nature portfolio

Peer Review File

Peripheral blood mononuclear cell DNA methylation signatures guide surgical decision-making in indeterminate pulmonary nodules

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have submitted a well-designed study on the power of PBMC DNA methylation patterns can differentiate between benign and malignant models, and furthermore, whether these patterns can distinguish between MIA vs. IAC. The manuscript is written well and is thoughtful. Please allow me to share the following thoughts about the study:
1. The validation cohort was not blinded or tested in an entirely different cohort, such as that at a different institution. The algorithms chosen can therefore constantly be tweaked or tested until they perform well in both the training as well as validation cohorts. This should be mentioned as a limitation of the study.
2. AUC was the primary criterion used to evaluate algorithm performance. As this is a translational study, the authors could consider picking a cut-off point and reporting sensitivity, specificity, positive predictive value, and negative predictive value for their models as an example of how their models might translate into the clinical setting.
3. Several clinical parameters were used in both the SurgMalig and ResectGuide models. The individual contribution of the DNA methylation patterns vs. clinical parameters is not clear. Often, clinical parameters are introduced when molecular features are not enough to generate clinically meaningful models. The authors should consider reporting model performance when just DNA methylation patterns are used as predictors. Otherwise, the paper is less convincing that there is something special about PBMC DNA methylation patterns that should be studied further.
4. The methods section indicates that $P<0.05$ was used as the significance cut-off value. As the authors recognize later in the manuscript, this may not be adequate to test >100 models. The authors later suggest that during DMC evaluation, a $P<0.001$ was used in pairwise comparisons. Determination of significance cut-off values should be clarified in the methods, as well as whether common methods, such as a Bonferroni correction, were used.

Reviewer #2

(Remarks to the Author)
The manuscript presents an innovative approach to the non-invasive classification of pulmonary nodules using DNA methylation signatures derived from peripheral blood mononuclear cells (PBMCs). In particular, in the context of malignant pulmonary nodules, it is crucial to distinguish minimally invasive adenocarcinoma (MIA) from early invasive adenocarcinoma (IAC), given their considerably different surgical management and prognostic implications. In this study, the authors employed Methylome-seq and targeted bisulfite sequencing (TBS) to profile DNA methylation patterns and identify differentially methylated cytosines (DMCs) and differentially methylated regions (DMRs) as candidate diagnostic biomarkers. Subsequently, the authors constructed two machine-learning models by integrating PBMC methylation signatures with conventional blood biomarkers. The SurgMalig model functions as a gatekeeper to identify nodules that warrant surgical resection, whereas the ResectGuide model provides guidance on the appropriate extent of surgery for malignant nodules.
The work demonstrates several notable strengths. First, the decision to focus on PBMC epigenetics rather than ctDNA represents a highly pragmatic and clinically feasible strategy, particularly in early-stage disease where ctDNA levels are often undetectable. The study design is also reinforced by a rigorous multi-cohort structure, including discovery, training, and independent test cohorts, which enhances the robustness and reproducibility of the findings. Methodologically, the combination of genome-wide methylation profiling, targeted bisulfite validation, and advanced machine-learning integration reflects a comprehensive and well-executed analytical framework. Finally, the consistent identification and validation of two key methylation features, DMC-16 and DMR-13, across multiple datasets underscores their potential as reliable biomarkers

for pulmonary nodule classification. Overall, the work aligns well with current efforts in the field to address an important unmet clinical need. However, while the manuscript is generally strong, several points require clarification or further discussion.
Major concerns:
1. PBMC heterogeneity and cell-type composition are not considered. PBMCs include multiple immune subsets (T cells, B cells, NK cells, monocytes), each characterized by distinct and well-established DNA methylation profiles. Consequently, differences in methylation signals may reflect shifts in cell-type proportions rather than true cancer-associated epigenetic remodelling. To strengthen the biological validity of the findings, the authors should, where possible, include additional analyses addressing cell-type composition or discuss this limitation more explicitly.
2. Limited sample size. As acknowledged by the authors in the Discussion section, the cohorts are relatively small. Given the heterogeneity of PBMC methylation and the complexity of the machine-learning models, larger patient sets would be necessary to ensure adequate statistical power and reduce the risk of overfitting. Expanding the sample size or validating the models in an external multicentre cohort would make the results more statistically robust. That said, despite the limited number of cases, the study still includes two independent sets for training and validating the models, and the work remains highly innovative; overall, it may be more appropriate to frame it as an exploratory study.
From Figure 1A, it also appears that the SurgMalig and ResectGuide models were trained directly on the Test Cohort, whereas the text and Figure 6 suggest a more logical workflow: training first, followed by testing. Which interpretation is correct?
3. Lack of functional characterization. Although ASIC2 and SPATS2 are mentioned in relation to cAMP signalling, no mechanistic interpretation or supporting literature is provided to explain how methylation changes in these loci within immune cells might influence lung tumorigenesis or systemic tumor-immune interactions. Expanding the discussion with plausible biological relevance or testable hypotheses would enhance the translational impact of the biomarkers.
4. Missing details on clinical variables. Some potential confounders are insufficiently detailed or controlled, for instance, radiologic features beyond basic nodule type and size, which may influence model performance, are not discussed. Smoking status is recorded only categorically, without quantitative measures such as pack-years, despite its known impact on methylation patterns. More granular clinical annotation would improve the clarity and interpretability of the findings. Moreover, patient age is a critical factor, as it is well established that immune-aging is associated with an elevated risk of cancer development. Unfortunately, this important information is not addressed in the current study.
In conclusion, the manuscript presents highly promising work that could significantly enhance the management of pulmonary nodules. The study is well executed and the findings are potentially impactful. However, clarification regarding PBMC cellular composition, confounding factors, and biological interpretation is needed to strengthen the manuscript and support its translational potential.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Thank you for your thoughtful corrections to the manuscript.

Reviewer #2

(Remarks to the Author)
Following the authors' revisions, the manuscript has been substantially improved and all major concerns have been adequately addressed. I therefore support its publication.

Replies to Reviewer #1

Comment 1: The validation cohort was not blinded or tested in an entirely different cohort, such as that at a different institution. The algorithms chosen can therefore constantly be tweaked or tested until they perform well in both the training as well as validation cohorts. This should be mentioned as a limitation of the study.

Answer: Thank you for your suggestion. The sample size was derived from a single center, which may limit the statistical robustness of our model. Future work will validate these results through multi-center cohorts with expanded participant recruitment. We have added these descriptions in the revised manuscript according to reviewer's suggestion (line 452-454).

Comment 2: AUC was the primary criterion used to evaluate algorithm performance. As this is a translational study, the authors could consider picking a cut-off point and reporting sensitivity, specificity, positive predictive value, and negative predictive value for their models as an example of how their models might translate into the clinical setting.

Answer: Thank you for your suggestion. In accordance with reviewer's comments, we have supplemented the descriptions of these metrics in Supplementary Table 8-10.

Supplementary Table 8. The sensitivity and specificity of various biomarkers in the Train cohort.

Distinction	Biomarker	Sensitivity	Specificity	PPV	NPV	Cutoff	AUC	95% CI
BPN vs MIA+eIAC	DMC-16	0.933	0.543	0.950	0.467	0.245	0.830	0.749 – 0.911
	DMR-13	0.933	0.543	0.950	0.467	0.519	0.775	0.686 – 0.865
	DMC-16+DMR-13	0.867	0.671	0.922	0.531	0.770	0.853	0.778 – 0.928
	CA19-9	0.933	0.286	0.909	0.359	13.350	0.556	0.441 – 0.672
	CA125	0.600	0.557	0.765	0.367	9.070	0.538	0.413 – 0.663
	NSE	0.600	0.500	0.745	0.340	11.750	0.510	0.386 – 0.635
	CEA	0.533	0.657	0.767	0.400	1.125	0.534	0.396 – 0.672
	Cyfra21-1	0.967	0.271	0.950	0.363	1.675	0.614	0.495 – 0.733
MIA vs eIAC	DMC-16	0.970	0.459	0.944	0.615	0.280	0.744	0.630 – 0.859
	DMR-13	0.727	0.595	0.710	0.615	0.542	0.666	0.538 – 0.794
	DMC-16+DMR-13	0.970	0.459	0.944	0.615	0.998	0.753	0.640 – 0.866
	CA19-9	0.545	0.514	0.559	0.500	8.050	0.475	0.337 – 0.612
	CA125	0.545	0.730	0.643	0.643	7.840	0.564	0.423 – 0.705
	NSE	0.394	0.730	0.574	0.565	10.150	0.526	0.388 – 0.664
	CEA	0.485	0.838	0.646	0.727	1.695	0.624	0.489 – 0.760
	Cyfra21-1	0.879	0.270	0.714	0.518	3.105	0.520	0.382 – 0.657

Supplementary Table 9. The sensitivity and specificity of various biomarkers in the Test cohort.

Distinction	Biomarker	Sensitivity	Specificity	PPV	NPV	Cutoff	AUC	95% CI
BPN vs MIA+eIAC	DMC-16	0.565	1.000	1.000	0.444	0.345	0.784	0.692 – 0.875
	DMR-13	0.797	0.708	0.887	0.548	0.612	0.772	0.662 – 0.883
	DMC-16+DMR-13	0.768	0.750	0.898	0.529	0.700	0.825	0.740 – 0.911
	CA19-9	0.435	0.750	0.833	0.316	11.500	0.530	0.395 – 0.666

	CA125	0.261	0.917	0.900	0.301	15.400	0.554	0.429 – 0.680
	NSE	0.464	0.667	0.800	0.302	12.950	0.518	0.377 – 0.658
	CEA	0.986	0.125	0.767	0.750	0.385	0.481	0.346 – 0.615
	Cyfra21-1	0.710	0.458	0.790	0.355	1.935	0.566	0.427 – 0.705
MIA vs eIAC	DMC-16	0.686	0.618	0.649	0.657	0.365	0.659	0.529 – 0.789
	DMR-13	0.686	0.765	0.750	0.703	0.664	0.705	0.580 – 0.831
	DMC-16+DMR-13	0.914	0.471	0.640	0.842	0.367	0.720	0.600 – 0.840
	CA19-9	0.314	0.912	0.583	0.667	5.600	0.561	0.422 – 0.699
	CA125	0.314	0.912	0.786	0.564	7.875	0.594	0.458 – 0.731
	NSE	0.771	0.353	0.551	0.600	10.700	0.512	0.372 – 0.651
	CEA	0.657	0.735	0.719	0.676	1.535	0.698	0.570 – 0.826
	Cyfra21-1	0.771	0.618	0.675	0.724	2.135	0.696	0.570 – 0.822

Supplementary Table 10. Performance of SurgMalig Model and ResectGuide Model in the Train and Test cohorts.

Model	Cohort	Algorithm	AUC	PRAUC	Accuracy	Classification Error	Sensitivity	Specificity	Precision	PPV	NPV	Recall	F1_Score
SurgMalig Model	Train	Lasso+Stepglm[forward]	0.926	0.970	0.860	0.140	0.843	0.900	0.952	0.952	0.711	0.843	0.894
SurgMalig Model	Test	Lasso+Stepglm[forward]	0.806	0.934	0.742	0.258	0.710	0.833	0.925	0.925	0.500	0.710	0.803
ResectGuide Model	Train	Enet[alpha=0.2]	0.862	0.881	0.814	0.186	0.636	0.973	0.955	0.955	0.750	0.636	0.764
ResectGuide Model	Test	Enet[alpha=0.2]	0.843	0.862	0.783	0.217	0.629	0.941	0.917	0.917	0.711	0.629	0.746

Comment 3: Several clinical parameters were used in both the SurgMalig and ResectGuide models. The individual contribution of the DNA methylation patterns vs. clinical parameters is not clear. Often, clinical parameters are introduced when molecular features are not enough to generate clinically meaningful models. The authors should consider reporting model performance when just DNA methylation patterns are used as predictors. Otherwise, the paper is less convincing that there is something special about PBMC DNA methylation patterns that should be studied further.

Answer: Thanks for the comments. We have supplemented the performance report when using only DNA methylation patterns as predictive factors (Fig. 5I-L; Supplementary Fig. 4; Supplementary Table 8-9). ROC curve results demonstrating the ability of DMC-16, DMR-13 and their combination score to distinguish patients with BPN who did not require surgical resection from patients with MIA and eIAC who did require surgical resection in the Train and Test cohorts, yet conventional serum tumor biomarkers were unavailable (Fig. 5I-J; Supplementary Fig. 4A-B). Besides, ROC

curve results demonstrating DMC-16, DMR-13 and their combination score were also able to differentiate to some extent between patients with MIA requiring only sublobectomy and patients with eIAC requiring lobectomy, and similarly conventional serum tumor biomarkers did not have the ability to differentiate (Fig. 5K-L; Supplementary Fig. 4C-D). The relevant metrics of these ROC curves were shown in Supplementary Table 8-9.

Comment 4: The methods section indicates that P<0.05 was used as the significance cut-off value. As the authors recognize later in the manuscript, this may not be adequate to test >100 models. The authors later suggest that during DMC evaluation, a P<0.001 was used in pairwise comparisons. Determination of significance cut-off values should be clarified in the methods, as well as whether common methods, such as a Bonferroni correction, were used.

Answer: Thank you for this insightful suggestion. We apologize for the confusion caused by the clerical error. While we presented the "q value" in Figure 1, we mistakenly wrote "p value" in the subsequent results section. In fact, during the identification of pulmonary nodule subtype-specific DMCs and DMRs via Methylome-seq, the p values were corrected for multiple hypothesis testing using the Benjamini-Hochberg (B-H) procedure to obtain a q value. $q < 0.05$ was used as the significance cut-off for DMRs. Although $q < 0.05$ was initially used as the significance threshold for DMCs, the large number of DMCs identified led us to subsequently filter for more significantly differential methylation by applying a stricter threshold of $q < 0.001$. As suggested by the reviewer, we have supplemented the relevant description in the Materials section (line 220-226).

Replies to Reviewer #2

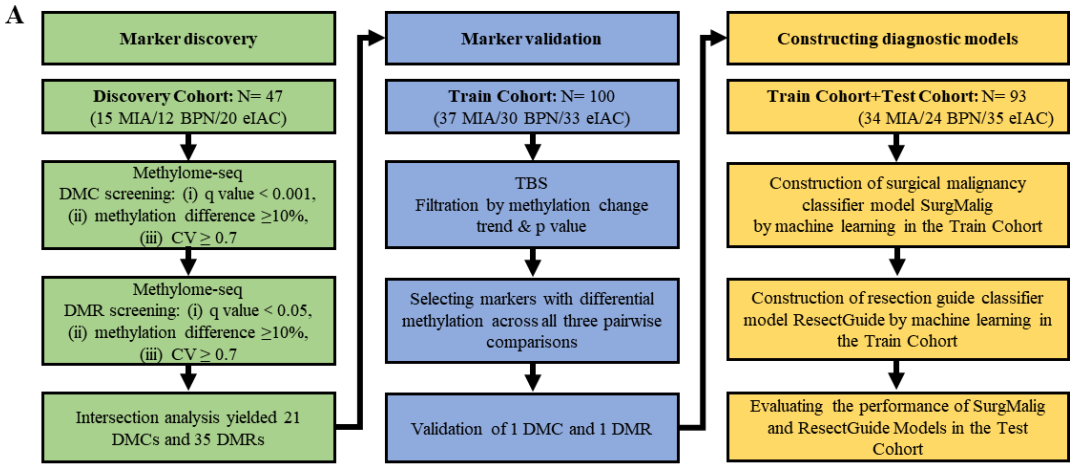
Comment 1: PBMC heterogeneity and cell-type composition are not considered. PBMCs include multiple immune subsets (T cells, B cells, NK cells, monocytes), each characterized by distinct and well-established DNA methylation profiles. Consequently, differences in methylation signals may reflect shifts in cell-type proportions rather than true cancer-associated epigenetic remodelling. To strengthen the biological validity of the findings, the authors should, where possible, include additional analyses addressing cell-type composition or discuss this limitation more explicitly.

Answer: Thank you for this insightful suggestion. It was indeed possible that the observed methylation alterations could stem from differences in PBMC cell-type proportions. To address this, we analyzed routine blood test data from the MIA, BPN, and eIAC groups in both the Train and Test sets. Our analysis showed no significant differences in the major PBMC components, monocytes and lymphocytes, across these groups (Supplementary Tables 2-3). These findings largely ruled out cell-type proportion differences as a primary driver of the methylation changes.

However, our results did not yet pinpoint in which specific cellular subpopulations these methylation alterations occur. Recently, research teams have begun developing single-cell methylome profiling technologies[1, 2]. Building on our preliminary conclusions, future studies could apply single-cell methylomics to further identify the specific cell subsets harboring these methylation changes. We have also included this limitation in the Discussion section (line 401-411).

Comment 2: Limited sample size. As acknowledged by the authors in the Discussion section, the cohorts are relatively small. Given the heterogeneity of PBMC methylation and the complexity of the machine-learning models, larger patient sets would be necessary to ensure adequate statistical power and reduce the risk of overfitting. Expanding the sample size or validating the models in an external multicentre cohort would make the results more statistically robust. That said, despite the limited number of cases, the study still includes two independent sets for training and validating the models, and the work remains highly innovative; overall, it may be more appropriate to frame it as an exploratory study. From Figure 1A, it also appears that the SurgMalig and ResectGuide models were trained directly on the Test Cohort, whereas the text and Figure 6 suggest a more logical workflow: training first, followed by testing. Which interpretation is correct?

Answer: Thank you for your suggestion. We have positioned this study as exploratory in the Discussion section, pending future prospective, multicenter validation (line 452-454). We apologize for the labeling error in Figure 1A. In fact, both the SurgMig and ResectGuide models were trained first and then tested. We have now revised Figure 1A accordingly.



Comment 3: Lack of functional characterization. Although ASIC2 and SPATS2 are mentioned in relation to cAMP signalling, no mechanistic interpretation or supporting literature is provided to explain how methylation changes in these loci within immune cells might influence lung tumorigenesis or systemic tumor-immune interactions. Expanding the discussion with plausible biological relevance or testable hypotheses would enhance the translational impact of the biomarkers.

Answer: Thanks for the comments. DMC-16 was located in the promoter region of the ASIC2 gene, whereas DMR-13 was situated within the gene body of SPATS2. ASIC2 was a member of the acid-sensing ion channel family[3]. Monocytes sensed extracellular acidification via ASIC2, and reduced expression of ASIC2 impairs their chemotactic migration and polarization, thereby attenuating its antitumor effects[4]. Single-cell sequencing data revealed that SPATS2 was significantly downregulated in infiltrating monocytes/macrophages within lung cancer tissues[5]. Given the established role of SPATS2 in regulating sperm cell differentiation, we speculated that it might also participate in macrophage polarization[6]. However, relevant studies remain limited, and further experimental investigation is warranted to explore this potential function. We have added these descriptions in the revised manuscript according to reviewer's suggestion (line 416-425).

Comment 4: Missing details on clinical variables. Some potential confounders are insufficiently detailed or controlled, for instance, radiologic features beyond basic nodule type and size, which may influence model performance, are not discussed. Smoking status is recorded only categorically, without quantitative measures such as pack-years, despite its known impact on methylation patterns. More granular clinical annotation would improve the clarity and interpretability of the findings. Moreover, patient age is a critical factor, as it is well established that immune-aging is associated with an elevated risk of cancer development. Unfortunately, this important information is not addressed in the current study.

Answer: Thanks for the comments. In designing our study, we carefully considered which clinical variables to include in our model by referencing several established lung cancer methylation screening studies. Whether based on cfDNA methylation or

leukocyte methylation, these published models incorporated only basic nodule type and size, without additional radiologic features[7-11]. Therefore, our model was designed to align with this precedent by including these two factors only.

Regarding smoking history, as this was a retrospective study, detailed data on smoking pack-years were largely missing for most patients. Consequently, our analysis was limited to the clearly documented smoking status.

We fully agree with the reviewer that patient age was a critical factor, given its established link to immunosenescence and increased cancer risk, as well as the documented association between age and DNA methylation in blood cells[12]. At the time this study was designed, the relationship between PBMC DNA methylation and lung cancer was unclear. To prevent potential confounding by age given our limited sample size, the three diagnostic groups within each cohort were intentionally well-matched for age. Now, based on the findings of this study, which confirm an association between PBMC methylation and lung cancer, future research with larger sample sizes can directly build upon our conclusions to investigate the specific influence of age on the model's performance. We have added these discussions in the revised manuscript (line 391-400).

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