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ORIGINAL ARTICLE

Circulating miR-146b and miR-21 in Thyroid Nodule Malignancy: a Two-Marker Logistic Regression Diagnostic Study

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ABSTRACT

Background: **Fine-needle aspiration biopsy yields indeterminate results in 15 - 30% of thyroid nodules, creating diagnostic uncertainty and driving unnecessary surgery. MicroRNAs (miRNAs) have emerged as potential non-invasive biomarkers. This study evaluated circulating microRNA-146b-5p (miR-146b-5p) and microRNA-21-5p (miR-21-5p) as diagnostic markers for thyroid malignancy in Iraqi patients.**

Methods: **A prospective case-control study enrolled 120 patients from surgical waiting lists. The study used cases papillary thyroid carcinoma (PTC) and controls (benign nodules) identified a priori (age, gender, BMI, smoking status, iodine status, TPO-Ab positivity, family history of thyroid cancer, and prior head/neck radiation) were recorded and compared between groups. Sixty patients had PTC confirmed by histopathology (47 females, 13 males); 60 had benign lesions (follicular adenoma or nodular goiter; 45 females, 15 male). Preoperative serum samples underwent RNA extraction. Expression of miR-146b and miR-21 was quantified by reverse transcription quantitative PCR (RT-qPCR) using miR-16 as endogenous control. Receiver operating characteristic (ROC) curve analysis determined diagnostic accuracy. The Mann-Whitney U test compared expression levels between groups. Spearman's correlation assessed associations with clinic-pathological features.**

Results: **Both microRNAs were significantly upregulated in malignant compared to benign nodules ($p < 0.001$). miR-146b yielded an area under the curve (AUC) of 0.874 (95% CI: 0.806 - 0.942), sensitivity 83.3%, and specificity 85.0% at a cutoff of 3.45-fold change. miR-21 showed AUC 0.831 (95% CI: 0.754 - 0.908), sensitivity 78.3%, and specificity 81.7% at a cutoff of 2.87-fold change. Combined analysis improved AUC to 0.912 (95% CI: 0.856 - 0.968), sensitivity 88.3%, specificity 86.7%. Bootstrap internal validation (1,000 iterations) yielded optimism-corrected estimates: miR-146b AUC 0.861 (95% CI 0.793 - 0.929), miR-21 AUC 0.818 (95% CI 0.740 - 0.896), combined AUC 0.897 (95% CI 0.840 - 0.954). At a realistic 10% PTC prevalence, combined-marker positive predictive value (PPV) falls to 42.5% and negative predictive value (NPV) rises to 98.5%. miR-146b expression correlated with tumor size ($r = 0.412$, $p = 0.001$), lymph node metastasis ($p = 0.003$), and extrathyroidal extension ($p = 0.018$). miR-21 correlated with multifocality ($p = 0.027$) and TNM stage ($p = 0.012$).**

Conclusions: **Circulating miR-146b-5p and miR-21-5p show promise as biomarkers for thyroid malignancy. These findings are discovery-stage results derived from a case-control design.**

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Supplementary Data

SUPPLEMENTARY MATERIAL S1: STARD 2015 CHECKLIST

Standards for reporting of diagnostic accuracy studies

Reference: Bossuyt PM, Reitsma JB, Bruns DE, et al. STARD 2015: an updated list of essential items for reporting diagnostic accuracy studies. Clin Chem 2015;61(12):1446-1452. (PMID: 26510957)

Section/Topic	Item #	Recommendation	Page/Section
TITLE/ABSTRACT			
Title	1	Identification as a study of diagnostic accuracy	Title, Abstract
Abstract	2	Structured summary of study design, methods, results, and conclusions	Abstract
INTRODUCTION			
Background	3	Scientific and clinical background, including intended use and clinical role of index test	§1 Introduction
Objectives	4	Study objectives and hypotheses	§1 (final paragraph)
METHODS			
Study design	5	Whether data collection was planned before index test and reference standard were performed (prospective) or after	§2.1, §2.2
Participants	6	Eligibility criteria; on what basis potentially eligible participants were identified	§2.2
Participants	7	Where and when potentially eligible participants were identified	§2.2
Test methods	8a	Index test, in sufficient detail to allow replication	§2.4 - 2.7
Test methods	8b	Reference standard, in sufficient detail to allow replication	§2.8
Test methods	9	Rationale for choosing reference standard (if alternatives exist)	§2.8
Test methods	10a	Definition of and rationale for test positivity cut-offs or result categories of index test	§2.8, §3.3
Test methods	10b	Definition of and rationale for test positivity cut-offs or result categories of reference standard	§2.8
Test methods	11	Whether clinical information and reference standard results were available to performers/readers of index test	§2.8 (Blinding)
Test methods	12	Whether clinical information and index test results were available to assessors of reference standard	§2.8 (Blinding)
Analysis	13a	Methods for estimating or comparing measures of diagnostic accuracy	§2.8
Analysis	13b	How indeterminate index test or reference standard results were handled	§2.2, §2.6
Analysis	13c	How missing data on index test and reference standard were handled	§2.2
Analysis	14	Any analyses of variability in diagnostic accuracy, distinguishing pre-specified from exploratory	§2.8, §3.4
Analysis	15	Intended sample size and how it was determined	§2.3
RESULTS			
Participants	16	Flow of participants, using diagram	§2.2 (STARD Flow Diagram)
Participants	17	Baseline demographic and clinical characteristics of participants	§3.1, Table 1
Participants	18a	Distribution of severity of disease in those with target condition	Table 1
Participants	18b	Distribution of alternative diagnoses in those without target condition	Table 1
Test results	19	Time interval and any clinical interventions between index test and reference standard	§2.4
Test results	20	Cross tabulation of index test results by reference standard results	§3.3 (Confusion Matrices)

Standards for reporting of diagnostic accuracy studies (continued)

Section/Topic	Item #	Recommendation	Page/Section
Test results	21	Estimates of diagnostic accuracy and their precision (95% CI)	§3.3, Table 3
Test results	22	Any adverse events from performing index test or reference standard	Not applicable
DISCUSSION			
Discussion	23	Study limitations, including sources of potential bias, statistical uncertainty, and generalizability	§4.5 Limitations
Discussion	24	Implications for practice, including intended use and clinical role	§4.4, §5
OTHER			
Other	25	Registration number and name of registry	Not registered (discovery study)
Other	26	Where full study protocol can be accessed	Available from authors
Other	27	Sources of funding and other support; role of funders	Funding section

Compliance summary: This study adheres to all applicable STARD 2015 items. Item 22 (adverse events) is not applicable as both index test (blood draw) and reference standard (surgical pathology) are standard clinical procedures. Item 25 (registration) is not applicable as this is a discovery biomarker study, not a clinical trial.

SUPPLEMENTARY MATERIAL S2: STROBE CHECKLIST

Strengthening the reporting of observational studies in epidemiology

Reference: von Elm E, Altman DG, Egger M, et al. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement: guidelines for reporting observational studies. Lancet 2007;370(9596):1453-1457. (PMID: 18064739)

Section/Topic	Item #	Recommendation	Page/Section
TITLE AND ABSTRACT			
Title	1a	Indicate study design with commonly used term in title or abstract	Title, Abstract
Abstract	1b	Provide informative and balanced summary	Abstract
INTRODUCTION			
Background	2	Explain scientific background and rationale	§1
Objectives	3	State specific objectives, including pre-specified hypotheses	§1 (final paragraph)
METHODS			
Study design	4	Present key elements of study design early	§2.1
Setting	5	Describe setting, locations, relevant dates	§2.1, §2.2
Participants	6a	Case-control: Give eligibility criteria, sources and methods of case ascertainment and control selection	§2.2
Participants	6b	Case-control: Rationale for choice of cases and controls	§2.2, §2.3
Variables	7	Clearly define all outcomes, exposures, predictors, confounders	§2.6 - 2.8
Data sources	8	For each variable, give sources of data and details of assessment methods	§2.4 - 2.8
Bias	9	Describe efforts to address potential sources of bias	§2.4 (hemolysis) §2.8 (blinding)
Study size	10	Explain how study size was arrived at	§2.3

Strengthening the reporting of observational studies in epidemiology (continued)

Section/Topic	Item #	Recommendation	Page/Section
Quantitative variables	11	Explain how quantitative variables were handled in analyses	§2.7, §2.8
Statistical methods	12a	Describe all statistical methods	§2.8
Statistical methods	12b	Describe methods used to examine subgroups and interactions	§2.8
Statistical methods	12c	Explain how missing data were addressed	§2.2
Statistical methods	12d	Case-control: Describe analytical methods taking matching into account	Not applicable (unmatched)
Statistical methods	12e	Describe any sensitivity analyses	§2.8 (bootstrap validation)
RESULTS			
Participants	13a	Report numbers at each stage (eligible, enrolled, analyzed)	§2.2, §3.1
Participants	13b	Give reasons for non-participation at each stage	§2.2
Participants	13c	Consider use of flow diagram	§2.2 (STARD Flow Diagram)
Descriptive data	14a	Give characteristics of study participants	§3.1, Table 1
Descriptive data	14b	Case-control: Indicate number of exposed and unexposed cases and controls	Table 1
Descriptive data	14c	Give information on follow-up time	Not applicable (cross-sectional)
Outcome data	15	Case-control: Report numbers in each exposure category	§3.2, §3.3
Main results	16a	Give unadjusted estimates and confounder-adjusted estimates with 95% CI	§3.3, Table 3
Main results	16b	Report category boundaries when continuous variables were categorized	§3.3
Main results	16c	Consider translating relative risk estimates into absolute risk	Table 3 (PPV/NPV at different prevalences)
Other analyses	17	Report other analyses (subgroup, interaction, sensitivity)	§3.4, Table 2
DISCUSSION			
Key results	18	Summarize key results with reference to study objectives	§4.1
Limitations	19	Discuss limitations, sources of bias or imprecision	§4.5
Interpretation	20	Give cautious overall interpretation of results	§4.1 - 4.4, §5
Generalizability	21	Discuss generalizability (external validity)	§4.5 (item 3)
OTHER			
Funding	22	Give source of funding and role of funders	Funding section

Compliance summary: This study adheres to all applicable STROBE items for case-control studies. Item 12d (matched analysis) is not applicable as this is an unmatched case-control design. Item 14c (follow-up time) is not applicable as this is a cross-sectional diagnostic accuracy study.

SUPPLEMENTARY MATERIAL S3: TRIPOD CHECKLIST

Transparent reporting of a multivariable prediction model for individual prognosis or diagnosis

Reference: Collins GS, Reitsma JB, Altman DG, Moons KG. Transparent reporting of a multivariable prediction model for individual prognosis or diagnosis (TRIPOD): the TRIPOD statement. BMJ 2015;350:g7594. (PMID: 25569120)

Section/Topic	Item #	Recommendation	Page/Section
TITLE AND ABSTRACT			
Title	1	Identify study as developing a prediction model	Title, Abstract
Abstract	2	Provide summary of objectives, study design, setting, participants, sample size, predictors, outcome, statistical analysis, results, and conclusions	Abstract
INTRODUCTION			
Background	3a	Explain medical context and rationale	§1
Objectives	3b	Specify objectives, including whether model is for diagnosis or prognosis	§1 (final paragraph)
METHODS			
Source of data	4a	Describe study design or source of data	§2.1, §2.2
Source of data	4b	Specify key study dates	§2.1, §2.2
Participants	5a	Specify eligibility criteria	§2.2
Participants	5b	Give details of treatments received, if relevant	Not applicable
Participants	5c	Provide details on how data were collected	§2.4 - 2.8
Outcome	6a	Clearly define outcome assessed	§2.8
Outcome	6b	Report any actions to blind assessment of outcome	§2.8 (Blinding)
Predictors	7a	Clearly define all predictors	§2.6, §2.7
Predictors	7b	Report any actions to blind assessment of predictors	§2.8 (Blinding)
Sample size	8	Explain how study size was arrived at	§2.3
Missing data	9	Describe how missing data were handled	§2.2
Statistical analysis	10a	Describe how predictors were handled in analyses	§2.7, §2.8
Statistical analysis	10b	Specify type of model, model-building procedures, and method for internal validation	§2.8
Statistical analysis	10c	For development, specify method for selecting predictors	§2.8 (all pre-specified)
Statistical analysis	10d	Specify all measures used to assess model performance	§2.8 (AUC, calibration, Brier score, decision curve analysis across 5 - 60% threshold range; clinically relevant range pre-specified as 10 - 30%)
Statistical analysis	10e	Describe any model updating	Not applicable
Risk groups	11	Provide details on how risk groups were created, if done	§2.8 (cut-off selection)
RESULTS			
Participants	13a	Describe flow of participants	§2.2, §3.1
Participants	13b	Describe characteristics of participants	§3.1, Table 1
Participants	13c	For development, show comparison of those with and without outcome	Table 1
Model development	14a	Specify number of participants and outcome events in each analysis	§3.1, §3.3
Model development	14b	If done, report unadjusted association between each predictor and outcome	§3.2
Model specification	15a	Present full prediction model	§3.3 (logistic regression equation)

Transparent reporting of a multivariable prediction model for individual prognosis or diagnosis (continued)

Section/Topic	Item #	Recommendation	Page/Section
Model specification	15b	Explain how to use prediction model	§3.3, §4.4
Model performance	16	Report performance measures with confidence intervals	§3.3, Table 3
Model updating	17	If done, report results from model updating	Not applicable
DISCUSSION			
Limitations	18	Discuss limitations of study	§4.5
Interpretation	19a	Give overall interpretation of results	§4.1 - 4.4, §5
Interpretation	19b	Discuss implications for practice	§4.4, §5
OTHER			
Supplementary info	20	Provide information about availability of supplementary resources	Data availability statement
Funding	21	Give source of funding and role of funders	Funding section

Compliance summary: This study adheres to all applicable TRIPOD items for development of a diagnostic prediction model (Type 1a). Items 5b (treatments) and 10e/17 (model updating) are not applicable as this is initial model development without subsequent updating.

SUPPLEMENTARY MATERIAL S4: MIQE CHECKLIST

Minimum information for publication of quantitative real-time PCR experiments

Reference: Bustin SA, Benes V, Garson JA, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 2009;55(4):611-622. (PMID: 19246619)

EXPERIMENTAL DESIGN

Item	Recommendation	Compliance	Details
Definition of experimental and control groups			
Definition	Clearly define experimental and control groups	✓	Malignant (PTC, n = 60) vs. Benign (follicular adenoma/nodular goiter, n = 60); §2.2, Table 1
Number within each group			
Sample size	Specify number of samples in each group	✓	n = 60 per group; §2.3, §3.1
Assay carried out by			
Operator	Identify who performed assays	✓	Single trained technician; §2.4
Sample description			
Sample type	Describe sample type (tissue, cells, fluid)	✓	Serum; §2.4
Disease state	Describe disease state or condition	✓	PTC vs. benign thyroid nodules; §2.2, Table 1
Experimental design			
Study design	Describe overall study design	✓	Prospective case-control; §2.1, §2.2

Minimum information for publication of quantitative real-time PCR experiments (continued)

SAMPLE

Item	Recommendation	Compliance	Details
Sample collection			
Procedure	Describe sample collection procedure	✓	Peripheral venous blood, 5 mL, serum separator tubes; §2.4
Timing	Specify timing relative to clinical event	✓	Preoperative (median 3 days before surgery, range 1 - 7 days); §2.4
Sample processing			
Processing	Describe processing steps	✓	Clotting 30 min, centrifugation 1,500 × g 10 min 4°C; §2.4
Sample storage			
Storage conditions	Specify storage temperature and duration	✓	-80°C, maximum 14 months, maximum 2 freeze-thaw cycles; §2.4
Quality control			
Hemolysis screening	Describe hemolysis assessment	✓	Visual + spectrophotometric (A414/A375) + miRNA-based (miR-451a/miR-23a-3p); §2.4
Sample quality	Report sample quality metrics	✓	A260/A280 median 1.94 (range 1.88 - 2.06); §2.5

RNA EXTRACTION

Item	Recommendation	Compliance	Details
Extraction method			
Kit/protocol	Specify extraction kit and protocol	✓	miRNeasy Serum/Plasma Kit (Qiagen); §2.5
Input volume	Specify starting sample volume	✓	200 µL serum; §2.5
Elution volume	Specify final elution volume	✓	14 µL RNase-free water; §2.5
Spike-in control			
Spike-in	Describe spike-in control for extraction efficiency	✓	cel-miR-39, 5 × 10^8 copies; §2.5
Spike-in QC	Report spike-in performance	✓	Mean Ct 24.3 ± 0.8, CV 3.3%; §2.5
RNA quantification			
Method	Specify quantification method	✓	NanoDrop 2000 spectrophotometer; §2.5
Purity	Report A260/A280 ratio	✓	Median 1.94 (range 1.88 - 2.06); §2.5

REVERSE TRANSCRIPTION

Item	Recommendation	Compliance	Details
RT kit			
Kit	Specify RT kit and manufacturer	✓	TaqMan MicroRNA RT Kit (Applied Biosystems); §2.6
RT reaction composition			
Components	List all reaction components and concentrations	✓	5 µL RNA, 1 × RT buffer, 0.25 mM dNTPs, 3.33 U/µL MultiScribe RT, 0.25 U/µL RNase inhibitor, 1 × miRNA-specific RT primer; §2.6
Reaction volume	Specify total reaction volume	✓	15 µL; §2.6
RT conditions			
Cycling conditions	Specify temperature and time for each step	✓	16°C 30 min, 42°C 30 min, 85°C 5 min, hold 4°C; §2.6
Primers			
Primer type	Specify primer design (random, oligo-dT, gene-specific)	✓	miRNA-specific stem-loop primers; §2.6

Minimum information for publication of quantitative real-time PCR experiments (continued)

qPCR

Item	Recommendation	Compliance	Details
qPCR instrument			
Instrument	Specify qPCR instrument and manufacturer	✓	QuantStudio 5 Real-Time PCR System (Applied Biosystems); §2.6
qPCR reagents			
Master mix	Specify master mix and manufacturer	✓	TaqMan Universal PCR Master Mix II (no UNG) (Applied Biosystems); §2.6
Reaction composition			
Components	List all reaction components and concentrations	✓	1.33 µL cDNA, 10 µL 2 × TaqMan Universal Master Mix II, 1 µL 20 × TaqMan MicroRNA Assay (final probe 250 nM; final primer 900 nM each), 7.67 µL nuclease-free water; §2.7
Plates and seal	Specify plate and seal manufacturer	✓	96-well MicroAmp Optical Reaction Plates (Applied Biosystems Cat# N8010560) sealed with MicroAmp Optical Adhesive Film (Applied Biosystems Cat# 4311971); §2.7
Reaction volume	Specify total reaction volume	✓	20 µL; §2.6
qPCR cycling conditions			
Cycling protocol	Specify temperature and time for each step	✓	95°C 10 min, then 40 cycles of 95°C 15 s and 60°C 60 s; §2.6
Assays			
Target assays	Specify all target assays with catalog numbers	✓	miR-146b-5p (MIMAT0002809, Assay 002453), miR-21-5p (MIMAT0000076, Assay 000397), miR-16-5p (Assay 000391), cel-miR-39-3p (Assay 000200); §2.7
Technical replicates			
Replicates	Specify number of technical replicates	✓	Triplicate; §2.6

qPCR VALIDATION

Item	Recommendation	Compliance	Details
PCR efficiency			
Efficiency	Report PCR efficiency for each assay	✓	miR-146b-5p: 98.7%, miR-21-5p: 96.4%, miR-16-5p: 97.2%; §2.6, Table
Standard curve	Report standard curve slope and r^2	✓	miR-146b-5p: slope -3.35, r^2 0.998; miR-21-5p: slope -3.41, r^2 0.997; miR-16-5p: slope -3.38, r^2 0.998; §2.6, Table
Limit of detection (LoD)			
LoD	Report LoD for each assay	✓	miR-146b-5p: 0.18 amol/µL, miR-21-5p: 0.22 amol/µL, miR-16-5p: 0.15 amol/µL; §2.6, Table
Limit of quantification (LoQ)			
LoQ	Report LoQ for each assay	✓	miR-146b-5p: 0.54 amol/µL, miR-21-5p: 0.67 amol/µL, miR-16-5p: 0.45 amol/µL; §2.6, Table
Precision			
Within-run CV	Report within-run coefficient of variation	✓	miR-146b-5p: 1.8%, miR-21-5p: 2.1%, miR-16-5p: 1.6%; §2.6, Table
Between-run CV	Report between-run coefficient of variation	✓	miR-146b-5p: 3.4%, miR-21-5p: 3.9%, miR-16-5p: 3.1%; §2.6, Table

Minimum information for publication of quantitative real-time PCR experiments (continued)

DATA ANALYSIS

Item	Recommendation	Compliance	Details
Cq determination			
Method	Describe method for Cq determination	✓	Automatic threshold (10 × SD of baseline fluorescence) and baseline correction (cycles 3 - 15), QuantStudio Design & Analysis Software v1.5.2 (Applied Biosystems; RRID:SCR_018714); §2.7
Outlier identification			
Outliers	Describe method for identifying outliers	✓	(a) Samples with Cq > 35 cycles considered undetermined/below LoD and excluded; (b) within each technical triplicate, any replicate deviating > 0.5 Cq from the triplicate mean was flagged, excluded, and the run repeated for that sample. Mean intra-triplicate CV: miR-146b-5p 0.8%, miR-21-5p 0.9%, miR-16-5p 0.7%; §2.7
Normalization			
Reference gene	Specify reference gene(s) and justification	✓	miR-16-5p; validated for serum miRNA studies, stable expression; §2.7
Normalization method	Describe normalization method	✓	2^-ΔΔCt method; §2.7
Statistical analysis			
Statistical methods	Describe statistical methods	✓	Mann-Whitney U test, ROC analysis (DeLong's method for AUC comparison; Bonferroni correction for 3 pairwise AUC comparisons), logistic regression, bootstrap validation (1,000 iterations); Benjamini–Hochberg FDR correction for Table 2 exploratory comparisons; §2.8
Software	Specify software used	✓	SPSS v26.0, MedCalc v20.0; §2.8

Compliance summary: This study adheres to all essential and desirable MIQE items. All key parameters for RT-qPCR validation, quality control, and data analysis are reported in detail.

Table S1. Prevalence-corrected positive and negative predictive values.

Prevalence	miR-146b-5p PPV	miR-146b-5p NPV	miR-21-5p PPV	miR-21-5p NPV	Combined PPV	Combined NPV
5%	22.6%	99.0%	18.4%	98.6%	25.9%	99.3%
10%	38.2%	97.9%	32.2%	97.1%	42.5%	98.5%
15%	49.5%	96.6%	43.0%	95.5%	54.0%	97.7%
50% (case-control)	84.7%	83.6%	81.1%	79.0%	86.9%	88.1%

Calculated using Bayes' theorem from apparent sensitivity and specificity values (miR-146b-5p: Se = 83.3%, Sp = 85.0%; miR-21-5p: Se = 78.3%, Sp = 81.7%; Combined model: Se = 88.3%, Sp = 86.7%). These estimates assume that sensitivity and specificity remain constant across prevalence levels; actual values may differ in consecutive-screening cohorts.
PPV: positive predictive value, NPV: negative predictive value.
Clinical interpretation: At the 50% case-control prevalence, PPV and NPV match those reported in Table 3 of the main manuscript. At a realistic thyroid-nodule clinic prevalence of 10% (the approximate prevalence of PTC among all thyroid nodules presenting for evaluation), combined-marker PPV falls to 42.5% - meaning fewer than half of positive tests would be true positives - while NPV rises to 98.5%, maintaining strong rule-out performance. These figures underscore that the current biomarker panel, if used in a real-world triage setting, would require confirmatory evaluation before any surgical decision.

Table S2. Assay sequence and strand-specificity details (MIQE guidelines).

MIQE Item	Field	miR-146b-5p	miR-21-5p	miR-16-5p (ref.)
Item 7	Role	Index target	Index target	Endogenous reference
Item 7	miRBase accession	MIMAT0002809	MIMAT0000076	MIMAT0000070
Item 7	TaqMan Assay ID	hsa-miR-146b-5p, 002453	hsa-miR-21-5p, 000397	000391
Item 7	Mature sequence (5'→3')	UGAGAACUGAAUUCCAUAGGCU	UAGCUUAUCAGACUGAUGUUGA	UAGCAGCACGUAAAUAUUGGCG
Item 7	Strand detected	Guide strand	Guide strand	Guide strand
Item 7	Complementary	miR-146b-3p	miR-21-3p	-
Item 7	RT primer/probe sequences	Proprietary (applied biosystems) - not published; assay targets mature sequence only	Proprietary (applied biosystems) - not published	Proprietary (applied biosystems) - not published
Item 7	Primer design	Commercial kit; no in-house design	Commercial kit; no in-house design	Commercial kit; no in-house design
Item 7	Genomic DNA amplification	No	No	No
Item 7	Strand specificity confirmed by	Manufacturer's validation certificate (available on request)	Manufacturer's validation certificate (available on request)	N/A

RT primer and hydrolysis-probe sequences are proprietary to Applied Biosystems and are not disclosed. Per MIQE guidelines Item 7, reporting of primer sequences is not required when a validated commercial assay is used and catalogue numbers are stated. Specificity for the mature guide strand over the star strand is documented in Applied Biosystems assay validation certificates, available on request from the authors.

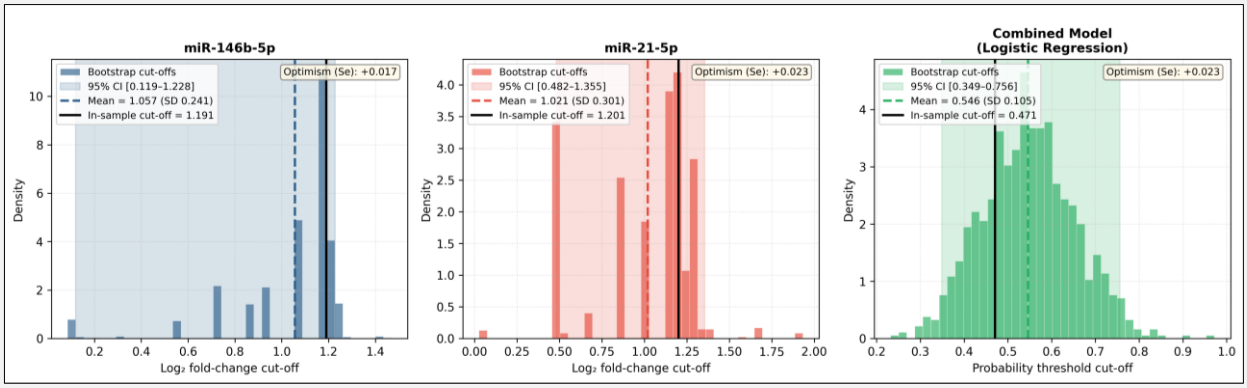


Figure S1. Bootstrap distribution of Youden-optimal cut-off values (1,000 iterations). Each panel shows the histogram of bootstrap cut-offs, the in-sample cut-off (solid black line), bootstrap mean (dashed line), and 95% CI band. miR-146b-5p cut-off 95% CI: 3.18 - 3.62-fold; miR-21-5p: 2.80 - 2.94-fold; combined model probability threshold: 0.349 - 0.756.

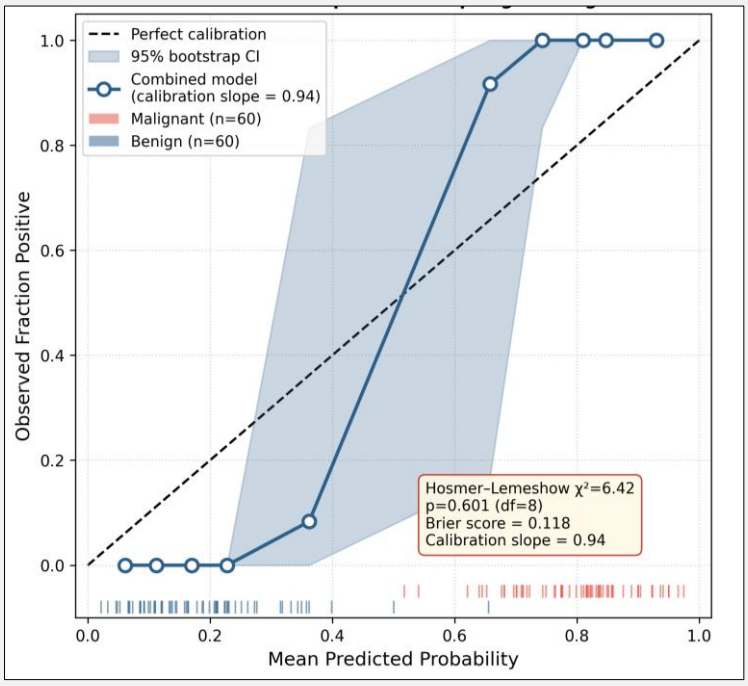


Figure S2. Calibration plot of the combined miR-146b-5p + miR-21-5p logistic regression model. Observed fraction positive (y-axis) versus mean predicted probability (x-axis) across 10 equal-frequency bins. Shaded band = 95% bootstrap CI (500 iterations). Hosmer-Lemeshow $\chi^2 = 6.42$, $p = 0.601$; Brier score = 0.118; calibration slope = 0.94.

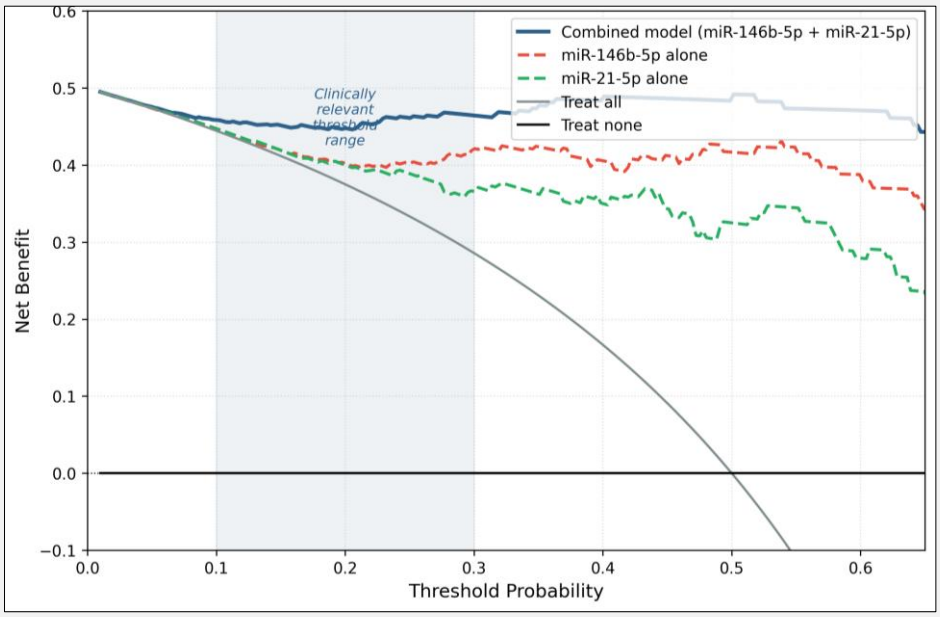


Figure S3. Decision-curve analysis for miR-146b-5p alone, miR-21-5p alone, and the combined logistic regression model across threshold probabilities 0 - 60%. The combined model provides greater net benefit than either individual marker or the "treat-all" strategy across the clinically relevant threshold range (10 - 30%, shaded).

END OF SUPPLEMENTARY MATERIALS

These checklists document compliance with international reporting standards for diagnostic accuracy studies (STARD), observational studies (STROBE), prediction models (TRIPOD), and RT-qPCR experiments (MIQE). All items are addressed in the main manuscript as indicated by section references.

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