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Machine learning-based epigenetic classifiers for axillary staging of patients with ER-positive early-stage breast cancer

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Abstract

Introduction: In the era of molecular stratification and effective multimodality therapies, surgical staging of the axilla is becoming less relevant for patients with estrogen receptor (ER)-positive early-stage breast cancer (EBC). Therefore, a non-surgical method for accurately predicting lymph node disease is the next step in the de-escalation of axillary surgery. This study sought to identify epigenetic signatures in the primary tumor that accurately predict lymph node status.

Methodology: We selected a cohort of patients in The Cancer Genome Atlas (TCGA) with ER-positive, HER2-negative invasive ductal carcinomas, and clinically-negative axillae (n=127). Clinicopathological nomograms from MSKCC and MDACC were calculated. DNA methylation (DNAm) patterns from primary tumor specimens were compared between pN0 and >pN0 patients. The cohort was divided into training (n=85) and validation (n=42) sets. Random Forest was employed to obtain the combinations of DNAm features with the highest accuracy for stratifying >pN0 patients. The most efficient combinations were selected according to the Area Under the Curve (AUC).

Results: Clinicopathological models displayed a modest predictive potential for identifying >pN0 disease (MSKCC AUC=0.76, MDACC AUC=0.69, p=0.15). Differentially methylated sites (DMS) between pN0 and >pN0 patients were identified (n=1,656). DMS showed a

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similar performance to the MSKCC model (AUC=0.76, p=0.83). Machine learning approaches generated five epigenetic classifiers, which showed higher discriminative potential than the clinicopathological variables tested (AUC>0.88, p<0.05).

Conclusions: Epigenetic classifiers based on primary tumor characteristics can efficiently stratify patients with no lymph node involvement from those with axillary lymph node disease, thereby providing an accurate method of staging the axilla.

INTRODUCTION:

Surgical staging of the axilla is becoming less relevant in the modern era of molecular stratification and effective multimodality therapies for patients with early-stage breast cancer (EBC). Current clinical guidelines consider surgical staging optional for patients with favorable tumors, older patients, those whose treatment decisions will not be impacted, or those with severe comorbidities.[1,2] Based on the ACOSOG Z0011 and RxPONDER trials, a positive sentinel node no longer drives surgical or adjuvant chemotherapy decision-making, especially in postmenopausal patients.[3,4] Nodal status, however, still maintains a role in providing information regarding prognosis and stage and may influence the delivery of radiation therapy. Although the morbidity of sentinel lymph node biopsy (SLNB) is significantly reduced compared to axillary lymph node dissection (ALND), no surgical procedure is without risk.[5,6] Therefore, an alternate method for accurately predicting lymph node disease would support the omission of the surgical staging of the axilla in patients with EBC.

Several mathematical models have been developed to predict whether breast cancer (BC) has spread to the axillary lymph nodes. The Memorial Sloan Kettering Cancer Center (MSKCC) and the MD Anderson Cancer Center (MDACC) nomograms are online tools that consider patient and tumor variables such as age, tumor location, tumor size, histology, grade, multifocality/multicentricity, lymphovascular invasion (LVI), and hormone receptor expression in their prediction models. However, the performance of these nomograms, confirmed by subsequent external validation studies, ranges from 67 to 78%, which has precluded widespread clinical adoption.[7–10]

Currently, machine learning methods significantly improve the sensitivity and specificity of predictive algorithms. In previous studies, we and others have demonstrated that epigenetic classifiers identified through machine learning of DNA methylation (DNAm) profiles are highly informative and reliable for disease stratification, precision diagnosis, and BC prognosis.[11–14] Therefore, applying machine-learning methods to epigenetic and clinicopathological data can improve the accuracy of predictive algorithms and thus produce more robust decision-making assays for routine clinical practice. In this study, we constructed a machine learning classifier based on DNAm profiles of primary tumors to provide a BC subtype-specific predictive tool that accurately identifies patients who may safely omit surgical staging of the axilla.

METHODS:

Patient Selection

We manually curated and cataloged the clinicopathological data from all patients (n=1,108) with breast cancer in The Cancer Genome Atlas (TCGA-BRCA) from the National Cancer Institute Genomic Data Commons (NCI/GDC) portal.[15] Only female patients with histologically confirmed invasive ductal carcinoma with clinically-negative axillae (cN0) at presentation were included. Male patients, female patients with inoperable BC (tumor with direct extension to the chest wall and/or to the skin, inflammatory BC, or *de novo* stage IV BC), or those who received neoadjuvant therapy, and patients without axillary surgery data were excluded. Patients with estrogen receptor (ER)-negative and/or HER2-positive tumors were also excluded. Finally, specimens with tumor purity lower than 65% or with missing DNAm data were removed from the analyses. The final cohort included 127 patients between 2002 and 2013, 70 with pathologically negative lymph nodes (pN0) and 57 with positive lymph nodes (>pN0) (Figure 1).

Clinical nomograms evaluation

The variables used to calculate the nomograms from MSKCC and MDACC included age at diagnosis, tumor location and size (cm), LVI, multifocality/multicentricity, histologic type, histologic grade, and hormone receptors and HER2 status. The nomograms were calculated using the available online tools.[16,17] Three trained reviewers generated the scores for all the patients independently. The cases showing discrepancies were reviewed and final scores were determined by consensus with a surgical oncologist.

DNA methylation data access and processing

DNAm data (HumanMethylation450 BeadChip array) were downloaded using the R/Bioconductor *TCGAbiolinks* package v2.16.4,[18] on April 13, 2021. Probes that did not pass the GenomeStudio quality controls (Illumina), with known cross-reactivity or missing data in any patient, were discarded from the analysis (final n=384,258). The effective tumor purity was evaluated using the consensus estimate purity (CPE) algorithm.[19] DNAm levels were reported as β-values and calculated using the signal intensity value for each CpG site, as we previously showed.[11,12] Differentially methylated sites (DMS) between patients with pN0 and >pN0 were identified using the Wilcoxon test. All CpG sites with differential methylation over 0.10 and *p*-value below 0.01 were considered DMS. Uniform Manifold Approximation and Projection for Dimension Reduction (UMAP) and hierarchical clustering were employed to visualize the stratification capacity of all DMS using R/*M3C* (v.1.10)[20] and R/*gplots* (v.3.1.1) packages. Genes affected by each DMS were obtained using the T-Gene algorithm.[21] Pathway enrichment of the selected genes was performed using the R/*GOfuncR* v.1.8.0 package.[22] Our analysis involved the gene and gene pathway analysis based on the three categories of gene ontology (biological process, cellular compartment, and molecular function).

Machine learning-based construction of classifiers for prediction of lymph node involvement

The cohort was divided into training (67%, n=85) and validation (33%, n=42) sets. Regions identified as differentially methylated (156 hypermethylated and 1500 hypomethylated sites) were employed as input for Machine Learning. In this study, we employed Random Forest (RF) in the training cohort to identify combinations of the minimum number of genomic regions with high accuracy for stratifying patients with pathological involvement of axillary lymph nodes. RF was only applied to epigenetic data from patients in the training cohort using the R/*varSelRF* package (v.0.7–8).[23] To ensure the reproducibility of the results, this process was iterated 150 times. The epigenetic signatures (EpiLN) selected by RF with high performance in the training cohort were then tested in the validation cohort. Finally, EpiLN signatures with high predictive performance in the validation cohort were evaluated in the entire cohort and reported. To ensure reproducibility of the machine learning data processing, we have included all the scripts for the open-source packages employed in this study at the GitHub repository (https://github.com/mensenyat/EpiLN).

Statistical analysis

Univariate comparisons were assessed using Pearson's chi-square and Fisher's exact tests for categorical variables, while Wilcoxon tests were used to examine continuous variables. All statistical calculations were performed using Stata, v.15.0 (StataCorp, College Station, TX) and SPSS, v.23 (IBM, Armonk, NY) software. The performance (sensitivity and specificity) of the clinicopathological nomograms (MSKCC and MDACC), all the DMS, and the top five best-performing signatures were assessed and represented by the Receiver Operating Characteristics (ROC) Curve/Area Under Curve (AUC) using the R/*ROCR* package (v.1.0–11).[24] The predictive ability of the different models was compared using DeLong's test for correlated ROC curves.[25]

RESULTS:

Patient Characteristics and clinicopathological nomograms

A total of 127 patients with ER-positive/HER2-negative, invasive ductal carcinoma were included in our study and divided into two groups: pN0 (n=70) and >pN0 (n=57). There were no statistical differences between age at diagnosis, tumor size, tumor laterality, grade, or tumor location in the overall cohort (Table 1) or the training and validation cohorts (Supplementary Table 1). The median number of lymph nodes examined in the pN0 and >pN0 groups was 2.0 and 14.0, respectively (p-value <0.001) in the overall cohort. The median number of positive nodes for patients in the >pN0 group was 2.0. As expected, the presence of LVI was significantly higher among >pN0 compared to the pN0 group (21% vs. 49%, p <0.005). Extranodal extension was present in 38% of patients with positive nodes.

We calculated the risk of lymph node (LN) metastases in patients (n=88) based on the clinicopathological variables from the two established nomograms. Similar to what has been previously reported, both models showed a modest prediction potential, and no statistical differences were detected between the two (MSKCC AUC=0.76 and MDACC AUC=0.69, p=0.15, Table 2).

Differential methylation according to pathological lymph node status

DMS between pN0 and >pN0 patients were identified (n=1,656, p<0.01). Notably, primary tumors from patients with positive LN disease showed 156 hypermethylated and 1,500 hypomethylated CpG sites (Figure 2A). Interestingly, we observed that genes associated with these DMS are involved in functions that may affect the ability of breast cancer cells to spread to lymph nodes. The most relevant differences involved cellular immune recognition, cell adhesion, cell proliferation, morphological reprogramming, chemotaxis, extracellular matrix remodeling, cell migration, and mesenchymal cell differentiation, among other functions and processes related to metastatic progression (Supplementary Table 2). Using these 1,656 DMS, there was a non-significant separation between patients with pN0 and >pN0 (Figures 2B–C). However, DMS displayed a similar predictive potential (AUC=0.76) to the MSKCC (p=0.84) and MDACC nomograms (p=0.31) for identifying patients with pathological LN involvement (Figure 2D, Table 2). Interestingly, pathways enriched in DMS in >pN0 versus pN0 appear to be involved in cellular adhesion, cell migration, extracellular matrix, and immune response.

Machine learning-based DNAm signatures to predict pathological lymph node status

Starting with all DMS, we used a Random Forest algorithm to generate epigenetic classifiers to predict pathological LN status. We identified five top-performing epigenetics signatures ranging from 15 to 37 genomic regions (*aka* "EpiLN" signatures). All signatures showed very good performance stratifying pN0 and >pN0 patients (AUC>0.88, Figures 3A–B). These epigenetic signatures demonstrated a significantly higher discriminative potential than the clinicopathological variables tested (p=0.02, Table 2). The EpiLN signatures represent efficient combinations of differentially methylated sites from various genomic regions with the potential to identify patients with >pN0. For example, the EpiLN-37, which showed the highest predictive potential (AUC=0.89), includes 37 genomic regions with 18 hypermethylated and 19 hypomethylated sites in >pN0 tumors, affecting different gene structures and non-coding genomic regions in 19 out of the 23 chromosomes (Supplementary Table 3).

DISCUSSION:

Only a third of patients with clinically negative axillae have positive sentinel nodes at biopsy,[26] yet axillary lymph node surgery is offered to most patients to complete pathologic staging for invasive breast cancer. Fortunately, with the adoption of SLNB, morbidity from axillary surgery has decreased tremendously.[6,27] And, with multimodality treatments improving locoregional control and overall survival, strategies to de-escalate axillary surgery even further have been at the forefront of care. The Society of Surgical Oncology released the Choosing Wisely guidelines in 2016 that advocated for the omission of SLNB in patients over age 70 with early-stage clinically node-negative ER-positive breast cancer.[1] Data from the ACOSOG Z0011 and RxPONDER trials further minimized the influence of the sentinel node in the decision making for completion of ALND or adjuvant chemotherapy in patients with ER-positive breast cancer.[3,4] As we continue to search for ways to safely de-escalate axillary surgery, non-surgical tools to predict nodal disease will be invaluable.

Clinical nomograms from MSKCC and MDACC provided the initial risk assessment tools to predict sentinel node positivity in patients with EBC.[16,17] Both models utilize clinicopathological data, including tumor size, tumor location, histologic type and grade, multifocality, hormone receptor status, and presence of lymphovascular invasion.[16,17] The performance of both clinical models has been validated externally, with the accuracy of predicting lymph node metastasis ranging from AUC 0.67 to 0.78.[7–10,28] The modest performance of these models in the different cohorts has been one of the primary limitations hindering routine clinical use of these classifiers. Furthermore, these classifiers were developed based on variables from surgical pathology specimens, rather than core biopsy tissue, raising concerns for the clinical applicability of these nomograms in the presurgical setting.

More recently, molecular classifiers have emerged as promising tools for classifying cancers based on DNA methylation (DNAm) signatures. DNAm profiling has been shown to accurately distinguish cancers of unknown primary,[29] primary brain tumors,[30,31] and other neoplasms.[32] We demonstrated that DNAm profiles can not only accurately differentiate primary from metastatic brain tumors, but also further define the origin of a metastatic tumor, including therapeutically-relevant breast cancer subtypes with sensitivity and specificity greater than 90%.[11,14] In the current study, we identified 1,656 DMS between pN0 and >pN0. Notably, primary tumors from patients with positive LN disease showed 156 hypermethylated and 1,500 hypomethylated CpG sites. This disproportion of hypomethylated sites in LN-positive primary tumors may reflect a higher transcriptional activity in tumors that are prone to lymph node invasion. However, the predictive potential of DMS demonstrated similar stratification accuracy as the clinical classifiers for lymph node disease (AUC=0.76).

Artificial intelligence algorithms, including machine and deep learning techniques, have fundamentally transformed the way we analyze and utilize data. Machine-learning approaches can integrate multiple layers of information to generate highly robust classifiers, as we and others have shown.[12,33,34] These methods select the most informative features, decreasing the need for large numbers of variables in the final classifiers and increasing the accuracy by removing non-relevant information. Notably, the Random Forest signatures generated in this study, which include only 15–37 features, have significantly better performance (AUC=0.88–0.89) than using all DMS. Perhaps, the main advantage of including only a small number of informative genomic regions in the DNAm classifiers is that these can be adapted and evaluated using quantitative PCR.[11] This technique, which is now widely used in low-complexity pathology laboratories, facilitates the near-term clinical adoption of machine learning-based signatures. Evaluating the feasibility of profiling these classifiers on core needle biopsies is an essential next step; indeed, one of the main advantages of these machine learning-based epigenetic signatures compared to the clinical nomograms is the ability to utilize presurgical tissue from core biopsies, thus identifying patients with negative nodes prior to surgery who could forego surgical staging of the axilla. However, the translation of these assays to routine practice is challenging, especially for core biopsy-derived DNA specimens, as the BeadChip microarray technology requires at least 200 ng of good-quality genomic DNA. In this regard, we and others have recently set up quantitative methylation-specific PCR assays to profile multiple genomic regions in

epigenetic signatures using minimum paraffin embedded archived tissue specimens from core biopsies and metastatic brain lesions.[11,35,36] However, before defining the optimal combination of genomic regions for targeted assays and given the relatively small number of cases employed in this study, additional genome-wide DNAm profiling of clinically-annotated cohorts may be necessary to improve the performance of our EpiLN signatures.

In conclusion, we have identified epigenetic classifiers based on primary tumor characteristics that can efficiently stratify patients with no lymph node involvement from those with axillary disease. These classifiers display better performance than current clinical-based nomograms. Identifying an accurate, non-invasive, and easily adoptable method for staging the axilla can provide the prognostic information needed without the morbidity of surgery. The small number of genomic regions employed for this analysis can facilitate the use of PCR-based assays, decreasing the cost compared to high-throughput technology, therefore allowing easier translation into clinical practice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS:

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SYNOPSIS:

Epigenetic classifiers represent a valuable non-invasive method to accurately predict axillary status as we continue to de-escalate axillary surgery in estrogen receptor-positive, HER2-negative early-stage breast cancer.
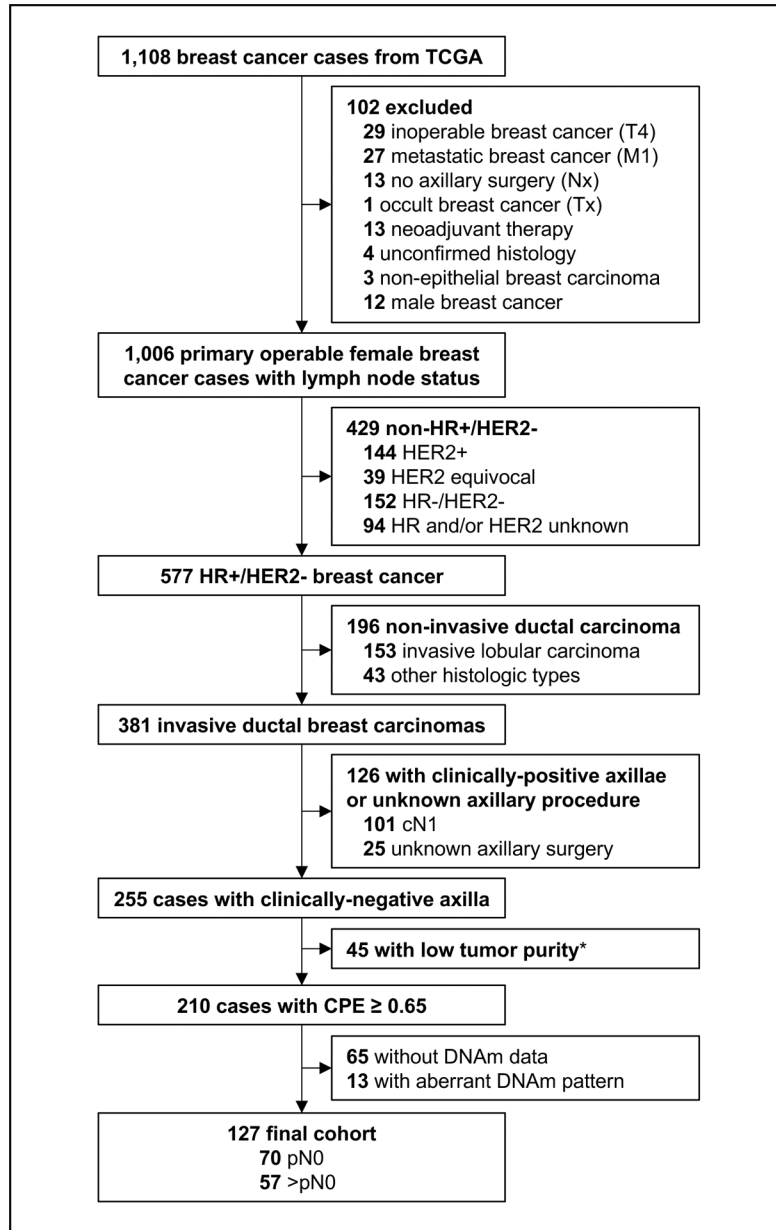
Figure 1: Patient selection based on clinical and DNA methylation features.
Patient selection flow chart based on inclusion criteria of ER-positive, HER2-negative invasive ductal carcinoma with clinically negative axilla. Exclusion criteria included unknown axillary surgery, *tumor purity <65% as estimated by the consensus estimate purity (CPE) algorithm,[19] incomplete or aberrant DNA methylation data.
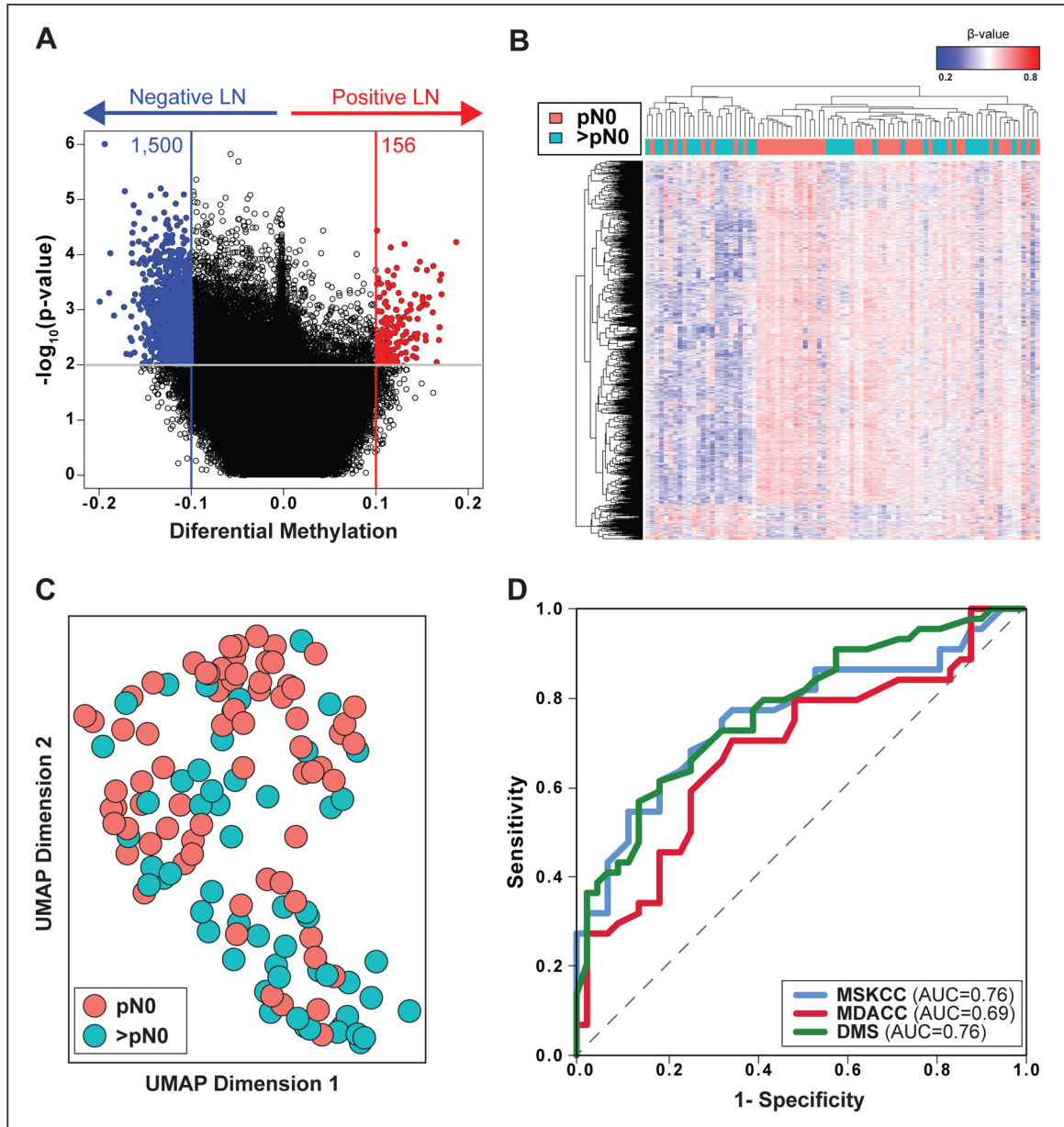
Figure 2: Differentially methylated site analysis between pN0 and >pN0 patients.
A. Volcano plot demonstrating differential methylation between pN0 and >pN0 BC patients
B. Heatmap showing clustering of BC patients using all DMS **C.** UMAP representation of
clustering of BC patients using all DMS **D.** ROC curve displaying the AUC of MSKCC,
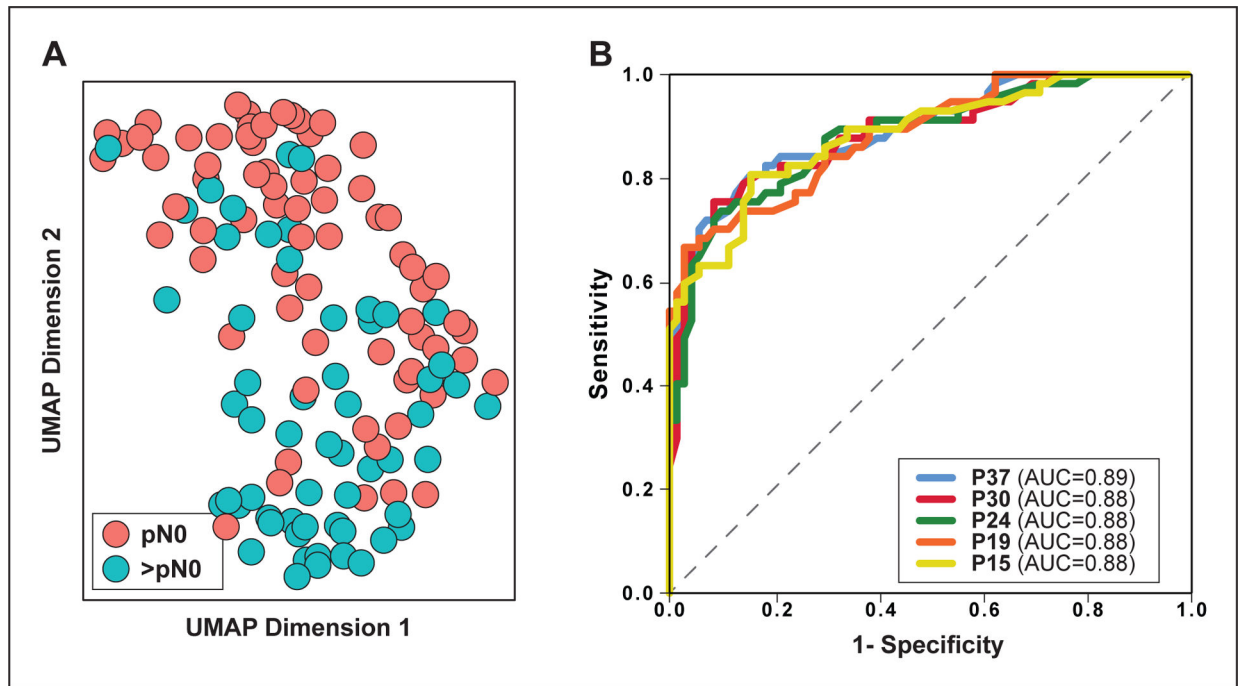MDACC, and all DMS that differentiates pN0 and >pN0 patients

Figure 3: Machine learning-based epigenetic classifiers.
A. UMAP representation of the stratification efficiency using a 37-genomic region DNAm classifier (EpiLN-37) in all patients. **B.** ROC curve created with the entire study population and the top five performing classifiers.

Table 1 –

Clinicopathological features of patients with pN0 and >pN0 disease

Characteristics	Overall	Node status		*p*-value
		pN0	>pN0	
No. of Patients	127	70	57	--
Age, median (IQR), (years)	55.0 (18.0)	55.5 (18.0)	54.0 (18.0)	0.32
Lymph Nodes Examined, median (IQR)	4.0 (11.0)	2.0 (3.0)	14.0 (14.0)	<0.001
Positive LN (H&E), median (IQR)	--	--	2.0 (1.0)	--
Tumor Size, median (IQR), (cm)	2.3 (1.1)	2.1 (1.0)	2.4 (1.8)	0.08
Tumor Size				
T1	47 (37%)	28 (40%)	19 (33%)	
T2	67 (53%)	34 (49%)	33 (58%)	0.66
T3	6 (5%)	3 (4%)	3 (5%)	
Missing	7 (5%)	5 (7%)	2 (4%)	
Laterality, No (%)				
Left	66 (52%)	37 (53%)	29 (51%)	0.82
Right	61 (48%)	33 (47%)	28 (49%)	
Tumor Location, No (%)				
UOQ	60 (47%)	33 (47%)	27 (47%)	
Non-UOQ	58 (46%)	30 (43%)	28 (49%)	0.40
Missing	9 (7%)	7 (10%)	2 (4%)	
Histological grade, No (%)				
I or II	80 (63%)	44 (63%)	36 (63%)	
III	39 (31%)	21 (30%)	18 (32%)	0.96
Missing	8 (6%)	5 (7%)	3 (5%)	
LVI, No (%)				
Present	43 (34%)	15 (21%)	28 (49%)	
Absent	58 (46%)	38 (54%)	20 (35%)	0.005
Missing	26 (20%)	17 (24%)	9 (16%)	
LN ENE, No (%)				
Present	22 (17%)	--	22 (39%)	
Absent	92 (72%)	--	22 (39%)	--
Missing	13 (10%)	--	13 (22%)	

Abbreviations: IQR, interquartile range; UOQ, upper outer quadrant; LVI, lymphovascular invasion; LN, lymph-node; ENE, extranodal extension

Table 2 –

Predictive performance of different models

Models	AUC	95% CI	p-value[*‡]	p-value[*†]
MSKCC	0.76	0.65–0.86	ref	0.15
MDACC	0.69	0.58–0.80	0.15	Ref
DMS	0.76	0.68–0.84	0.84	0.31
EpiLN-37	0.89	0.84–0.95	0.019	0.0013
EpiLN-30	0.88	0.82–0.94	0.032	0.0022
EpiLN-24	0.88	0.82–0.94	0.037	0.0028
EpiLN-19	0.88	0.82–0.94	0.025	0.0016
EpiLN-15	0.88	0.82–0.94	0.026	0.0019

Abbreviations: MSKCC, Memorial Sloan Kettering Cancer Center; MDACC, MD Anderson Cancer Center; DMS, Differentially methylated sites.

[*] The predictive ability of the different models was compared using DeLong's test.

[‡] MSKCC is used as a reference.

[†] MDACC is used as a reference.