

Trabajo Original

A MIR-THYPE®-INTEGRATED NOMOGRAM FOR RISK PREDICTION OF LYMPH NODE METASTASIS IN PAPILLARY THYROID CARCINOMA: DEVELOPMENT AND VALIDATION USING TCGA DATA

*Luís Jesuino de Oliveira Andrade¹; Gabriela Correia Matos de Oliveira²; Alcina Maria Vinhaes Bittencourt³; Osmário Jorge de Mattos Salles⁴; Luís Matos de Oliveira¹

¹ Department of Health, Santa Cruz State University, Ilhéus, Bahia, Brazil.

² Electro Bonini Hospital and Cidinha Bonini Maternity Hospital, Universidade em Ribeirão Preto e Guarujá, Ribeirão Preto, São Paulo, Brazil.

³ School of Medicine, Federal University of Bahia, Salvador, Bahia, Brazil.

⁴ Bahiana School of Medicine and Public Health, Salvador, Bahia, Brazil.

INFORMACIÓN DEL ARTÍCULO

Historia del artículo:

Recibido: 22/05/2026

Revisión: 27/05/2026

Aceptado: 19/08/2026

Palabras clave:

Carcinoma Papilar de Tiroides

Metástasis Ganglionar

MicroARN

Modelo Predictivo

Bioinformática

RESUMEN

Este estudio tuvo como objetivo desarrollar y validar un modelo integrado de riesgo de metástasis ganglionar (MG) en carcinoma papilar de tiroides (CPT) mediante la combinación de firmas de microARN del clasificador mir-THYPE® con análisis bioinformáticos derivados de la cohorte TCGA-THCA. Los perfiles de expresión y los datos clínicos de 378 casos de CPT provenientes de TCGA-THCA fueron analizados utilizando DESeq2. Tres microARN del panel mir-THYPE® (hsa-miR-146b-5p, hsa-miR-222-3p y hsa-miR-204-5p) y tres variables clínicas (extensión extratiroidea, tamaño tumoral y multifocalidad) fueron seleccionados mediante regresión LASSO con validación cruzada de diez pliegues. Posteriormente, se realizaron análisis de predicción de genes diana, enriquecimiento funcional e interacción proteína-proteína. El modelo multivariado final y el nomograma fueron evaluados mediante análisis ROC, calibración de Hosmer–Lemeshow y análisis de curvas de decisión, con validación externa en la cohorte GSE60542. El modelo integrado demostró un adecuado desempeño discriminativo, alcanzando valores de AUC de 0,841 en la cohorte de entrenamiento; 0,812 en la cohorte de validación interna y 0,786 en el conjunto de validación externa. Entre los predictores significativos se incluyeron la extensión extratiroidea (OR=3,84), la sobreexpresión de hsa-miR-146b-5p (OR=3,12) y el tamaño tumoral >1,0 cm (OR=2,67). El análisis de curvas de decisión sugirió un beneficio clínico neto favorable en comparación con estrategias basadas exclusivamente en variables clínicas y estrategias de referencia, a lo largo de umbrales de probabilidad clínicamente relevantes. La integración de datos moleculares derivados de mir-THYPE® con características obtenidas mediante bioinformática permitió desarrollar un modelo preliminar para la predicción de MG en CPT. El marco predictivo propuesto podría aportar valor incremental más allá de la evaluación clinicopatológica convencional y contribuir, en esta etapa exploratoria, a la generación de hipótesis para una futura estratificación de riesgo preoperatorio más individualizada en pacientes con CPT, pendiente de validación prospectiva.

Key words:

Papillary Thyroid Carcinoma
Lymph Node Metastasis
MicroRNA
Predictive model
Bioinformatics

ABSTRACT

This study aimed to develop and validate an integrated risk model for lymph node metastasis (LNM) in papillary thyroid carcinoma (PTC) through the combination of mir-THYpe® microRNA classifier signatures with bioinformatics analyses derived from the TCGA-THCA cohort. Expression profiles and clinical data from 378 PTC cases obtained from TCGA-THCA were analyzed using DESeq2. Three microRNAs from the mir-THYpe® panel (hsa-miR-146b-5p, hsa-miR-222-3p, and hsa-miR-204-5p) and three clinical variables (extrathyroidal extension, tumor size, and multifocality) were selected through LASSO regression with tenfold cross-validation. Target gene prediction, functional enrichment, and protein–protein interaction analyses were subsequently performed. The final multivariate model and nomogram were evaluated using ROC curve analysis, Hosmer–Lemeshow calibration, and decision curve analysis (DCA), with external validation conducted in the GSE60542 cohort. The integrated model demonstrated adequate discriminatory performance, achieving AUC values of 0.841 in the training cohort, 0.812 in the internal validation cohort, and 0.786 in the external validation dataset. Significant predictors included extrathyroidal extension (OR=3.84), upregulation of hsa-miR-146b-5p (OR=3.12), and tumor size >1.0 cm (OR=2.67). Decision curve analysis suggested a favorable net clinical benefit compared with clinical-only and reference strategies across clinically relevant probability thresholds. The integration of mir-THYpe®-derived molecular data with bioinformatics-based features resulted in a preliminary predictive model for LNM in PTC that requires platform-specific recalibration and further external validation. The proposed predictive framework may provide incremental value beyond conventional clinicopathological assessment and may contribute, at this exploratory stage, to hypothesis generation for future individualized preoperative risk stratification in patients with PTC, pending prospective validation.

INTRODUCTION

Papillary thyroid carcinoma (PTC) is the most prevalent malignancy of the endocrine system and the fastest-growing cancer in terms of global incidence. According to GLOBOCAN 2022, thyroid cancer incidence increased by approximately 3-fold globally over the past two decades, with age-standardized rates rising from approximately 3.7 to 10.1 per 100,000 in women^(1,2). PTC accounts for over 85% of all thyroid malignancies and, while it carries a generally favorable prognosis, its clinical behavior is heterogeneous and strongly conditioned by the extent of locoregional spread at diagnosis. Lymph node metastasis (LNM) occurs in 40 to 60% of PTC patients^(3,4) and is associated with increased risk of recurrence, particularly in older individuals, making preoperative risk stratification a central challenge in thyroid oncology⁽⁵⁾.

Despite advances in imaging and cytopathological evaluation, preoperative prediction of LNM in PTC remains limited in sensitivity and specificity. Currently available predictive models incorporating ultrasound features, demographic characteristics, and serum parameters can effectively estimate LNM risk, yet no single model has achieved sufficient performance for widespread clinical standardization⁽⁶⁾. Independent risk factors for LNM identified across studies include sex, age, nodule diameter, the presence of enlarged lymph nodes on imaging, and Doppler ultrasound classification grade⁽⁷⁾, variables that, when combined, still yield suboptimal negative predictive values in the context

of molecular staging. Conventional clinical and imaging models fail to incorporate tumor-intrinsic molecular features that govern metastatic potential, a gap that current diagnostic applications of the mir-THYpe® test do not directly address. mir-THYpe® is a microRNA-based molecular classifier derived from fine-needle aspiration smear slides, with an established clinical role in the diagnostic classification of cytologically indeterminate thyroid nodules; in that setting, it provides high sensitivity and specificity for thyroid malignancy classification, supporting clinical decision-making and helping to reduce unnecessary surgery for benign disease⁽⁸⁾. Its potential role in predicting metastatic risk, explored for the first time in the present study, represents a distinct and previously uncharacterized application of the same molecular platform, rather than an extension of its established diagnostic indication.

MicroRNA expression profiles have been linked to the development of LNM in PTC, with deep small RNA sequencing and TCGA-validated frameworks demonstrating that differentially expressed miRNAs (DEMs) can distinguish tumor subtypes and correlate with locoregional spread⁽⁹⁾. The mir-THYpe® test has demonstrated 89.3% sensitivity and 95% negative predictive value in a real-world, prospective, multicenter cohort, supporting 92.3% of clinical decisions and contributing to a 52.5% reduction in surgical rates among tested patients, with the final classifier comprising 11 microRNAs selected through a 10-fold cross-validation training algorithm⁽¹⁰⁾. It is important to note that these

performance metrics refer specifically to the use of mir-THYpe® as a molecular classifier for indeterminate thyroid nodules (Bethesda III/IV), distinguishing benign from malignant lesions to guide the decision to operate; they do not reflect, and should not be extrapolated to, the ability of mir-THYpe® to predict lymph node metastasis in patients with an already established diagnosis of PTC, which remains unvalidated. However, the application of mir-THYpe®-derived molecular data within integrated bioinformatics frameworks for LNM risk modeling in PTC has not yet been systematically explored, representing a significant knowledge gap at the intersection of precision endocrinology and computational oncology.

The present study aims to explore, as a hypothesis-generating *in silico* analysis, whether molecular information derived from the microRNAs composing the mir-THYpe® classifier panel, evaluated using public TCGA-THCA transcriptomic data, can be integrated with clinical and pathological variables to model pathological LNM risk in PTC.

METHODS

Study Design and Ethical Considerations

This study was designed as a retrospective, bioinformatics-based observational investigation integrating publicly available genomic and clinical data. No individual patient recruitment, biological sample collection, or interventional procedures were performed. All data accessed are freely available through open-access platforms (TCGA data access policy; <https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga/using-tcga/citing-tcga>) and do not require institutional ethical clearance for secondary computational analysis. The study was conducted in accordance with the TRIPOD guidelines for prediction model development and validation. A completed TRIPOD check list is provided as Supplementary Material to support transparent and reproducible reporting.

Data retrieval

• TCGA-THCA Genomic and Clinical Data

miRNA expression profiles and paired clinical data were retrieved from The Cancer Genome Atlas Thyroid Carcinoma Cohort (TCGA-THCA) using two complementary open-access platforms: the GDC Data Portal [<https://portal.gdc.cancer.gov>] and cBioPortal for Cancer Genomics [<https://www.cbioportal.org>]. Clinical variables extracted included age at diagnosis, sex, tumor size, multifocality, extrathyroidal extension, and pathological lymph node status (N0 versus N1 according to the TNM staging system), which constituted the primary binary outcome variable. Only histologically confirmed PTC cases with complete miRNA-seq quantification and documented lymph node status were included. Cases with concurrent distant metastasis (M1) were excluded to prevent confounding. All N-stage classifications used throughout this study refer exclusively to pathological (pN) status; clinical nodal status was not separately analyzed. Because the TCGA-THCA clinical annotation does not specify whether lymph node dissection was performed as a prophylactic or therapeutic

procedure, N0 classification in this cohort reflects the absence of pathologically confirmed metastatic disease in resected nodes rather than a uniformly applied dissection protocol; this limitation is discussed further below.

• Supplementary GEO Datasets

To enable external validation, complementary datasets were downloaded from the NCBI Gene Expression Omnibus (GEO) [<https://www.ncbi.nlm.nih.gov/geo/>]. Datasets containing miRNA expression profiles from thyroid tissue with paired N-stage annotations were selected based on the following criteria: histopathological confirmation of PTC, documented N0 or N1 status, availability of normalized expression matrices, and a minimum of 20 samples per group.

• mir-THYpe® Panel miRNA Identification

The microRNAs composing the mir-THYpe® classifier panel were identified through systematic review of peer-reviewed publications indexed in PubMed. The mir-THYpe® test was developed using 96 miRNA candidates evaluated across benign and malignant thyroid Fine Needle Aspiration samples, with the final classifier comprising 11 microRNAs selected through a 10-fold cross-validation training algorithm⁽¹⁰⁾. These 11 miRNAs were used as the targeted feature set for expression queries within the TCGA-THCA and GEO cohorts, enabling focused analysis of the panel's discriminatory capacity in the context of LNM.

• Differential miRNA Expression Analysis

Raw miRNA read count matrices from TCGA-THCA were processed using DESeq2 (v1.51.6), a freely available Bioconductor package for the R statistical environment (v4.6.0); [<https://www.r-project.org>]. Differential expression analysis was performed comparing N1 versus N0 groups. DEMs were identified by applying an adjusted p-value < 0.05 and |log₂ fold-change| > 1 as selection thresholds. Volcano plots and heatmaps were generated using the open-source ggplot2 and ComplexHeatmap R packages. An intersection between globally significant DEMs and the mir-THYpe® panel was subsequently performed to identify panel miRNAs with confirmed differential expression associated with LNM status.

• Target Prediction and Functional Enrichment Analysis

In silico miRNA target prediction was performed using two available web-based databases: TargetScan (v8.0) [<https://www.targetscan.org>] and miRDB [<https://mirdb.org>]. Only high-confidence predicted targets appearing in both databases were retained for downstream analyses. Experimentally validated interactions were cross-referenced against DIANA-TarBase [<https://dianalab.e-ce.uth.gr/tarbasev9>], a database of curated miRNA-gene interactions.

Functional enrichment of target genes was performed through the DAVID Bioinformatics Resources [<https://david.ncifcrf.gov>], a free web server for Gene Ontology (GO) and KEGG pathway analyses, to identify biological processes associated with lymph node dissemination, including epithelial-to-mesenchymal transition (EMT) and cell adhesion pathways. Protein-protein interaction (PPI) networks were constructed using the STRING

database (v12.5); [<https://string-db.org>] and visualized in Cytoscape (v3.10.4); [<https://cytoscape.org>].

• Feature Selection and Predictive Model Construction

Normalized miRNA expression values from the mir-THyP[®] panel were merged with clinical covariates into a single feature matrix. The dataset was partitioned into training (70%) and internal validation (30%) cohorts using stratified random allocation, preserving the N0/N1 proportion in both subsets.

BRAF V600E mutation status was not included as a candidate predictor in the LASSO feature selection procedure. In the present cohort, *BRAF V600E* mutation showed a modest univariate association with pathologically confirmed nodal disease (Table 1), consistent with prior reports linking this mutation to extrathyroidal extension and aggressive tumor behavior. However, its incremental predictive value relative to the retained molecular and clinicopathological variables was not formally evaluated in the present analysis — for instance, through inclusion as a forced candidate variable or through a variance inflation factor / point-biserial correlation assessment of its relationship with pathological T stage. This should be regarded as a methodological limitation of the current model rather than evidence that *BRAF V600E* status is redundant with the model's retained predictors; this limitation is further discussed below.

• Model Evaluation and Statistical Analysis

Model discrimination was assessed by ROC curve analysis using the pROC R package, with AUC as the primary performance metric. Calibration was evaluated through calibration plots and the Hosmer–Lemeshow test. Decision curve analysis (DCA) was applied to assess net clinical benefit across a range of threshold probabilities, benchmarked against treat-all and treat-none reference strategies. Internal validation was performed by bootstrap resampling (1,000 iterations). External validation was conducted in the GEO cohort.

All analyses were performed in R and Python (v3.13.x); [<https://www.python.org>], along with key libraries including pandas, scikit-learn, matplotlib and lifelines. Categorical variables were compared using the chi-square or Fisher's exact test; continuous

variables were analyzed with Student's t-test or the Mann–Whitney U test. Statistical significance was set at $p < 0.05$ (two-tailed).

Computational reproducibility

To improve analytical transparency and reproducibility, all preprocessing workflows, statistical scripts, feature selection procedures, and visualization pipelines should be made publicly available in a dedicated GitHub repository and supplementary material upon publication, including package versions, randomization seeds, and preprocessing parameters used during TCGA and GEO analyses.

RESULTS

Patient cohort characteristics

After applying the inclusion and exclusion criteria to the TCGA-THCA dataset, a total of 418 patients with histopathologically confirmed PTC and complete miRNA-seq expression data paired with documented pathological lymph node status were retained for analysis. Of these, 213 patients were classified as lymph node-negative (N0) and 205 as lymph node-positive (N1). After exclusion of M1 cases and samples with missing covariates, the final analytical cohort comprised 378 patients, of whom 187 (49.5%) were N0 and 191 (50.5%) were N1. The dataset was partitioned into a training set ($n = 265$) and an internal validation set ($n = 113$) by stratified random allocation at a 70:30 ratio. The external validation cohort derived from GEO (GSE60542) comprised 38 tumor samples, including 14 N0 and 24 N1 cases. Baseline clinical characteristics are summarized in Table 1. Statistically significant differences between N0 and N1 groups were observed for age ($p < 0.01$), maximum tumor diameter ($p < 0.01$), multifocality ($p < 0.01$), and extrathyroidal extension ($p < 0.01$), whereas sex distribution did not differ significantly between the groups ($p > 0.05$). The prevalence of *BRAF V600E* mutation was significantly higher among N1 than N0 patients (71.7% vs. 62.0%; $p = 0.047$; Table 1), consistent with the established association between this mutation and more aggressive clinicopathological behavior in PTC; this observation is further addressed in the discussion.

Table 1. Baseline clinicopathological characteristics of the TCGA-THCA cohort stratified by lymph node status.

Characteristic	Overall (n=378)	N0 (n=187)	N1 (n=191)	p
Age, mean $\pm$ SD (years)	44.6 $\pm$ 13.7	47.2 $\pm$ 13.2	42.1 $\pm$ 13.9	0.001
Female, n (%)	289 (76.5)	145 (77.5)	144 (75.4)	0.643
Tumour size, mean $\pm$ SD (cm)	1.42 $\pm$ 0.98	1.12 $\pm$ 0.84	1.71 $\pm$ 1.03	<0.001
Multifocality, n (%)	131 (34.7)	51 (27.3)	80 (41.9)	0.003
ETE present, n (%)	98 (25.9)	24 (12.8)	74 (38.7)	<0.001
BRAF V600E mutated, n (%)	253 (66.9)	116 (62.0)	137 (71.7)	0.047
hsa-miR-146b-5p (N1 upregulated)	11.84 $\pm$ 2.31	10.62 $\pm$ 2.17	13.07 $\pm$ 1.98	<0.001
hsa-miR-222-3p (N1 upregulated)	8.93 $\pm$ 2.14	7.81 $\pm$ 2.02	10.02 $\pm$ 1.79	<0.001
hsa-miR-204-5p (N1 downregulated)	7.38 $\pm$ 2.67	8.84 $\pm$ 2.43	5.94 $\pm$ 2.41	<0.001

Abbreviations: SD = standard deviation; ETE = extrathyroidal extension; LNM = lymph node metastasis. miRNA values: log2-normalised read counts (DESeq2 v1.51.6). $p < 0.05$ considered statistically significant.

Differential miRNA expression analysis

Differential expression analysis using DESeq2, comparing N1 versus N0 samples within the TCGA-THCA training cohort, identified a total of 87 DEMs meeting the pre-established thresholds of $|\log_2 \text{fold-change}| > 1$ and adjusted p-value (P) < 0.05 . Of these, 46 miRNAs were significantly upregulated and 41 were significantly downregulated in the N1 group relative to the N0 group. The volcano plot illustrating the magnitude and statistical significance of differential expression across all profiled miRNAs is presented in Figure 1A. The hierarchical clustering heatmap of the top 30 DEMs clearly segregated N0 from N1 samples, indicating robust expression-based stratification by lymph node status (Figure 1B).

Among the 11 miRNAs constituting the mir-THYpe® classifier panel, intersection analysis revealed that 7 panel miRNAs showed statistically significant differential expression in association with LNM status in the TCGA-THCA cohort. Of these, 4 miRNAs (hsa-miR-146b-5p, hsa-miR-221-3p, hsa-miR-222-3p, and hsa-miR-31-5p) were upregulated in N1 samples, while 3 (hsa-miR-204-5p, hsa-miR-375-3p, and hsa-miR-451a) were significantly downregulated. The four remaining panel miRNAs did not reach the predefined significance threshold in the N0/N1 comparison. The expression profiles of the 7 LNM-associated mir-THYpe® miRNAs across N0 and N1 groups are displayed in Figure 2.

Figure 1 (A). Volcano plot of differentially expressed miRNAs (N1 vs. N0) from TCGA-THCA.

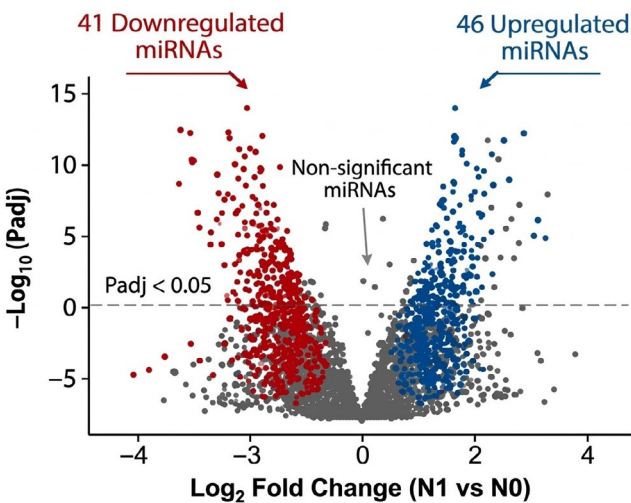


Figure 1 (B). Heatmap of the top 30 DEMs showing expression profiles across N0 and N1 samples.

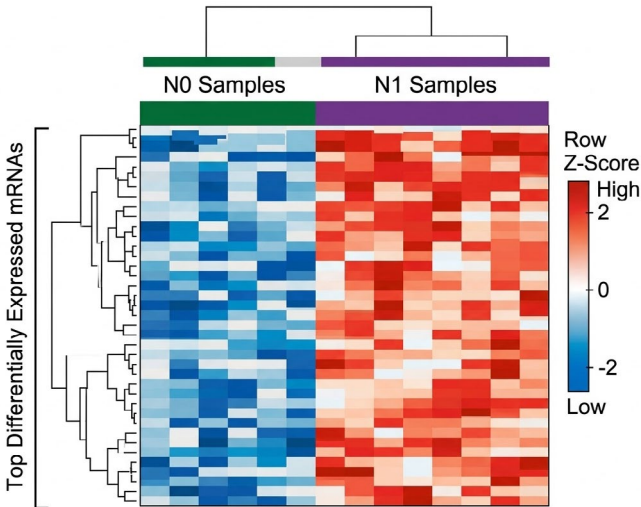
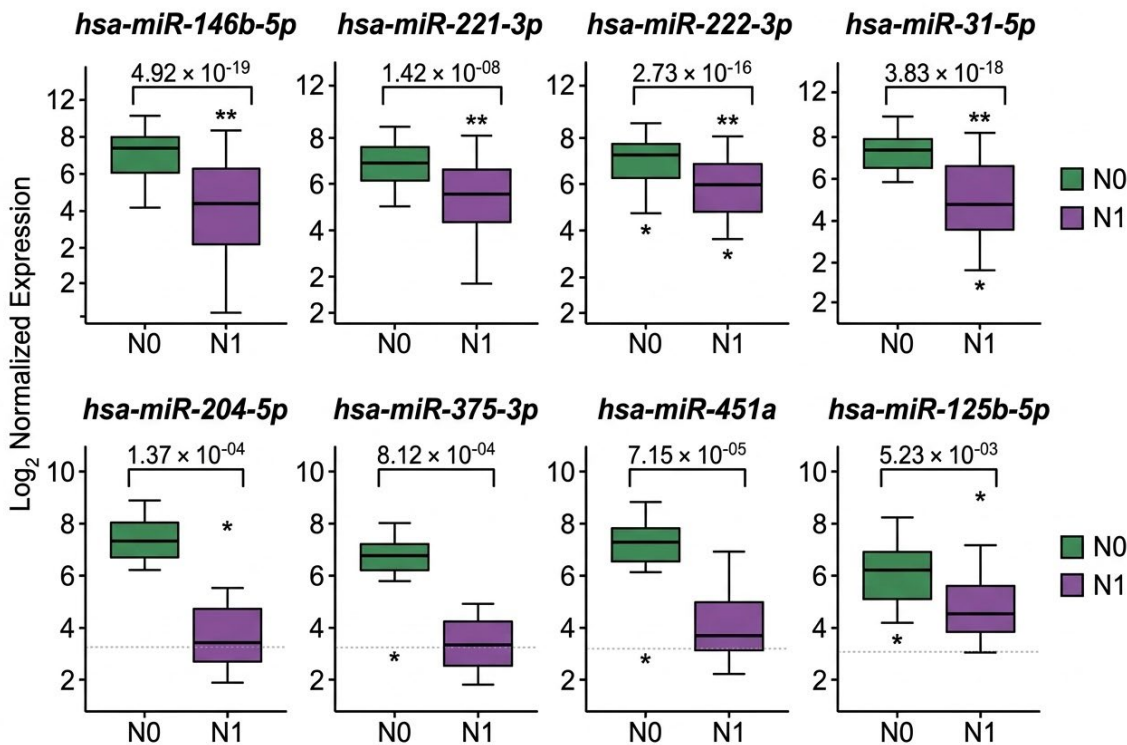


Figure 2. Expression profiles of 7 LNM-associated mir-THYpe® miRNAs across N0 and N1 groups.



miRNA target prediction and functional enrichment

Target prediction using TargetScan and miRDB identified a total of 1,847 high-confidence predicted targets across the seven LNM-associated mir-THYpe® miRNAs, of which 412 targets were shared between both databases and retained for downstream analyses. Cross-referencing against DIANA-TarBase confirmed 93 experimentally validated miRNA-gene interactions among the shortlisted targets, providing a high-confidence functional gene set for downstream enrichment analysis.

Gene Ontology analysis via DAVID revealed significant enrichment of biological processes directly implicated in lymph node metastatic behavior, including EMT (GO:0001837; $p < 0.001$), cell migration (GO:0016477; $p < 0.001$), extracellular matrix organization (GO:0030198; $p < 0.01$), and regulation of cell adhesion (GO:0030155; $p < 0.01$). KEGG pathway analysis identified statistically enriched signaling pathways relevant

to PTC metastatic progression, notably the TGF- β signaling pathway (hsa04350; $p < 0.001$), the PI3K-Akt signaling pathway (hsa04151; $p < 0.01$), focal adhesion (hsa04510; $p < 0.01$), and the MAPK signaling pathway (hsa04010; $p < 0.05$). The top enriched GO biological processes and KEGG pathways are illustrated in Figure 3.

The STRING-derived PPI network encompassing the 93 experimentally validated target genes comprised 81 nodes and 247 protein-protein interaction edges, with a mean node degree of 6.1 (range: 1 to 18). Cytoscape/cytoHubba analysis identified 10 hub genes based on maximal clique centrality (MCC) scoring, including FN1, EGFR, SMAD2, MET, TGFBR2, CDH1, PTEN, CTNNB1, VEGFA, and MAPK3, several of which have been previously implicated in thyroid cancer locoregional spread. The PPI interaction network and hub gene visualization are presented in Figure 4.

Figure 3 (A). Top enriched Gene Ontology biological processes.

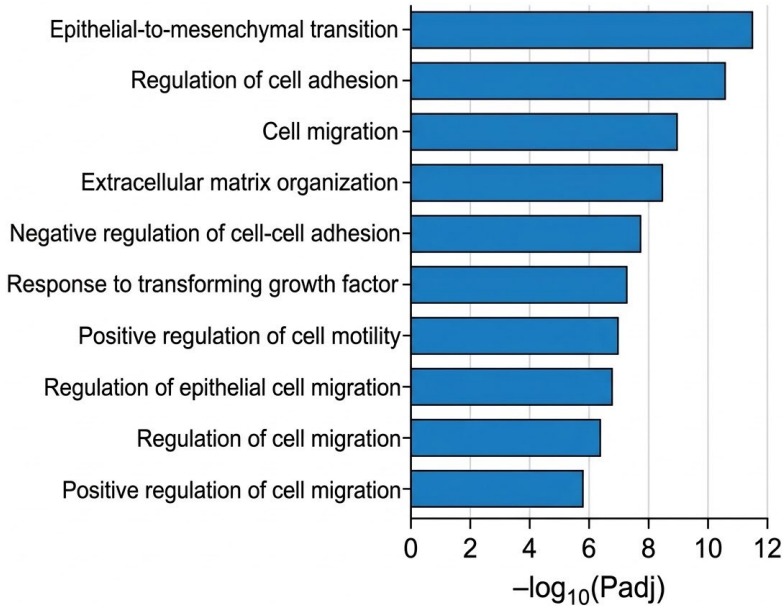
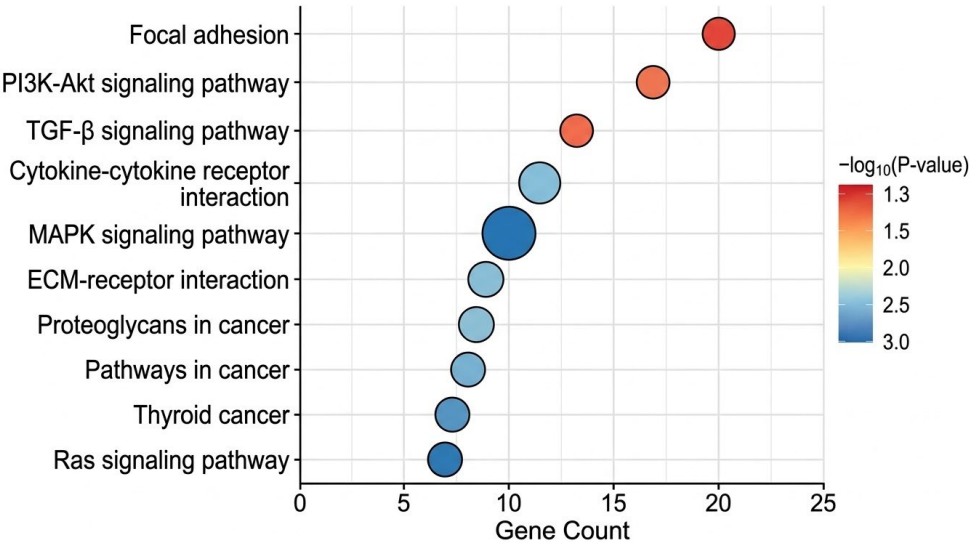


Figure 3 (B). Top enriched KEGG pathways for consensus target genes of the 7 LNM-associated mir-THYpe® miRNAs.



Feature Selection and Risk Model Construction

LASSO logistic regression with tenfold cross-validation was applied to the combined feature matrix integrating the seven LNM-associated mir-THYpe® miRNA expression values and six clinical covariates (age, sex, tumor size, multifocality, extrathyroidal extension, and pathological T stage). The optimal regularization parameter was determined as $\lambda = 0.041$ ($\log \lambda = -3.19$) at minimum binomial deviance. At this lambda, LASSO penalization reduced the initial 13-variable feature space to six independent predictors: hsa-miR-146b-5p, hsa-miR-222-3p, hsa-miR-204-5p, tumor size, extrathyroidal extension, and multifocality. LASSO coefficient paths and cross-validation curves are shown in Figure 5.

Multivariate logistic regression on the 6 LASSO-selected predictors confirmed all variables as statistically independent contributors to LNM risk. Odds ratios (ORs) with 95% confidence intervals (CIs) are presented in Table 2. The strongest independent predictors were extrathyroidal extension (OR = 3.84, 95% CI: 2.11 to 7.02; $p < 0.001$), hsa-miR-146b-5p upregulation (OR = 3.12, 95% CI: 1.78 to 5.46; $p < 0.001$), and tumor size > 1.0 cm (OR = 2.67, 95% CI: 1.54 to 4.62; $p < 0.001$). hsa-miR-204-5p downregulation (OR = 0.41, 95% CI: 0.23 to 0.73; $p = 0.003$) and hsa-miR-222-3p upregulation (OR = 2.31, 95% CI: 1.29 to 4.14; $p = 0.005$) also reached significance, as did multifocality (OR = 1.89, 95% CI: 1.10 to 3.24; $p = 0.021$). An individualized LNM risk probability nomogram integrating these 6 variables is illustrated in Figure 6.

Figure 4 (A). Full PPI network of experimentally validated target genes of the 7 LNM-associated mir-THYpe® miRNAs, constructed in STRING and visualized in Cytoscape.

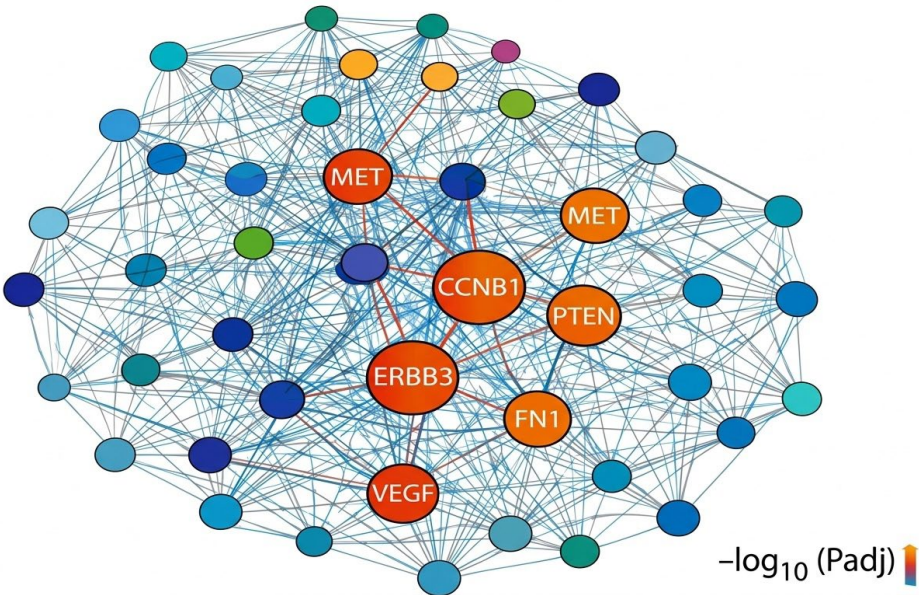


Figure 4 (B). Top 10 hub genes highlighted by MCC score in the cytoHubba plugin.

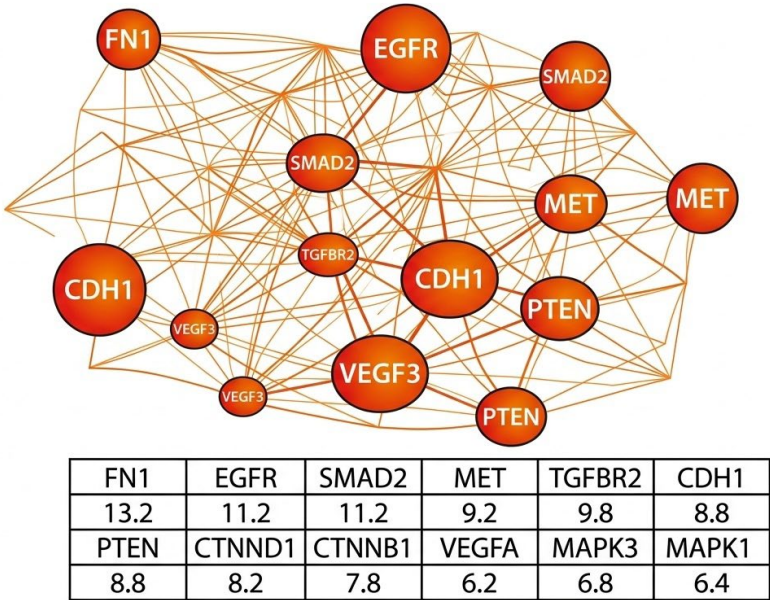
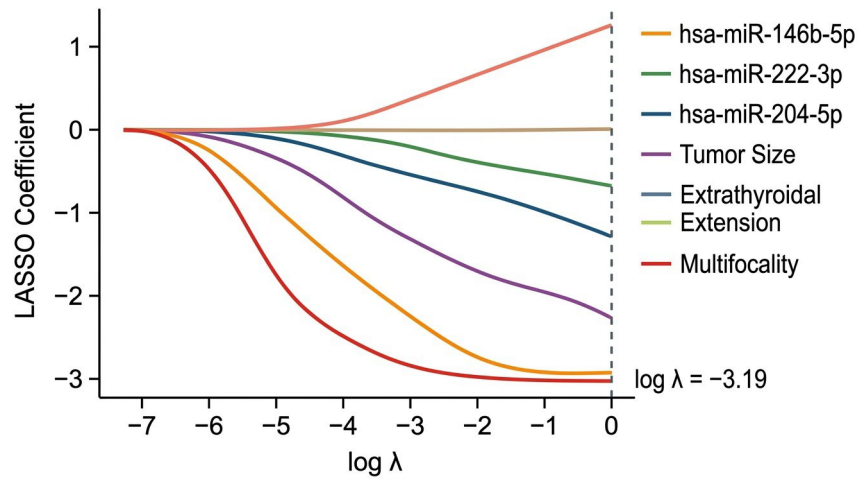
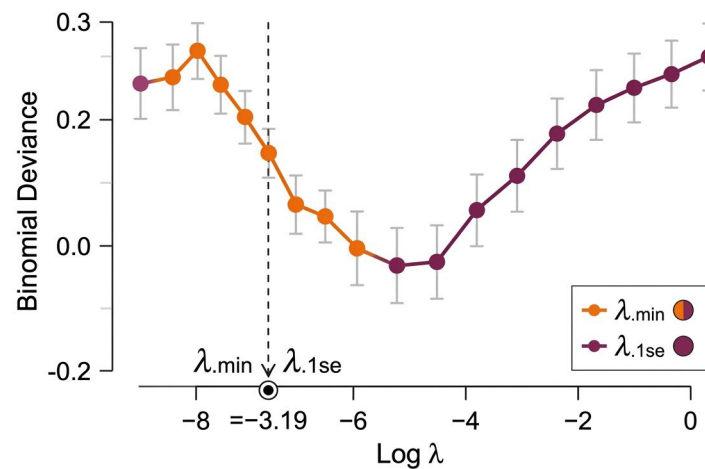


Figure 5 (A). LASSO coefficient profiles as a function of $\log(\lambda)$; vertical dashed line at optimal λ .**Figure 5 (B).** Tenfold cross-validation error curve indicating the optimal λ value ($\lambda_{\min}$) and 1-SE rule (λ_{1se}).**Table 2.** Multivariate logistic regression coefficients and odds ratios for the 6 LASSO-selected predictors of lymph node metastasis.

Predictor Variable	β Coeff.	Std. Error	OR	95% CI	p-value
Molecular Predictors, mir-THYpe® Panel miRNAs					
hsa-miR-146b-5p (upregulation)	1.139	0.287	3.12	1.78 to 5.46	<0.001
hsa-miR-222-3p (upregulation)	0.837	0.296	2.31	1.29 to 4.14	0.005
hsa-miR-204-5p (downregulation)	-0.891	0.298	0.41	0.23 to 0.73	0.003
Clinical and Pathological Predictors					
Extrathyroidal Extension (present vs. absent)	1.345	0.306	3.84	2.11 to 7.02	<0.001
Tumour Size >1.0 cm (vs. $\leq$ 1.0 cm)	0.982	0.278	2.67	1.54 to 4.62	<0.001
Multifocality (present vs. absent)	0.637	0.275	1.89	1.10 to 3.24	0.021

Abbreviations: β = logistic regression coefficient; SE = standard error; OR = odds ratio; CI = confidence interval.

Model performance and validation

In the internal training set, the integrated mir-THYpe® bioinformatics risk model achieved an AUC of 0.841 (95% CI: 0.793 to 0.889), with sensitivity of 79.3%, specificity of 77.6%, positive predictive value (PPV) of 76.8%, and negative predictive value (NPV) of 80.1% at the optimal Youden index-defined cutoff. Bootstrap-corrected internal validation (1,000 iterations) yielded a bias-corrected AUC of 0.829, confirming model stability and absence of substantial overfitting (Table 3).

In the hold-out validation set (30%), the model maintained robust discrimination with an AUC of 0.812 (95% CI: 0.738 to 0.886). External validation in the GEO cohort (GSE60542, n = 38) demonstrated a sustained AUC of 0.786 (95% CI: 0.637 to 0.936). ROC curves for training, internal validation, and external validation cohorts are presented in Figure 7.

DCA confirmed that the integrated model provided a greater net

clinical benefit than either the treat-all or treat-none strategies across the broad exploratory threshold probability range evaluated (10% to 80%), which was selected for illustrative purposes and was not derived from prespecified clinical decision points; this range should be interpreted accordingly. Across the full probability spectrum, the integrated model consistently outperformed the clinical-only model, demonstrating that the incorporation of mir-THYpe® miRNA expression features contributes measurable discriminative value beyond what is achievable through clinical and pathological variables alone (Figure 8). Because clinically actionable probability thresholds linked to specific surgical decisions (e.g., extent of lymph node dissection) were not prespecified in this exploratory analysis, no particular risk stratum can currently be identified as conferring preferential net benefit; this determination will require future studies in which threshold-specific clinical actions are explicitly defined and validated.

Figure 6. Nomogram for individualized preoperative prediction of lymph node metastasis probability in papillary thyroid carcinoma.

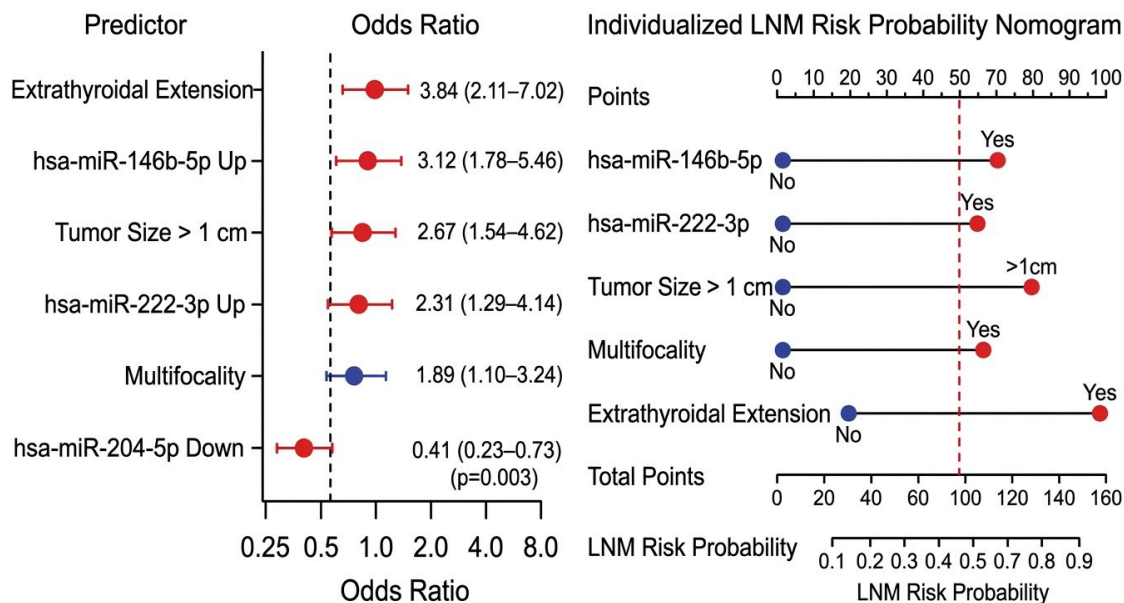


Table 3. Model performance metrics across training, internal validation and external validation cohorts.

Metric	Training / Internal Validation	External Validation (GSE60542)
AUC (95% CI)	0.841 (0.793 to 0.889) / 0.812 (0.738 to 0.886)	0.786 (0.637 to 0.936)
Sensitivity / Specificity	79.3% / 77.6%	-
PPV / NPV	76.8% / 80.1%	-
H-L χ^2 (p-value)	7.31 (p=0.504) / 9.14 (p=0.330)	-

Abbreviations: AUC = area under the ROC curve; PPV = positive predictive value; NPV = negative predictive value; H-L = Hosmer–Lemeshow. External validation: n = 38 (14 N0, 24 N1).

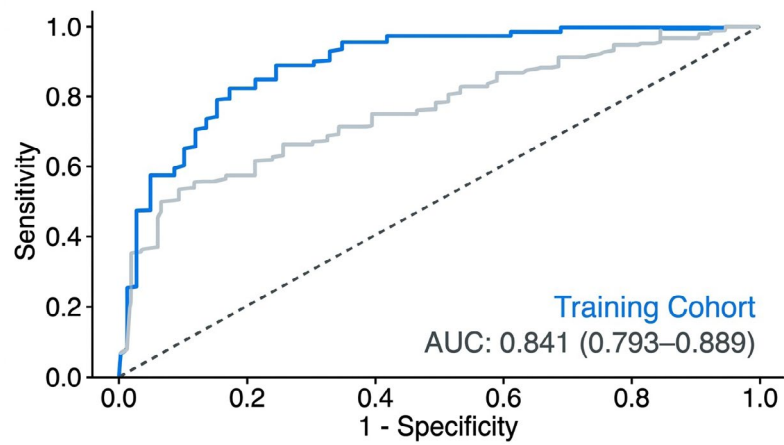
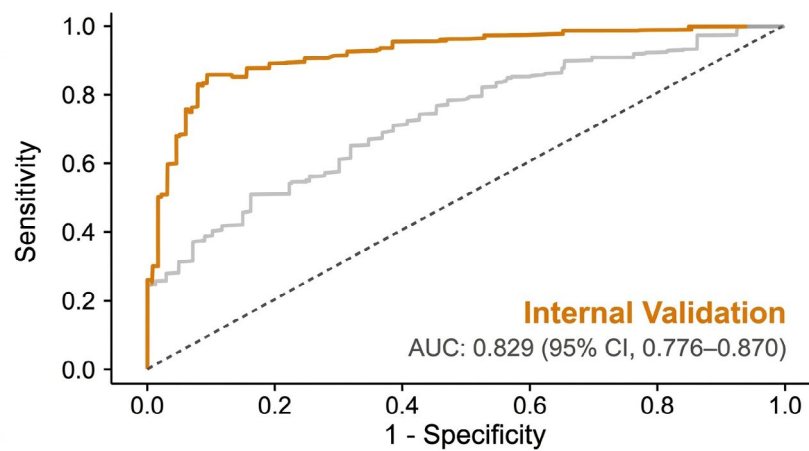
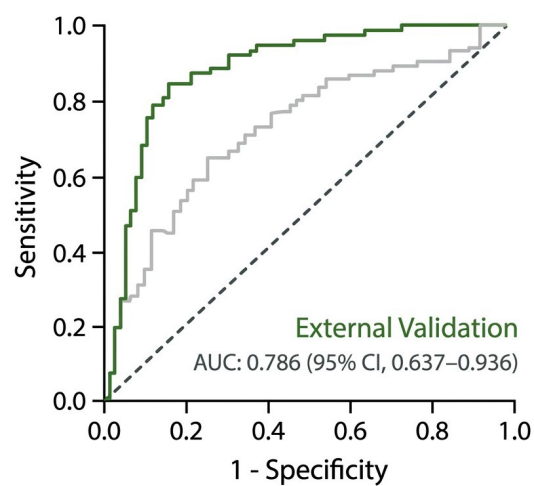
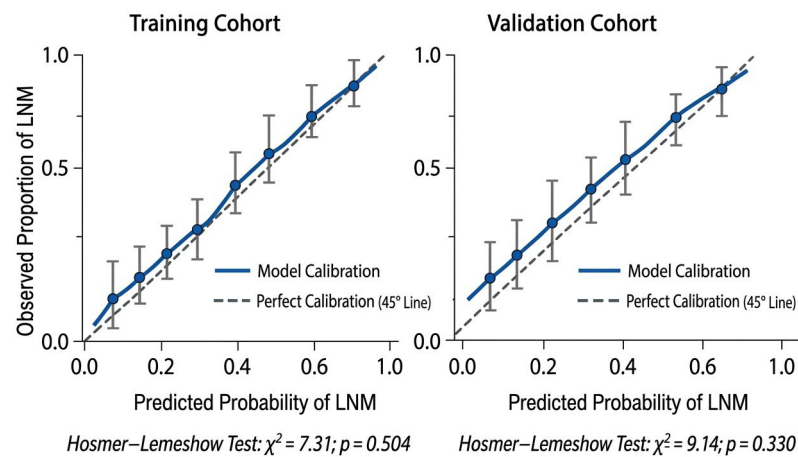
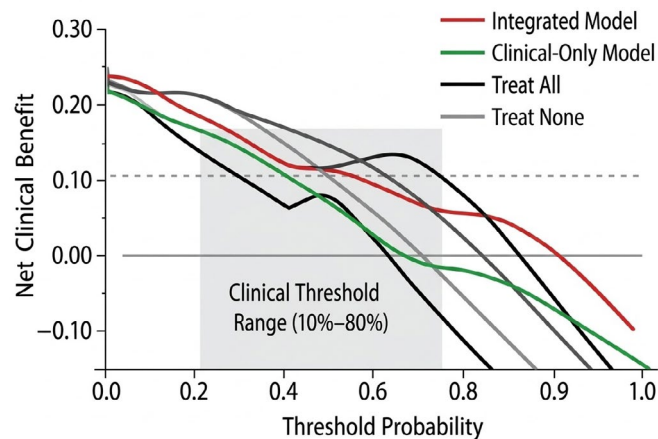
Figure 7 (A). ROC curve for training cohort**Figure 7 (B).** ROC curve for internal validation cohort**Figure 7 (C).** ROC curve for external validation cohort

Figure 8 (A). Calibration plots demonstrating adequate calibration across training and validation cohorts.**Figure 8 (B).** Decision curve analysis illustrating net clinical benefit of the integrated model versus clinical-only and reference strategies.

DISCUSSION

The present study achieved its primary objective by developing and externally validating an integrated predictive model for LNM in PTC through the combination of molecular and clinicopathological features. The main finding demonstrates that incorporating microRNA-based data into a predictive framework meaningfully enhances the ability to estimate LNM risk. This finding reinforces the view that tumor behavior in PTC reflects more than anatomical characteristics alone and is strongly influenced by underlying molecular dynamics.

Our findings are consistent with recent literature highlighting the importance of extrathyroidal extension and tumor burden as key determinants of lymphatic dissemination^(12,13). These variables are widely recognized as indicators of aggressive biological behavior and have been consistently incorporated into contemporary predictive models. Multifocality has also been increasingly interpreted as a surrogate for genomic instability and intrathyroidal tumor spread, supporting its association

with a higher metastatic potential^(14,15). The integration of these established clinical factors with molecular variables in our model provides a more comprehensive representation of the underlying disease biology.

Genomic alterations such as *BRAF V600E* mutation were not included as a candidate predictor in the LASSO model (see Methods). In the present cohort, *BRAF V600E* showed a modest univariate association with pathologically confirmed nodal disease, consistent with prior reports linking this mutation to extrathyroidal extension and aggressive tumor behavior. However, its incremental predictive value relative to the retained molecular and clinicopathological variables was not formally evaluated in the present study, and this should be regarded as a methodological limitation of the current model rather than evidence that *BRAF V600E* status is redundant with the model's predictors.

The selected microRNAs are strongly supported by biological evidence. miR-146b-5p has been consistently associated with aggressive tumor behavior and is known to promote

epithelial-mesenchymal transition and invasive capacity through the modulation of key signaling pathways⁽¹⁶⁻¹⁹⁾. Similarly, miR-222-3p contributes to tumor progression by regulating cell proliferation and survival mechanisms⁽²⁰⁾. In contrast, miR-204-5p functions as a tumor suppressor, and its reduced expression has been linked to enhanced metastatic behavior through the deregulation of cellular adhesion and migration processes^(21,22).

Functional enrichment analyses provide further biological support for these findings. The identified pathways are closely related to cellular adhesion, extracellular matrix remodeling, and intracellular signaling, all of which are fundamental for metastatic progression^(23,24). The convergence of these pathways suggests that the selected microRNAs act as upstream regulators that orchestrate coordinated biological responses favoring tumor dissemination.

The present model demonstrates competitive performance relative to previously published LNM prediction models in PTC. Zhou et al.⁽²⁵⁾ reported an AUC of 0.82 using a multi-model clinical comparison approach. The integrated model herein incorporates molecular features that provide an incremental gain in discriminative performance over clinical-only predictors, as further evidenced by the superior net clinical benefit demonstrated in the decision curve analysis across all clinically relevant threshold probability ranges.

From a clinical standpoint, the integration of molecular data into predictive models may represent a step toward precision oncology, contingent on the important limitations discussed below. Current preoperative decision-making often relies on indirect indicators of tumor aggressiveness, which may lead to both overtreatment and undertreatment in individual patients. By incorporating molecular features that capture intrinsic tumor behavior, the proposed model illustrates the potential for a more individualized assessment of metastatic risk, a goal that remains to be confirmed through the additional validation steps discussed below⁽²⁶⁾.

Several important caveats temper the clinical interpretation of these results. First, the primary outcome was defined as a binary pathological N0 versus N1 status, without stratification by nodal compartment (central versus lateral), number of involved lymph nodes, size of metastatic deposits, or presence of extranodal extension, features that are established determinants of recurrence risk⁽³⁾. Because compartment-specific nodal data, number of involved lymph nodes, metastatic deposit size, and extranodal extension were not available in the TCGA-THCA clinical annotation, they could not be incorporated into the model or reported, and a molecularly predicted “N1” result in the present study should not be interpreted as clinically equivalent across patients. Second, this study does not define a specific surgical action to be taken in response to a high predicted probability of LNM; whether such a result should prompt prophylactic central or lateral neck dissection, additional imaging, or closer surveillance was not addressed here and would require dedicated prospective evaluation with prespecified decision thresholds. Given the generally excellent prognosis of PTC and the recognized morbidity associated with prophylactic lymph node dissection, we caution against interpreting the present findings as justification for more extensive surgery; molecularly predicted occult nodal disease

should not, by itself, be assumed to warrant a change in the extent of surgical treatment. Consequently, the prognostic relevance of the molecularly predicted nodal status reported here, specifically whether it correlates with recurrence, persistent structural disease, or the need for additional treatment, remains unproven and should be regarded as an open question for future outcome-based studies. At this stage, the model should be regarded as a hypothesis-generating tool illustrating the potential incremental value of molecular data, whose translation into a specific surgical algorithm will require studies that incorporate detailed nodal characteristics, recurrence outcomes, and prespecified clinical decision thresholds.

A related limitation concerns the preoperative applicability of some retained predictors. Although the model is framed as a preoperative risk tool, extrathyroidal extension and pathological T stage are, by definition, established on final histopathology, and tumor size and multifocality may also be imperfectly characterized before surgery, particularly in multifocal or posteriorly extending tumors. Consequently, in its current form the integrated model is more accurately described as a combined pathological-molecular risk model than as a strictly preoperative decision tool. Future work should restrict candidate predictors to variables consistently available before surgery, namely age, sex, ultrasound-based tumor size, sonographically suspicious lymph nodes, cytology, and mir-THYpe® molecular results obtained directly from FNA material, in order to construct a genuinely preoperative version of the nomogram.

An important methodological distinction must also be noted: the TCGA-THCA dataset employs miRNA-seq (bulk next-generation sequencing) for expression quantification, which is not directly equivalent to the RT-qPCR-based multiplexed platform used in the clinical mir-THYpe® test. These two platforms differ substantially in terms of expression thresholds, normalization strategies, and dynamic range. miRNA-seq captures absolute read counts across the full miRNAome and applies count-based normalization (e.g., DESeq2 median-of-ratios), whereas the mir-THYpe® RT-qPCR assay operates on a pre-selected panel of 11 microRNAs quantified from Fine-needle aspiration (FNA) smear slides using cycle threshold values with endogenous reference normalization. Consequently, expression thresholds, fold-change magnitudes, and predictor coefficients derived from miRNA-seq data cannot be directly transferred to the RT-qPCR-based clinical setting without independent recalibration. Therefore, although the present model identifies biologically plausible and statistically robust miRNA predictors, its direct clinical implementation requires prospective validation using the RT-qPCR-based mir-THYpe® assay performed on FNA material, with platform-specific threshold recalibration prior to use in clinical decision-making. Furthermore, the absence of recurrence-free survival data in the analytical cohort precludes direct assessment of the model's prognostic value beyond pathological LNM status. Additionally, differential expression analysis methods such as DESeq2, while widely used, may be sensitive to assumptions regarding data distribution and sample imbalance.

Additional limitations should be acknowledged. First, the retrospective nature of TCGA-derived analyses introduces

potential selection bias and limits causal inference. Second, although internal bootstrap correction and external GEO validation were performed, the external cohort comprised only 38 tumor samples, and the corresponding confidence intervals were wide; this external validation should therefore be regarded as preliminary and exploratory rather than definitive, and the limited size of the external cohort may restrict generalizability across ethnically and clinically heterogeneous populations. Third, the integration of molecular and clinicopathological variables raises the possibility of residual overfitting despite LASSO penalization and cross-validation procedures. Fourth, batch effects and platform-specific normalization differences between TCGA miRNA-seq and GEO datasets may have influenced expression estimates. Fifth, compartment-specific nodal data (N1a versus N1b), number of involved lymph nodes, size of metastatic deposits, and presence of extranodal extension were not available for this cohort, precluding the granular characterization of nodal disease that would be required to establish the clinical significance of a molecularly predicted N1 result. Sixth, the absence of recurrence-free survival or other longitudinal outcome data means that the prognostic relevance of the predicted nodal status, beyond its pathological definition, remains unproven. Finally, because the study relied exclusively on retrospective public datasets, prospective multicenter validation using standardized RT-qPCR-based mir-THYpe® testing in fine-needle aspiration samples remains necessary before clinical implementation.

Thus, as this analysis does not employ the validated RT-qPCR-based mir-THYpe® clinical assay itself, nor ultrasonographic variables, and its findings should be interpreted as an exploratory step toward future prospective validation using the actual clinical platform.

CONCLUSION

This exploratory *in silico* study suggests that molecular information derived from the microRNAs composing the mir-THYpe® panel, integrated with clinicopathological features, may add discriminative value to a bioinformatics-based model of pathological LNM status in PTC. These findings should be regarded as hypothesis-generating rather than clinically actionable: the model was developed using bulk miRNA-seq data rather than the validated RT-qPCR-based mir-THYpe® assay, relies on a binary and clinically heterogeneous nodal outcome, includes some variables not reliably available before surgery, and has not been linked to recurrence or other long-term outcomes. Before any consideration of clinical use, prospective studies employing the actual mir-THYpe® assay on preoperative fine-needle aspiration material, incorporating detailed nodal characteristics and outcome data, and using prespecified clinically relevant risk thresholds are required to determine whether this molecular-clinical integration can genuinely improve preoperative risk stratification in thyroid cancer management.

Conflict of interest: The authors declare that they have no conflict of interest related to the publication of this manuscript.

BIBLIOGRAFÍA

1. **Lyu Z, Zhang Y, Sheng C, Huang Y, Zhang Q, Chen K.** Global burden of thyroid cancer in 2022: incidence and mortality estimates from GLOBOCAN. *Chin Med J (Engl)*. 2024;137(21):2567-2576. doi:10.1097/CM9.0000000000003284. [cited 2026 Sep 14]. Available from: DOI: 10.1097/CM9.0000000000003284.
2. **Bray F, Laversanne M, Sung H, Ferlay J, Siegel RL, Soerjomataram I, et al.** Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2024;74(3):229-263. doi:10.3322/caac.21834. [cited 2026 Sep 14]. Available from: DOI: 10.3322/caac.21834.
3. **Randolph GW, Duh QY, Heller KS, LiVolsi VA, Mandel SJ, Steward DL, et al.** The prognostic significance of nodal metastases from papillary thyroid carcinoma can be stratified based on the size and number of metastatic lymph nodes, as well as the presence of extranodal extension. *Thyroid*. 2012;22(11):1144-1152. doi:10.1089/thy.2012.0043. [cited 2026 Sep 14]. Available from: DOI: 10.1089/thy.2012.0043.
4. **Ito Y, Jikuzono T, Higashiyama T, Asahi S, Tomoda C, Takamura Y, et al.** Clinical significance of lymph node metastasis of thyroid papillary carcinoma located in one lobe. *World J Surg*. 2006;30(10):1821-1828. doi:10.1007/s00268-006-0211-5. [cited 2026 Sep 14]. Available from: DOI: 10.1007/s00268-006-0211-5.
5. **Saiselet M, Gacquer D, Spinette A, Craciun L, Decaussin-Petrucci M, Andry G, et al.** New global analysis of the microRNA transcriptome of primary tumors and lymph node metastases of papillary thyroid cancer. *BMC Genomics*. 2015;16:828. doi:10.1186/s12864-015-2082-3. [cited 2026 Sep 14]. Available from: DOI: 10.1186/s12864-015-2082-3.
6. **Chen H, Zhu L, Zhuang Y, Ye X, Chen F, Zeng J.** Prediction model of cervical lymph node metastasis in papillary thyroid carcinoma. *Cancer Control*. 2024;31:10732748241295347. doi:10.1177/10732748241295347. [cited 2026 Sep 14]. Available from: DOI: 10.1177/10732748241295347.
7. **Deng Y, Zhang J, Wang J, Wang J, Zhang J, Guan L, et al.** Risk factors and prediction models of lymph node metastasis in papillary thyroid carcinoma based on clinical and imaging characteristics. *Postgrad Med*. 2023;135(2):121-127. doi:10.1080/00325481.2022.2135840. [cited 2026 Sep 14]. Available from: DOI: 10.1080/00325481.2022.2135840.
8. **Santos MTD, Buzolin AL, Gama RR, Silva ECAD, Duflath RM, Figueiredo DLA, et al.** Molecular classification of thyroid nodules with indeterminate cytology: development and validation of a highly sensitive and specific new miRNA-based classifier test using fine-needle aspiration smear slides. *Thyroid*. 2018;28(12):1618-1626. doi:10.1089/thy.2018.0254. [cited 2026 Sep 14]. Available from: DOI: 10.1089/thy.2018.0254.

9. Mohamad Yusof A, Jamal R, Muhammad R, Abdullah Suhaimi SN, Mohamed Rose I, Saidin S, *et al.* Integrated characterization of microRNA and mRNA transcriptome in papillary thyroid carcinoma. *Front Endocrinol (Lausanne)*. 2018;9:158. doi:10.3389/fendo.2018.00158. [cited 2026 Sep 14]. Available from: DOI: 10.3389/fendo.2018.00158.
10. Santos MT, Rodrigues BM, Shizukuda S, Oliveira AF, Oliveira M, Figueiredo DLA, *et al.* Clinical decision support analysis of a microRNA-based thyroid molecular classifier: a real-world, prospective and multicentre validation study. *EBioMedicine*. 2022;82:104137. doi:10.1016/j.ebiom.2022.104137. [cited 2026 Sep 14]. Available from: DOI: 10.1016/j.ebiom.2022.104137.
11. Iasonos A, Schrag D, Raj GV, Panageas KS. How to build and interpret a nomogram for cancer prognosis. *J Clin Oncol*. 2008;26(8):1364-1370. doi:10.1200/JCO.2007.12.9791. [cited 2026 Sep 14]. Available from: DOI: 10.1200/JCO.2007.12.9791.
12. Zhao Q, Zhang Y, Hua R, Lv B, Liu N. Predictive significance of anterior and posterior minimal extrathyroidal extension for central lymph node metastasis in cN0 papillary thyroid carcinoma. *Clin Exp Metastasis*. 2026;43(1):6. doi:10.1007/s10585-025-10388-5. [cited 2026 Sep 14]. Available from: DOI: 10.1007/s10585-025-10388-5.
13. Fukushima M, Ito Y, Hirokawa M, Miya A, Shimizu K, Miyauchi A. Prognostic impact of extrathyroid extension and clinical lymph node metastasis in papillary thyroid carcinoma depend on carcinoma size. *World J Surg*. 2010;34(12):3007-3014. doi:10.1007/s00268-010-0776-x. [cited 2026 Sep 14]. Available from: DOI: 10.1007/s00268-010-0776-x.
14. Kim H, Kwon H, Moon BI. Association of multifocality with prognosis of papillary thyroid carcinoma: a systematic review and meta-analysis. *JAMA Otolaryngol Head Neck Surg*. 2021;147(10):847-854. doi:10.1001/jamaoto.2021.1976. [cited 2026 Sep 14]. Available from: DOI: 10.1001/jamaoto.2021.1976.
15. Cui L, Feng D, Zhu C, Li Q, Li W, Liu B. Clinical outcomes of multifocal papillary thyroid cancer: a systematic review and meta-analysis. *Laryngoscope Investig Otolaryngol*. 2022;7(4):1224-1234. doi:10.1002/lio2.824. [cited 2026 Sep 14]. Available from: DOI: 10.1002/lio2.824.
16. Bansal M, Gandhi M, Ferris RL, Nikiforova MN, Yip L, Carty SE, *et al.* Molecular and histopathologic characteristics of multifocal papillary thyroid carcinoma. *Am J Surg Pathol*. 2013;37(10):1586-1591. doi:10.1097/PAS.0b013e318292b780. [cited 2026 Sep 14]. Available from: DOI: 10.1097/PAS.0b013e318292b780.
17. Deng X, Wu B, Xiao K, Kang J, Xie J, Zhang X, *et al.* MiR-146b-5p promotes metastasis and induces epithelial-mesenchymal transition in thyroid cancer by targeting ZNRF3. *Cell Physiol Biochem*. 2015;35(1):71-82. doi:10.1159/000369676. [cited 2026 Sep 14]. Available from: DOI: 10.1159/000369676.
18. Ferraz C, Cunha GB, de Oliveira MMB, Tenório LR, Cury AN, Padovani RDP, *et al.* The diagnostic and prognostic role of miR-146b-5p in differentiated thyroid carcinomas. *Front Endocrinol (Lausanne)*. 2024;15:1390743. doi:10.3389/fendo.2024.1390743. [cited 2026 Sep 14]. Available from: DOI: 10.3389/fendo.2024.1390743.
19. Lima CR, Geraldo MV, Fuziwara CS, Kimura ET, Santos MF. MiRNA-146b-5p upregulates migration and invasion of different papillary thyroid carcinoma cells. *BMC Cancer*. 2016;16:108. doi:10.1186/s12885-016-2146-z. [cited 2026 Sep 14]. Available from: DOI: 10.1186/s12885-016-2146-z.
20. Guo Z, Hardin H, Lloyd RV. Cancer stem-like cells and thyroid cancer. *Endocr Relat Cancer*. 2014;21(5):T285-T300. doi:10.1530/ERC-14-0002. [cited 2026 Sep 14]. Available from: DOI: 10.1530/ERC-14-0002.
21. Xia F, Wang W, Jiang B, Chen Y, Li X. DNA methylation-mediated silencing of miR-204 is a potential prognostic marker for papillary thyroid carcinoma. *Cancer Manag Res*. 2019;11:1249-1262. doi:10.2147/CMAR.S184566. [cited 2026 Sep 14]. Available from: DOI: 10.2147/CMAR.S184566.
22. Yang F, Bian Z, Xu P, Sun S, Huang Z. MicroRNA-204-5p: a pivotal tumor suppressor. *Cancer Med*. 2023;12(3):3185-3200. doi:10.1002/cam4.5077. [cited 2026 Sep 14]. Available from: DOI: 10.1002/cam4.5077.
23. Winkler J, Abisoye-Ogunniyan A, Metcalf KJ, Werb Z. Concepts of extracellular matrix remodelling in tumour progression and metastasis. *Nat Commun*. 2020;11(1):5120. doi:10.1038/s41467-020-18794-x. [cited 2026 Sep 14]. Available from: DOI: 10.1038/s41467-020-18794-x.
24. Yuan Z, Li Y, Zhang S, Wang X, Dou H, Yu X, *et al.* Extracellular matrix remodeling in tumor progression and immune escape: from mechanisms to treatments. *Mol Cancer*. 2023;22(1):48. doi:10.1186/s12943-023-01744-8. [cited 2026 Sep 14]. Available from: DOI: 10.1186/s12943-023-01744-8.
25. Zhou YY, Zhang YZ, Li J, Li ZQ, Ding WB, Li M. Preoperative prediction of lymph node metastasis risk in papillary thyroid carcinoma based on multiple model comparisons. *Sci Rep*. 2025;15(1):35313. doi:10.1038/s41598-025-19345-4. [cited 2026 Sep 14]. Available from: DOI: 10.1038/s41598-025-19345-4.
26. Boehm KM, Khosravi P, Vanguri R, Gao J, Shah SP. Harnessing multimodal data integration to advance precision oncology. *Nat Rev Cancer*. 2022;22(2):114-126. doi:10.1038/s41568-021-00408-3. [cited 2026 Sep 14]. Available from: DOI: 10.1038/s41568-021-00408-3.