers for predicting response or resistance to treatment (Fig 1)

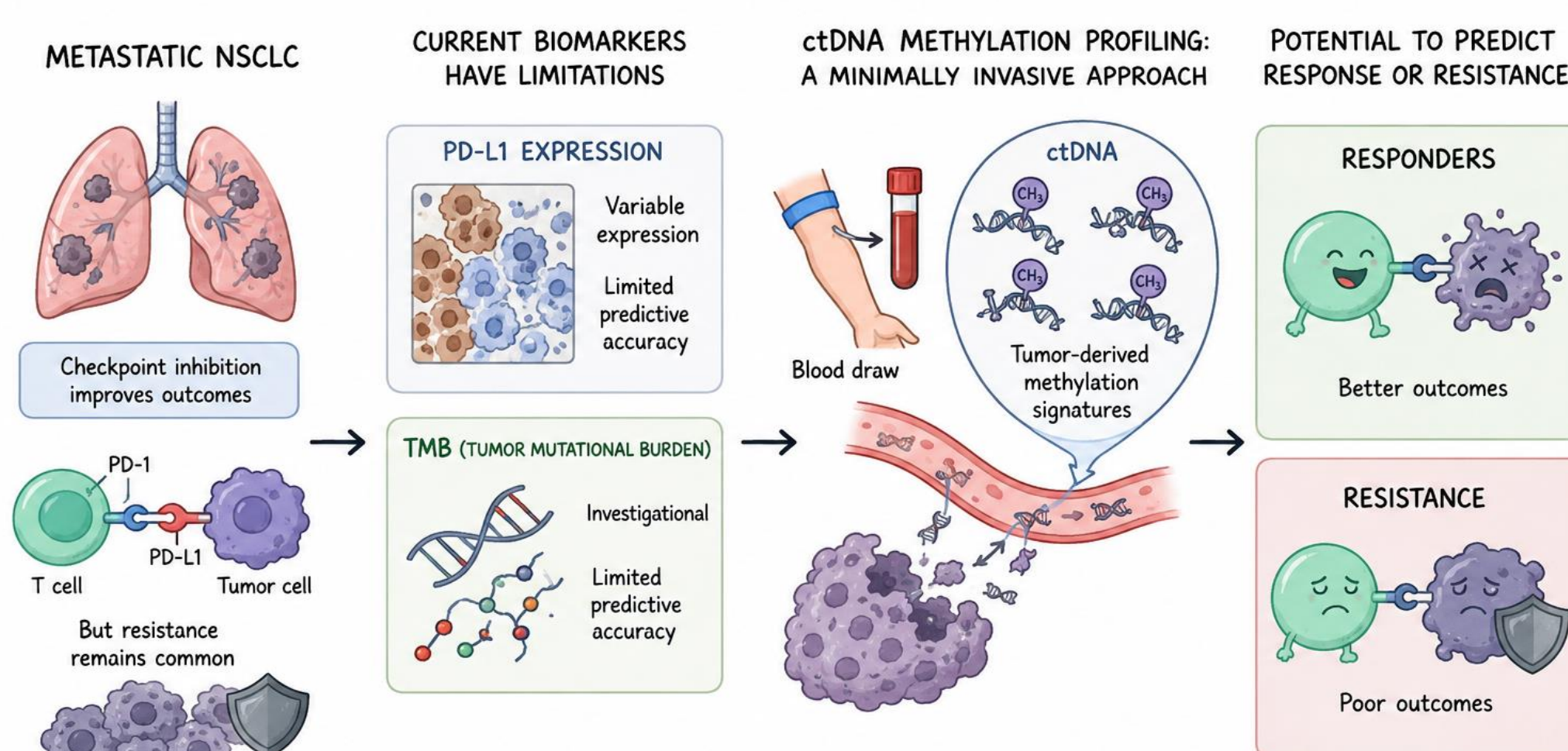


Figure 1. Schematic overall of the study's aims

AIMS AND METHODS

Aims of study

- To characterize the cell-free DNA Methylome of Stage IV NSCLC patients
- To understand if cell free DNA methylation patterns are associated with clinical and molecular features of patients
- To identify methylation targets associated with response or resistance to IO treatment.

Methods

This study was embedded in TRACKER (1), a prospective, multicentre cohort study in Australia of patients with stage IV NSCLC treated with standard-of-care immune checkpoint inhibition-based regimens, including chemo-immune checkpoint inhibition. Plasma samples were collected at baseline and at disease progression. Responders were defined as patients who achieved a radiological response for ≥ 6 months after treatment initiation.

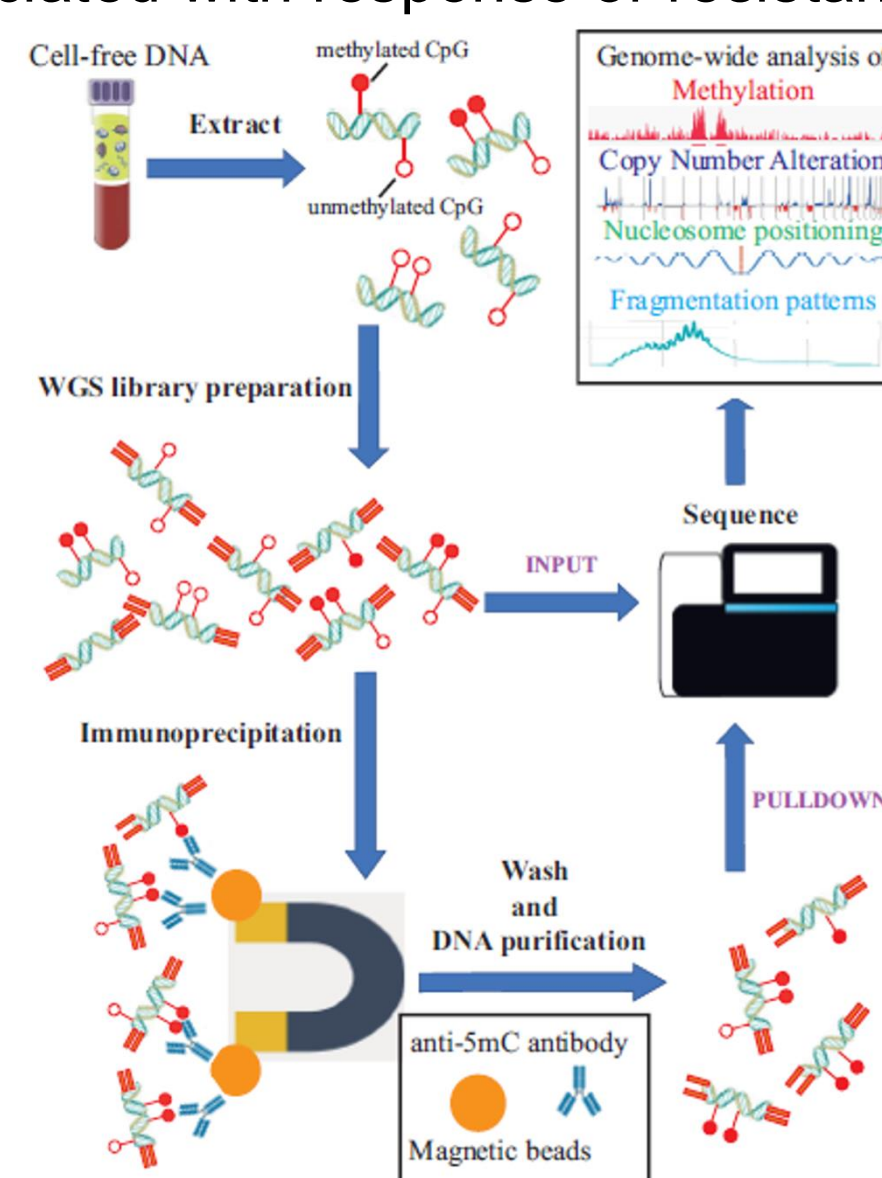


Figure 2. Analysis of DNA methylation was performed via cfMeDIP-seq.

Cell-free DNA underwent whole-genome sequencing and cell-free methylated DNA immunoprecipitation sequencing (cfMeDIP-seq) (Fig 2). ctDNA tumor fraction was estimated using ichorCNA, and differential methylation analysis between responders and primary non-responders to IO was assessed using MEDIPS, which quantifies cfMeDIP-seq signal in genomic windows and tests for differential coverage using edgeR.

DISCUSSION AND CONCLUSION

The NSCLC plasma DNA methylation landscape

- 29 patients were included in the analysis including 5 patients treated with targeted therapy and 24 patients treated with immunotherapy. Median age was 68 years (range 47-81). For IO treated patients, this comprised of 12 responders, 11 non-responders and 1 patient currently not assessable for response (Fig 3).
- Methylation profiles from NSCLC patients were compared with healthy donors, identifying significant differentially methylated regions (DMRs), including both hypo- and hypermethylated regions, predominantly concentrated in promoter regions. Distinct methylation patterns clearly separated NSCLC from healthy donors, in particularly those with higher tumour purity clustered together, demonstrating that plasma methylation signatures can reliably discriminate cancer patients from healthy controls (Fig 4).
- Differential methylation analysis between NSCLC patients and healthy controls showed global hypomethylation in lung cancer patients compared to healthy controls (Fig 5A) and enrichment of hypermethylated regions in the promoter regions of NSCLC patients (Fig 5B).

DNA Methylation changes associated with clinical features

- AHRR & F2RL3 hypomethylation was a robust, dose-dependent smoking biomarkers and showed greater hypomethylation in current vs. past smokers (2) (Fig 6).
- There were no statistically significant differential methylation differences associated with age or gender.

Genomic and DNA Methylation features associated with response and resistance to IO

- Whole-genome sequencing revealed higher ctDNA tumor fractions at baseline in non-responders compared to responders to IO, reflecting greater disease burden in non-responders. (Fig 7).
- cfMeDIP-seq analysis showed hypermethylated promoter regions (gene silencing) converged on genes involved in Immune evasion antigen presentation pathways and T-cell engagement machinery (Fig 8).
- Analysis of three responders showed acquired methylation changes at the time of disease progression (Fig 9).

Future Directions

- This study provides an initial characterization of ctDNA abundance and methylation profiles in metastatic NSCLC patients treated with checkpoint inhibition
- Ongoing work integrating these data within a multi-omics framework aims to improve prediction of response to checkpoint inhibition and inform personalized treatment strategies.

REFERENCES

- Yeo S, Wong SQ, Atashrazm F, et al. Lung cancer biobanking in Australia: challenges and future directions. *Med J Aust.* 2025;223(4):180–184. doi:10.5694/mja2.70012
- Fasanelli F, et al. Hypomethylation of smoking-related genes is associated with future lung cancer in four prospective cohorts. *Nat Commun.* 2015;6:10192. doi:10.1038/ncomms10192

RESULTS

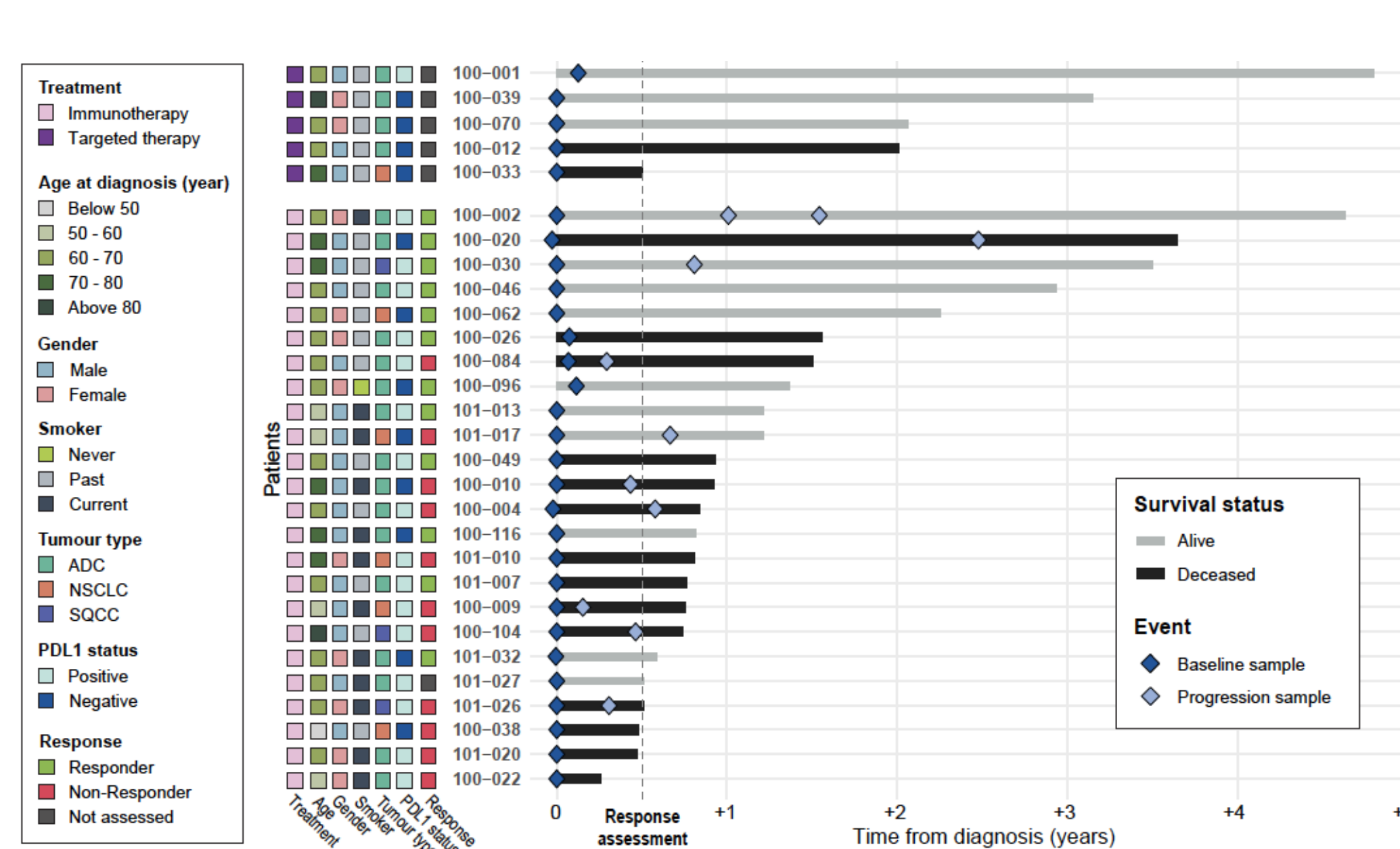


Figure 3. Swimmer plot of patients analysed using cfMeDIP-seq

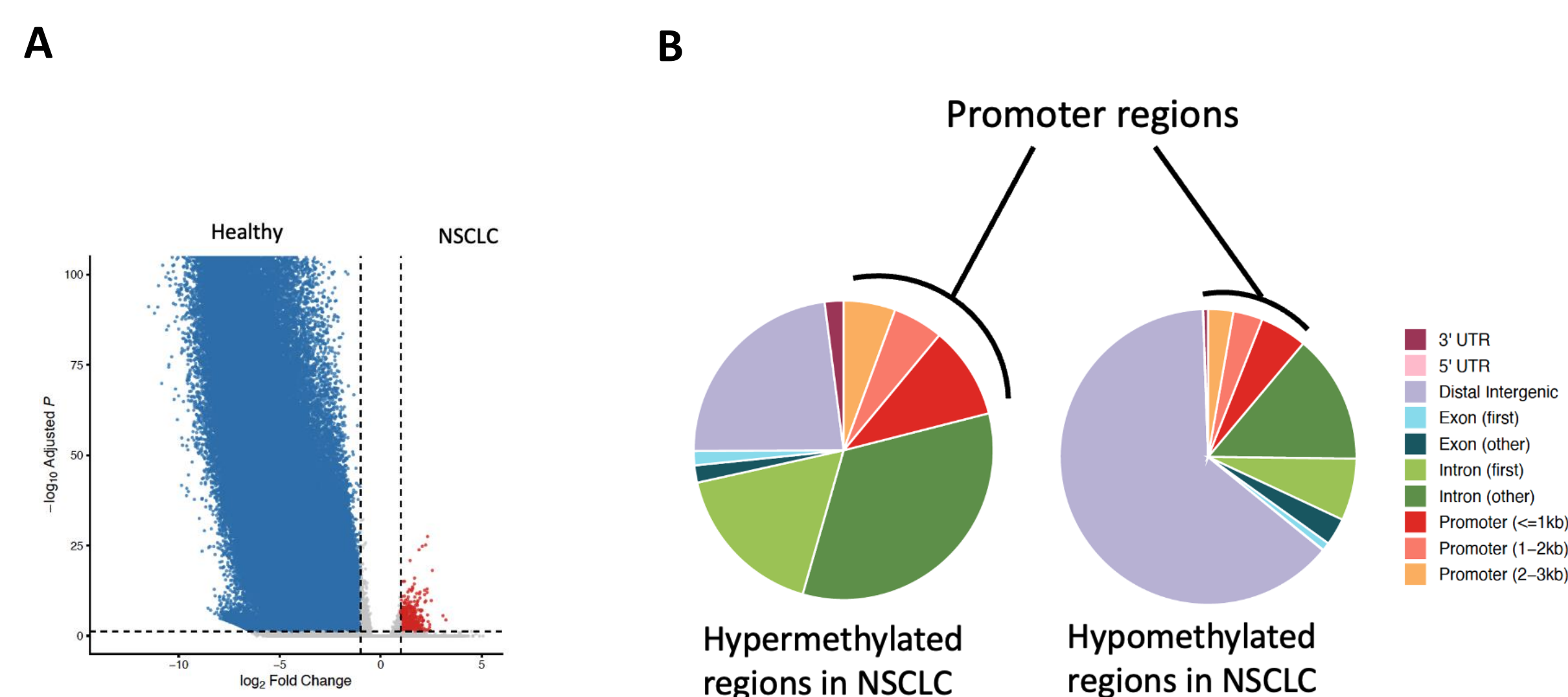


Figure 5. A) Differential analysis of methylated regions between NSCLC patients and healthy donor controls B) Genomic context of hyper- and hypo- methylated regions for the NSCLC cohort.

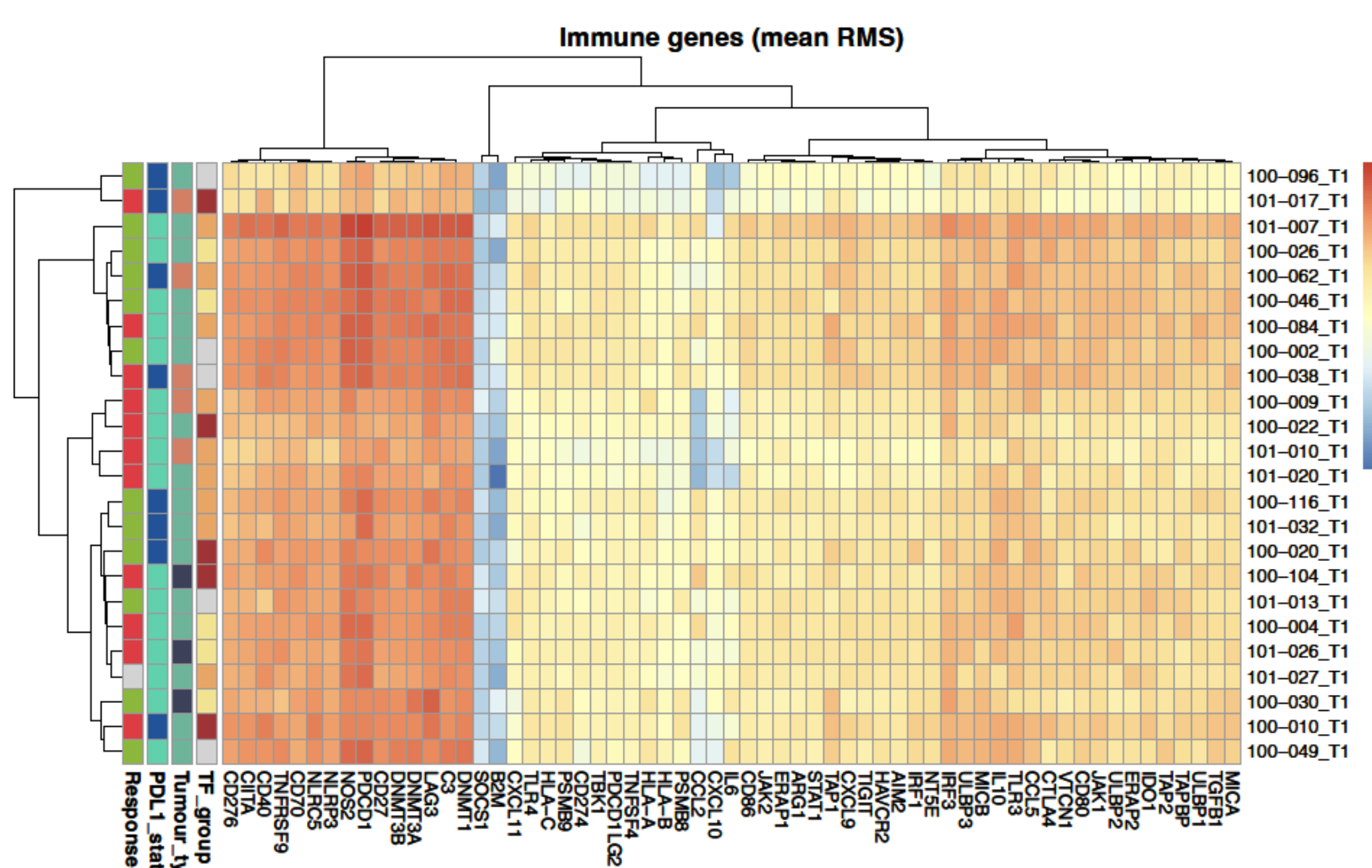


Figure 8. Methylation patterns associates with known immunomodulatory gene. Plot shows methylation levels in genes at promoter regions.

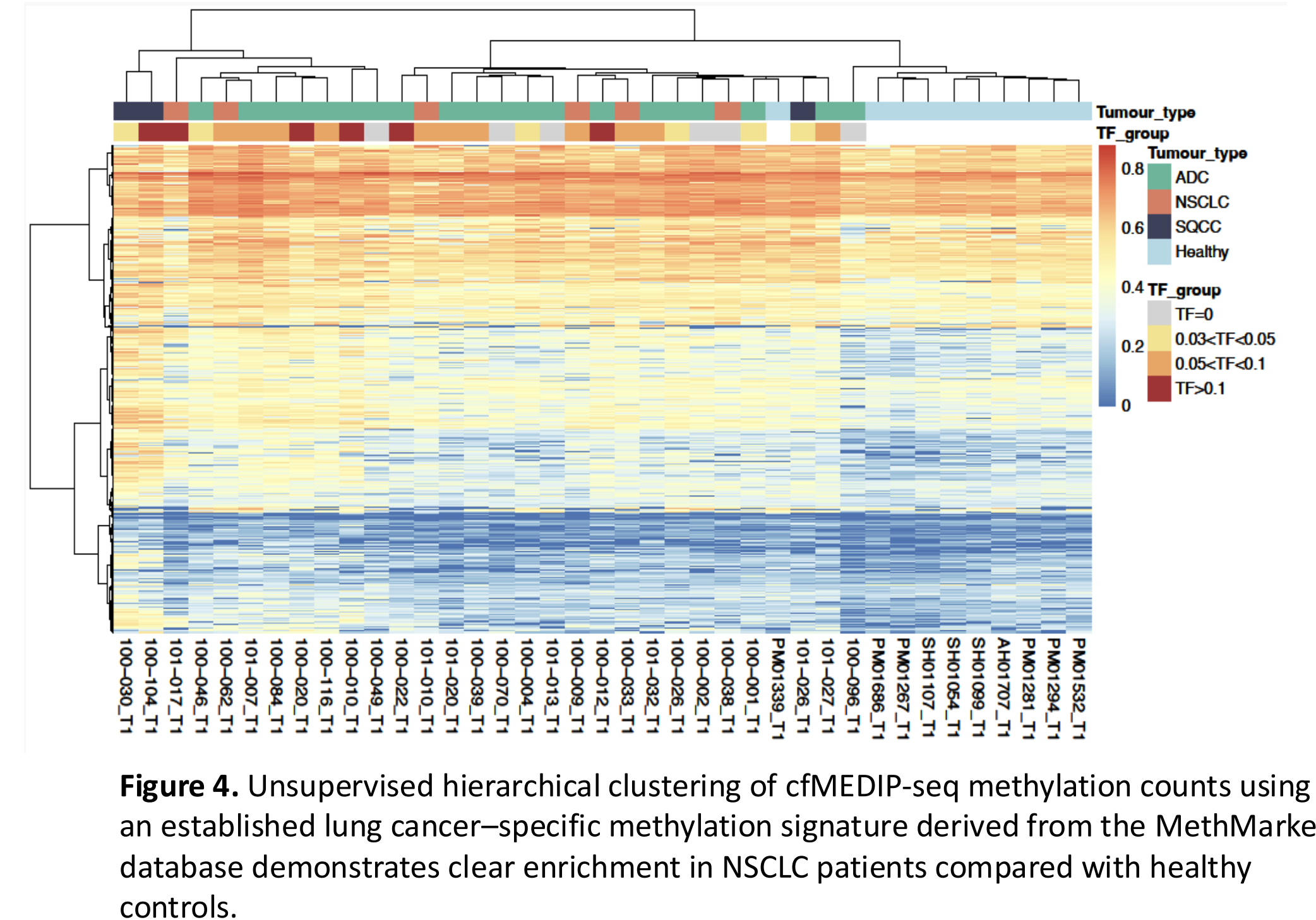


Figure 4. Unsupervised hierarchical clustering of cfMeDIP-seq methylation counts using an established lung cancer-specific methylation signature derived from the MethMarker database demonstrates clear enrichment in NSCLC patients compared with healthy controls.

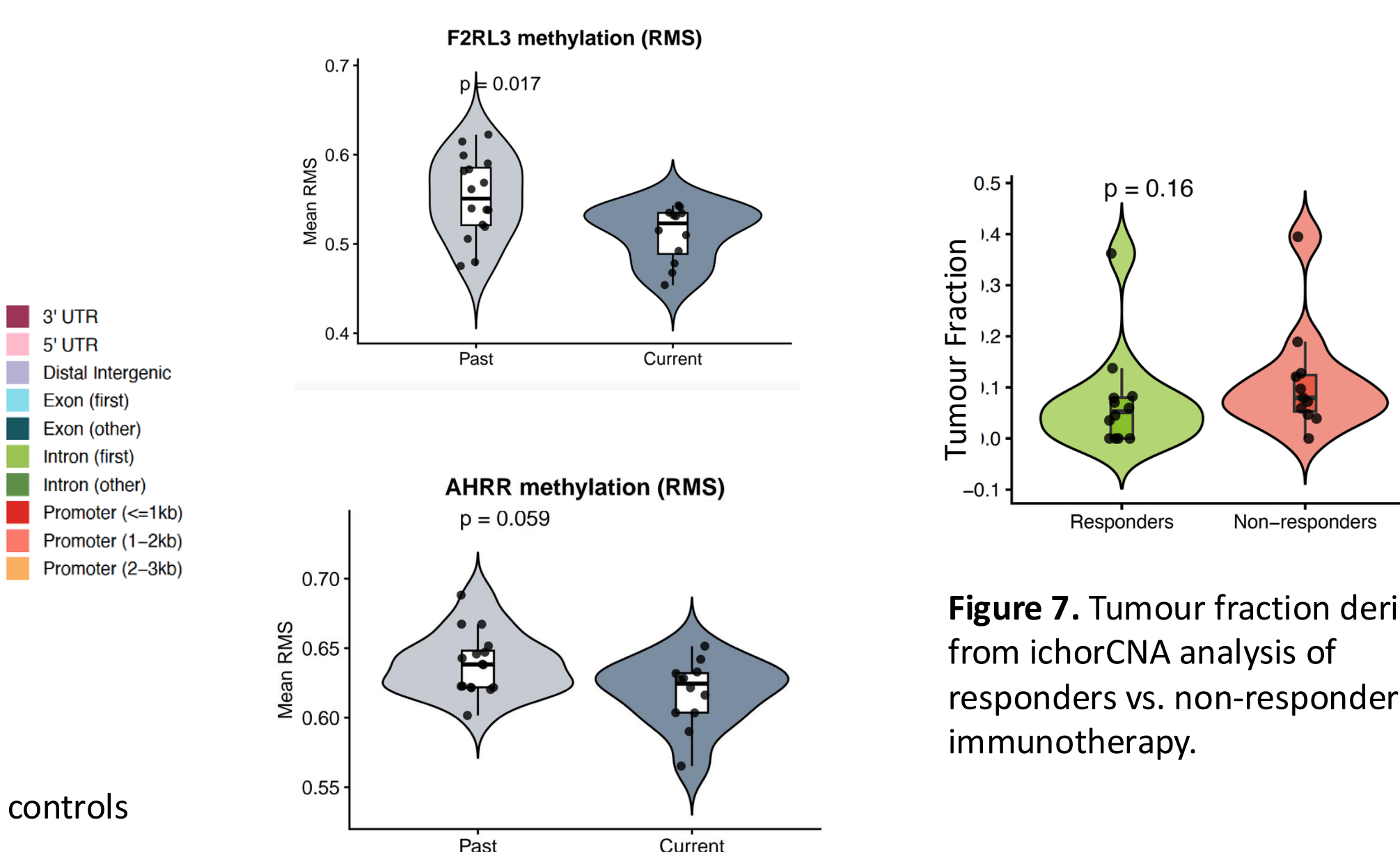


Figure 7. Tumour fraction derived from ichorCNA analysis of responders vs. non-responders to immunotherapy.

Figure 6. AHRR & F2RL3 methylation levels in past vs. current smokers.

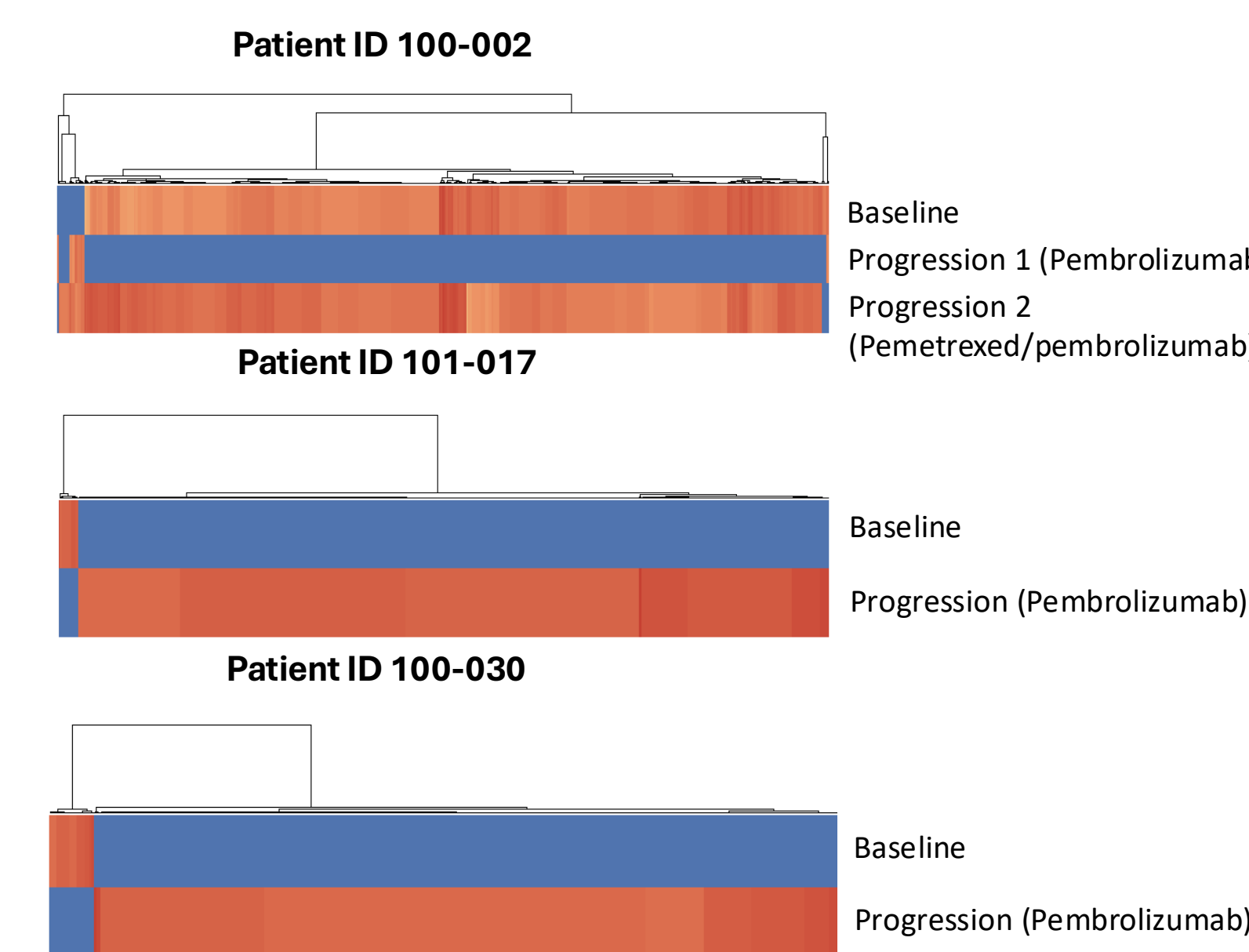


Figure 9. Longitudinal Analysis of DNA methylation following treatment in three patients with acquired resistance to immunotherapy. The top 1000 most variable windows are shown for each patient.