Nipple Aspirate Cell-Free DNA Methylation Signatures for Early Breast Cancer Detection

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Background

Cell-free DNA (cfDNA), shed by malignant tumor cells into extracellular fluid, provides valuable epigenetic information indicative of cancer status. Nipple aspirate fluid (NAF), a noninvasive liquid biopsy from at-risk women, contains nucleic acid and protein biomarkers from adjacent cancer cells, showing promise for breast cancer (BrC) detection. However, despite its potential, the application of cfDNA in NAF for BrC screening is still underexplored. This study aims to assess the utility and sensitivity of NAF cfDNA methylation signatures as biomarkers for early detection and risk stratification of BrC.

Methods and Materials

A low-input cfDNA bisulfite sequencing (cfBS) protocol was developed and optimized for profiling genome-wide DNA methylation at single-base resolution using nanogram-scale cfDNA from NAF (Figure 1). A total of 76 NAF samples (17 BrC and 59 benign) were sequenced and analyzed. An epithelial-mesenchymal transition (EMT) methylation scoring metric was developed to quantify tumor phenotype. CpG methylation regions were identified using an in-house mean shift-based machine learning algorithm. Differentially methylated regions (DMRs) were identified using one-way ANOVA stratified by clinical stage. Predictive modeling was performed using random forest, LASSO regression, and support vector machine (SVM) classifiers.

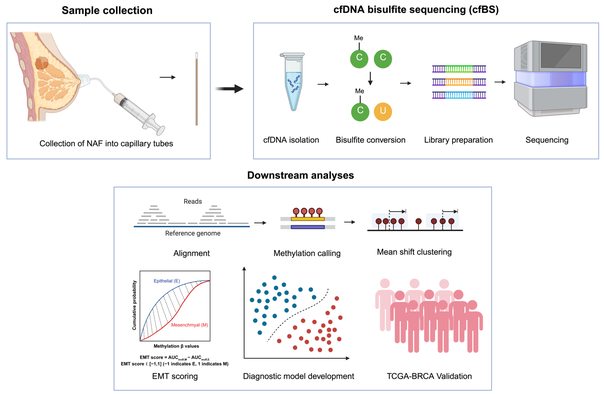


Figure 1. Schematic of the NAF cfDNA methylation profiling workflow.

Sample Description

Table 1. Sample information and distribution of risk scores in the NAF cohort.

Risk score	Description	Sample size	Category
0	Healthy, no biopsy	30	Low
1	Family history of BrC	9	
2	Hyperplasia without atypia	7	Intermediate
2b	Papilloma	2	
2c	Fibroadenoma	2	
3	Contralateral breast	7	
4	Atypical hyperplasia	2	
6	DCIS	5	High
7	Invasive	12	

*DCIS = Ductal Carcinoma In Situ

Results

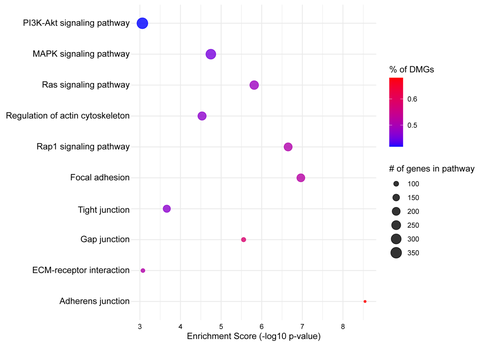


Figure 2. EMT-associated pathways identified from pathway analysis of differentially methylated genes.

Results

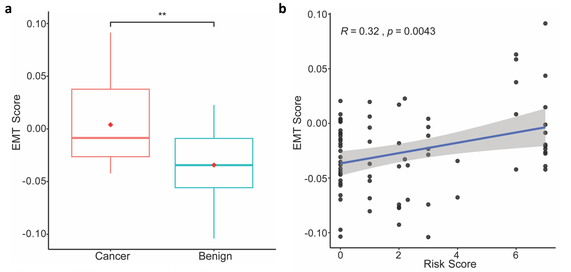


Figure 3. EMT is activated in BrC.
a EMT score between benign and cancer samples (** p-val < 0.01). **b** Correlation between EMT score and risk score.

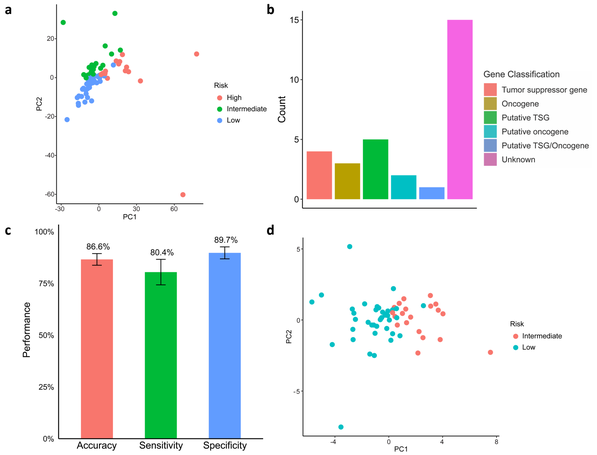


Figure 4. Performance of the 30-gene panel for early detection in NAF cfDNA.
a Scatterplot of the first 2 PCs derived from ANOVA-derived CpG regions. **b** Distribution of 30 genes across functional categories. **c** Classification performance of the 30-marker panel in distinguishing low vs. intermediate risks. Results represent the average prediction accuracy over 1,000 iterations of 5-fold cross-validation, reported as mean ± SD. **c** PCA of the 30 markers demonstrating separation between low- and intermediate-risk samples.

Results

Classification	Accuracy	Sensitivity	Specificity
Normal vs. Stage I tumors	0.976	0.968	0.988
Stage I tumors vs. Stage II–IV tumors	0.838	1	0

Table 2. Performance of the 30-gene panel in classifying early-and late-stage breast tumors in TCGA-BRCA.

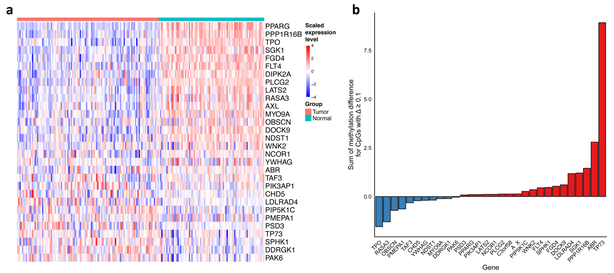


Figure 5. TCGA validation of cfDNA methylation markers for early detection.
a Heatmap of expression for the 30 genes in normal and primary tumors. **b** Cumulative methylation differences (Stage I tumor − normal) for CpG sites within each gene with $\Delta\beta \geq 0.1$. Positive values (red bars) indicate overall hypermethylation in Stage I tumors, whereas negative values (blue bars) indicate overall hypomethylation.

Conclusion

Our findings demonstrate that cfDNA methylation profiling from NAF is both technically feasible and biologically informative, offering a highly sensitive and noninvasive strategy for early BrC detection. By capturing EMT-associated epigenetic alterations, NAF cfBS has the potential to identify aggressive phenotypes at early stages. The high predictive accuracy achieved through machine learning models highlights its clinical utility as a complementary tool to existing screening modalities, especially for women with inconclusive mammograms, which is especially common in women with dense breast tissue. These results strongly support further validation in larger, prospective cohorts and suggest that NAF-based liquid biopsy could be integrated into personalized screening frameworks to improve early detection, reduce unnecessary biopsies, and guide preventative care.

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References

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