Title: Multiomics Study of Biological Behavior of Lymph Node Metastasis in Papillary Thyroid Carcinoma

NCT number:

Date: 11/26/2024

Protocol

This protocol has been provided by the authors to give readers additional information about the work.

Subtitle:Multi-omics integration of metabolomics, proteomics, ultrasound radiomics for prediction of central lymph node metastasis in stage T1 papillary thyroid carcinoma： A real-world study

version number： 2.0

version data：12/1/2024

Research Centers：

Hangzhou Traditional Chinese Medical Hospital

Summary of protocol

Study name: Multi-omics integration of metabolomics, proteomics, ultrasound radiomics for prediction of central lymph node metastasis in stage T1 papillary thyroid carcinoma

The expected total duration of the trial:2024.12-2026.12

Study Objective

Primary Objective: This study design aims to enhance the understanding of CLNM predictions using a multi-omics approach and aims to improve clinical decision-making in managing PTC patients.

Study Design

This study is a multicenter, observational cohort study aimed at assessing the accuracy and effectiveness of the ThyMPR-CLNM multi-omics model in predicting CLNM in patients diagnosed with stage T1 PTC. The design incorporates the following critical components:

The study enrolled 2000 patients diagnosed with stage T1 PTC from Hangzhou Traditional Chinese Medical Hospital, affiliated with Zhejiang Chinese Medical University, between Dec.2024 and Dec.2026. Fresh frozen tumor tissue, serum samples, and preoperative ultrasound images were collected from participants. These samples were utilized for comprehensive multi-omics analyses, including metabolomic and proteomic profiling, as well as ultrasound radiomic feature extraction. To minimize selection bias and balance covariates, propensity score matching was performed in two rounds, establishing a discovery set and a validation set with matched groups based on the propensity scores calculated through logistic regression. This ensured comparable groups for subsequent analyses. The study involved analyzing the collected samples through advanced techniques such as liquid chromatography-mass spectrometry (LC-MS) for metabolomic and proteomic analyses, and Pyradiomics for extracting radiomics features from ultrasound images. Differentially expressed metabolites,

proteins, and radiomic features were identified and integrated for the development of the ThyMPR-CLNM prediction model. The Least Absolute Shrinkage and Selection Operator (LASSO) regression technique was utilized to construct the ThyMPR-CLNM model based on identified features from the multi-omics analyses. The model's performance was subsequently validated using an independent dataset. Statistical evaluations were performed using R software to determine the model's accuracy, sensitivity, specificity, and AUC values. Comparisons with conventional diagnostic methods were conducted to highlight the ThyMPR-CLNM model's advantages.

SOMMAIRE / TABLE OF CONTENT

I. INTRODUCTION AND RATIONALE OF THE STUDY

Thyroid cancer is one of common endocrine malignancies and its global incidence seems to be rising [1]. Papillary thyroid carcinoma (PTC), the predominant form, constitutes over 90% of all thyroid cancer cases [2]. Initially, Stage T1 PTC, commonly defined as a tumor less than 2 cm in size without extrathyroidal extension, comprises 80% of PTC cases [3]. At diagnosis, 25-80% of this part of patients exhibit cervical lymph node metastasis (LNM), with central lymph node metastasis (CLNM) being notably prevalent [4-6]. A minority of patients with PTC develop locoregional recurrence, including cervical lymph node metastases, which eventually leads to mortality in some patients [7]. Conventionally, PTC patients presenting with clinical lymph node positivity (cN1) undergo thyroidectomy and therapeutic lymph node dissection [2]. However, the utility of prophylactic central neck dissection (pCND) in clinically lymph node-negative (cN0) patients remains a subject of debate. Guidelines from China and Asia-Pacific regions support pCND in cN0 PTC patients, referencing studies that detected occult LNM during dissections in these cases [8, 9]. In contrast, the 2015 American Thyroid Association (ATA) guidelines recommend against routine pCND in cN0 PTC [10]. This divergence in guidelines underscores the need for more evidence to inform surgical decisions on an individual basis. Preoperative, precise evaluation of CLNM is crucial to tailor appropriate treatment strategies for PTC, aiming to minimize risks such as recurrent laryngeal nerve damage and postoperative hypoparathyroidism, and to avert treatment delays or secondary surgery risks [11].

Proteins, fundamental organic components of cells, play critical roles in biological processes [12]. Moreover, small molecules such as sugars, amino acids, and lipids, products of cellular metabolism, reflect the dynamic metabolic adaptations of living systems to various external stimuli or perturbations, including diseases, pharmaceutical interventions, dietary changes, or environmental conditions [13]. Recent advancements in proteomics and metabolomics have significantly enhanced the diagnosis and treatment of diseases [14]. Concurrently, radiomics, an emerging imaging analysis technique, has gained widespread application in tumor diagnostics, post-treatment evaluations, and prognostic assessments [15]. Furthermore, the integration of big data

and multi-omics approaches is increasingly supporting precision medicine. For instance, Vanguri et al. have provided a quantitative basis for utilizing multi-modal features through expert-guided machine learning to enhance the prediction of immunotherapy responses in patients with non-small cell lung cancer [16].

In this study, we collected and analyzed metabolomic, proteomic, and ultrasound radiomic data from stage T1 PTC patients. We then developed a comprehensive multi-omics model designed to predict CLNM in PTC patients. This non-invasive model facilitates the precise prediction of CLNM in low-risk PTC cases and provides critical guidance for surgical strategies in thyroid cancer management, thereby minimizing the risks associated with overtreatment or undertreatment.

II. Study Objective

II.1. Primary Objective

The primary objective of this study is to evaluate the accuracy of the ThyMPR-CLNM model in predicting central lymph node metastasis (CLNM) in patients with stage T1 papillary thyroid carcinoma (PTC).

II.2. Secondary Objective

The secondary objectives are to compare the performance of the ThyMPR-CLNM multi-omics model and evaluate differences in sensitivity, specificity and area under the receiver operating characteristic curve (AUC) values.

III. Methodology

This is a real-world retrospective cohort study. This study enrolled patients from Hangzhou TCM Hospital between August 2017 and June 2022. Preoperative serum

samples and ultrasound data were systematically collected from all participants. In the discovery set, fresh frozen tumor tissues were obtained during surgical procedures.

III.1. Selection of patients

III.1.1 Inclusion criteria

(1) Pathological confirmation of PTC.
(2) Patients who underwent primary surgery accompanied by central neck lymph node dissection. (3) Tumors measuring less than 2 cm in diameter.
(4) Postoperative pathological reports including detailed information on the number of lymph nodes dissected and the number of metastatic lymph nodes.
(5) Availability of comprehensive preoperative thyroid ultrasound images for analysis.

III.1.2 Exclusion criteria

(1) Postoperative pathological diagnosis indicating sub-types of PTC.
(2) Tumor invasion into adjacent anatomic structures such as the sternothyroid muscle, surrounding soft tissues, trachea, esophagus, or laryngeal nerve.
(3) History of neck trauma, previous tumor surgery, or adjuvant chemoradiotherapy.
(4) Fewer than three lymph nodes dissected during surgery.
(5) Concurrent acute inflammatory conditions or other hematologic disorders.

III.2. Study termination and withdrawal criteria are as follows

1）The inclusion criteria are not met after enrollment.
2）The exclusion criteria are met after enrollment.
3) The ultrasound image is unclear.
4）Abnormalities in patient specimens, such as hemolysis or insufficient volume, etc.
5) Patients cannot comply with the test procedure.

III.3. **Metabolomics and proteomics analyses**

Each tumor sample and corresponding serum specimen were processed using pressure cycling technology (PCT). Metabolomic data were acquired through multiple reaction monitoring (MRM) mode analysis using triple quadrupole mass spectrometry, while proteomic data were gleaned via data-independent acquisition mass spectrometry (DIA-MS), with settings detailed in the supplementary materials. Metabolomic data were analyzed against a custom-built target standard product database, the Metware Database (MWDB), supplemented by a home-made metadata database and additional established metabolomic repositories such as MassBank, KNApSAcK, HMDB, and METLIN. Proteomic data were processed using DIA-NN software against a thyroid-specific spectral library.

III.4. **Habitat radiomics analysis**

Color Doppler ultrasound machines equipped with a real-time, high-frequency (5-10 MHz) linear-array probes are used in this study. Ultrasound images of all nodules are collected and stored according to the protocol specified in the guideline.

The extracted data comprise a set of radiomic features, including first-order statistics, intensity histograms, shape and size statistics, and textured features, both raw and filtered. These handcrafted features are extracted using an in-house program implemented via Pyradiomics (http://pyradiomics.readthedocs.io). The K-means clustering algorithm stratifies the tumor voxel cluster into optimal spatial divisions across all modalities. For each spatial division, Pyradiomics facilitates the extraction of texture features specific to that habitat region. Features from any given modality represent its respective characteristics and are subsequently fused to enhance the data's robustness.

III.5. Surgical procedures

All patients underwent open surgery where simultaneous thyroidectomy and cervical lymph node dissection were conducted. Central lymph nodes were consistently dissected as a standard procedure. Patients showing indications of lateral cervical lymph node metastases via fine-needle aspiration biopsy (FNA) or preoperative imaging underwent lateral cervical dissection. Critical structures, including the internal jugular vein, sternocleidomastoid muscle, and spinal accessory nerve, were preserved during the procedure. The number of dissected lymph nodes and identified metastases was meticulously quantified and independently assessed by two pathologists.

IV. Statistical methods

IV.1. Sample size estimation

In this study, we enrolled a total of 1,062 patients diagnosed with papillary thyroid carcinoma (PTC) from Hangzhou Traditional Chinese Medical Hospital, affiliated with Zhejiang Chinese Medical University, between August 2017 and June 2022. To construct the study cohorts, we established a discovery set and a validation set using two rounds of propensity score matching (PSM).

1. Initial Enrollment and Inclusion Criteria:

- The initial sample consisted of 1,062 PTC patients.
- Inclusion criteria were established to ensure that only patients meeting specific clinical and demographic characteristics were considered for the analysis.

2. Propensity Score Calculation:

- The propensity score for each patient was calculated using variables that were likely to influence the likelihood of central lymph node metastasis (CLNM) and treatment group assignment. This involved conducting a logistic regression analysis that included potential confounding factors, such as age, gender, tumor size, and other clinical characteristics.

3. Matching Process:

- Propensity score matching was conducted in two rounds to balance the discovery and validation cohorts.
- In the first round, patients were matched 1:1 based on their calculated propensity scores, aiming to achieve a balanced representation of both groups (e.g., CLNM positive versus negative patients).
- The second round of matching further refined the cohorts by using specific thresholds (calipers) to ensure that matched patients were similar in their characteristics.

4. Establishment of Discovery and Validation Sets:

- **Discovery Set:** Following the propensity score matching, the first subset of patients designated as the discovery set contained a balanced number of patients with CLNM and non CLNM, allowing for model development and validation.
- **Validation Set:** The-subsequent set served as an independent validation cohort, ensuring that the findings were robust and applicable across both groups. This set was essential for testing the predictive performance of the developed model.

5. Final Sample Size:

- The final count of patients included in the discovery and validation sets was determined after matching, ensuring a sufficient sample size for statistical power and generalizability of results.

In summary, the approach to sample size estimation involved strategically enrolling a large cohort of PTC patients and applying propensity score matching to create balanced discovery and validation sets, ensuring the integrity and validity of the study's findings.

IV.2. Statistical Analysis

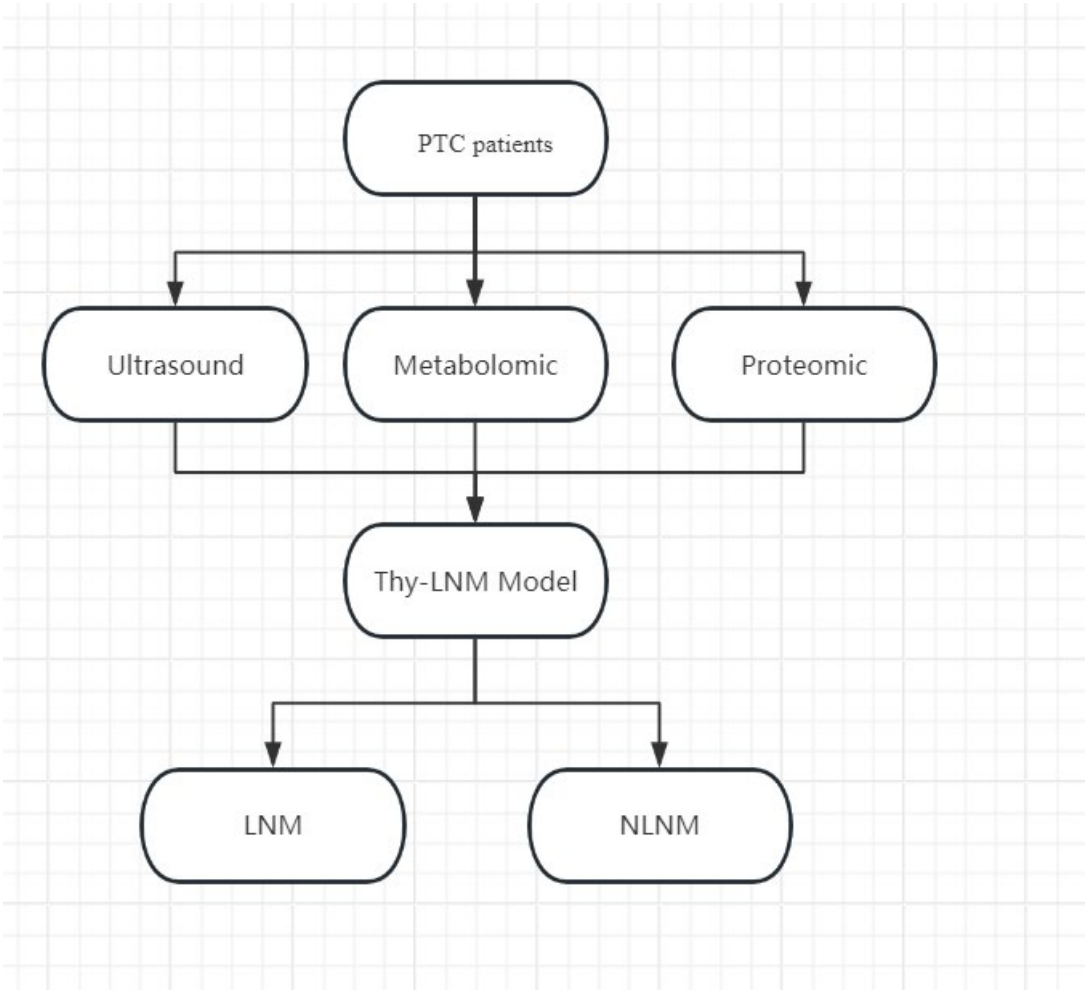
The statistical analyses were conducted using R software（version 4.3.1），with the utilization of various visual representation methods such as heatmaps, volcano plots, and receiver operating characteristic (ROC) curve plots. PSM analysis was conducted

to minimize the effects of potential confounders on selection bias. And PSM was performed as a 1:1 matching with a caliper value of 0.05. Differentially expressed metabolites (DEMs) and differentially expressed proteins (DEPs) were assessed utilizing the R package, with the significance threshold set at a P-value less than 0.05. The least absolute shrinkage and selection operator (LASSO) regression model was used on the discovery dataset training dataset for signature construction. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were computed following standardized procedures, each accompanied by a 95% Wilson confidence interval.

IV.3. Statistical analysts

The members of the research team consist of Hangzhou Traditional Chinese Medical Hospital, Affiliated to Zhejiang Chinese Medical University, Hangzhou, China.

V. Flow chart of the study implementation



VI. The plan of study schedule

2024.12-2025.08 Include patients, fill out case report forms, collect clinical data.

2025.08-2025.12 Patients are continued to be enrolled.

2025.12-2026.8 Patients are continued to be enrolled and data analysis.

2026.08-2026.12 Evaluate the diagnostic efficacy of ThyMPR-CLNM multi-omics model.

VII. Ethics approval

Potential subjects will be fully informed of the risks and requirements of the study, and will be provided with any new information during the study period that may affect their continued participation in the study. They will be told that participation in the study is voluntary, that they can withdraw at any time without any reason, and that they will not be penalized or lose any due benefits. Only subjects who fully understand the risks, benefits, and potential adverse events of the study will be admitted after they voluntarily sign an informed consent form.

VII.1. Local institutional review boards or independent ethics committees

Protocol and informed consent must be reviewed and approved by a reasonably constituted independent ethics committee/institutional Review Committee (IEC/IRB) prior to trial initiation. A signed and dated IEC/IRB approval document for the protocol and informed consent must be submitted to the principal investigator prior to trial initiation.

VII.2. Responsibility of the investigators

The principal investigator of each establishment concerned promises to conduct the clinical trial in conformity with the protocol which has been approved by the competent authority.

The principal investigator should not modify any aspect of the protocol without the approval of the proposed modifications by the competent authority.

The Principal Investigator is responsible for:

- providing the Sponsor with his/her CV as well as the investigators' ones,

-identifying members of his/her team participating in the trial and defining their responsibilities,

- recruiting patients after receiving the Sponsor's approval
- personally obtaining the informed consent form which has been dated and signed by the participant in the research prior to any specific trial selection procedure
- regularly completing the case report form (CRF) for each patient included in the trial
- dating, correcting and signing the corrections on the CRF for each patient included in the trial
- accepting regular visits from a CRA and possibly visits from auditors mandated by the Sponsor or inspectors from the regulatory authorities.

All documentation concerning the trial (protocol, consent form, case report form, investigator file), as well as the original documents (laboratory results, imaging studies, medical consultation reports, clinical examination reports, etc.) is considered confidential and should be kept in a safe place. The Principal Investigator should keep data as well as a list of patient-identifying data for at least 10 years after the end of the study.

VII.3. Information and consent of participants

Prior to the conduct of any procedure linked to biomedical research, any person wishing to participate in a research study gives his/her free, informed and written consent. This consent is obtained once the participant has been informed by the investigator during a consultation and after the person had been given sufficient time to think it over.

Having read the information notice the patient must date and sign the consent form) if he/she accepts to participate. This consent form must also be signed by the investigator. The original consent form must be kept in the study file by the investigator and the study participant should receive a copy.

VII.4. Confidentiality of personal data

Personal data collected and processed by study subjects will be limited to data necessary for the purpose of the study.

Adequate precautions must be taken to ensure confidentiality and comply with privacy laws and regulations when collecting and using such data. Appropriate technical and organizational measures must be in place to protect personal data from unauthorized disclosure or access, or accidental loss or alteration.

VIII. Research management and quality assurance

VIII.1. Revised plan

The principal investigator of each establishment concerned promises to conduct the clinical trial in conformity with the protocol which has been approved by the competent authority.

The principal investigator should not modify any aspect of the protocol without the approval of the proposed modifications by the competent authority.

VIII.2. Case Report Form

All case report form recording, correction, and modification must be done by the investigator or other authorized study center personnel. All case report forms and source documents supporting case report forms (e.g. Informed consent, laboratory reports, treatment history, procedure records, physical examination and treatment results, diagnosis, follow-up results) must be kept in the files of the responsible investigator for at least 5 years upon receipt of notification from the sponsor that all studies have been discontinued and completed.

VIII.3. Serious adverse event report

Fatal or life-threatening serious adverse events should be reported regardless of whether or not the study drug or the study process is causal. It is the responsibility of investigator to inform the principal investigator and the ETHICS committee of serious adverse events.

VIII.4. Data Collection

Researchers must maintain adequate and accurate records to allow for complete archiving of studies and subsequent validation of data. These documents will be divided into two distinct and independent categories: (1) investigator's study documents and (2) patient clinical source documents.

The investigator's study documentation will include protocol/revision protocol, case report and inquiry form, Ethics approval, sample informed consent, surgical records, staff history, and other relevant documents.

IX. Reference

[1] Siegel RL, Miller KD, Wagle NS, Jemal A. Cancer statistics, 2023. CA Cancer J Clin. 2023 Jan;73(1):17-48.

[2] Haddad RI, Bischoff L, Ball D, Bernet V, Blomain E, Busaidy NL, Campbell M, Dickson P, Duh QY, Ehya H, Goldner WS, Guo T, Haymart M, Holt S, Hunt JP, Iagaru A, Kandeel F, Lamonica DM, Mandel S, Markovina S, McIver B, Raeburn CD, Rezaee R, Ridge JA, Roth MY, Scheri RP, Shah JP, Sipos JA, Sippel R, Sturgeon C, Wang TN, Wirth LJ, Wong RJ, Yeh M, Cassara CJ, Darlow S. Thyroid Carcinoma, Version 2.2022, NCCN Clinical Practice Guidelines in Oncology. J Natl Compr Canc Netw. 2022 Aug;20(8):925-951.

[3] Sudoko CK, Jenks CM, Bauer AJ, Isaza A, Mostoufi-Moab S, Surrey LF, Bhatti TR, Franco A, Adzick NS, Kazahaya K. Thyroid Lobectomy for T1 Papillary Thyroid Carcinoma in Pediatric Patients. JAMA Otolaryngol Head Neck Surg. 2021 Nov 1;147(11):943-950.

[4] Xu S, Huang H, Huang Y, Qian J, Wang X, Xu Z, Liu S, Liu J. Comparison of Lobectomy vs Total Thyroidectomy for Intermediate-Risk Papillary Thyroid Carcinoma With Lymph Node Metastasis. JAMA Surg. 2023 Jan 1;158(1):73-79.

[5] van Dijk SPJ, Coerts HI, Gunput STG, van Velsen EFS, Medici M, Moelker A, Peeters RP, Verhoef C, van Ginhoven TM. Assessment of Radiofrequency Ablation for Papillary Microcarcinoma of the Thyroid: A Systematic Review and Meta-analysis. JAMA Otolaryngol Head Neck Surg. 2022 Apr 1;148(4):317-325.

[6] Chen DW, Lang BHH, McLeod DSA, Newbold K, Haymart MR. Thyroid cancer. Lancet. 2023 May 6;401(10387):1531-1544.

[7] Xiao L, Zhou J, Tan W, Liu Y, Zheng H, Wang G, Zheng W, Pei X, Yang A, Liu L. Contrast-enhanced US with Perfluorobutane to Diagnose Small Lateral Cervical Lymph Node Metastases of Papillary Thyroid Carcinoma. Radiology. 2023 May;307(4):e221465.

[8] Hyun SM, Song HY, Kim SY, Nam SY, Roh JL, Han MW, Choi SH. Impact of combined prophylactic unilateral central neck dissection and hemithyroidectomy in patients with papillary thyroid microcarcinoma. Ann Surg Oncol. 2012; 19:591–96.

[9] Kim SK, Woo JW, Lee JH, Park I, Choe JH, Kim JH, Kim JS. Prophylactic Central Neck Dissection Might Not Be Necessary in Papillary Thyroid Carcinoma: Analysis of 11,569 Cases from

a Single Institution. J Am Coll Surg. 2016; 222:853–64.

[10] Haugen BR, Alexander EK, Bible KC, Doherty GM, Mandel SJ, Nikiforov YE, Pacini F, Randolph GW, Sawka AM, Schlumberger M, Schuff KG, Sherman SI, Sosa JA, et al. 2015 American Thyroid Association Management Guidelines for Adult Patients with Thyroid Nodules and Differentiated Thyroid Cancer: The American Thyroid Association Guidelines Task Force on Thyroid Nodules and Differentiated Thyroid Cancer. Thyroid. 2016; 26:1–133

[11] Cao XJ, Liu J, Zhu YL, Qi L, Liu G, Wang HL, Wang ZH, Zhou Y, He JF, Guo JQ, Shi LL, Jian M, Shataer A, Yan GZ, Zhao ZL, Wei Y, Peng LL, Li Y, Che Y, Wang SR, Yu MA. Efficacy and Safety of Thermal Ablation for Solitary T1bN0M0 Papillary Thyroid Carcinoma: A Multicenter Study. J Clin Endocrinol Metab. 2021 Jan 23;106(2):e573-e581.

[12] Sun Y, Selvarajan S, Zang Z, et al. Artificial intelligence defines protein-based classification of thyroid nodules. Cell Discov 2022; 8(1): 85.

[13] Alseekh S, Aharoni A, Brotman Y, Contrepois K, D'Auria J, Ewald J, C Ewald J, Fraser PD, Giavalisco P, Hall RD, Heinemann M, Link H, Luo J, Neumann S, Nielsen J, Perez de Souza L, Saito K, Sauer U, Schroeder FC, Schuster S, Siuzdak G, Skirycz A, Sumner LW, Snyder MP, Tang H, Tohge T, Wang Y, Wen W, Wu S, Xu G, Zamboni N, Fernie AR. Mass spectrometry-based metabolomics: a guide for annotation, quantification and best reporting practices. Nat Methods. 2021 Jul;18(7):747-756.

[14] Wang LB, Karpova A, Gritsenko MA, Kyle JE, et al. Proteogenomic and metabolomic characterization of human glioblastoma. Cancer Cell. 2021 Apr 12;39(4):509-528.e20.

[15] Zhou T, Hu T, Ni Z, Yao C, Xie Y, Jin H, Luo D, Huang H. Comparative analysis of machine learning-based ultrasound radiomics in predicting malignancy of partially cystic thyroid nodules. Endocrine. 2024 Jan;83(1):118-126.

[16] Vanguri RS, Luo J, Aukerman AT, Egger JV, Fong CJ, Horvat N, Pagano A, Araujo-Filho JAB, Geneslaw L, Rizvi H, Sosa R, Boehm KM, Yang SR, Bodd FM, Ventura K, Hollmann TJ, Ginsberg MS, Gao J; MSK MIND Consortium; Hellmann MD, Sauter JL, Shah SP. Multimodal integration of radiology, pathology and genomics for prediction of response to PD-(L)1 blockade in patients with non-small cell lung cancer. Nat Cancer. 2022 Oct;3(10):1151-1164.

Statistical analysis plan –trial Final Version

1.Sample size justifications

The trial is designed as a diagnostic comparation trial to answer the question whether the accuracy of thyroid Artificial Intelligence (AI) is non-inferior to that in fine needle aspiration (FNA) biopsy group.

In a retrospective cohort, we perform a stratified test that the data is split into ten sets. Then, the accuracy of each layer is calculated separately. Classification accuracy is improved significantly as sample size increased initially, reaching above 70% at 349 samples, and then plateaued with a further increase of sample size.

In a prospective cohort, the sensitivity and specificity of AI are 0.96% and 89%, and the sensitivity and specificity of control group are 87% and 96%, respectively. Bilateral test A =0.05 and 90% certainty are set. The sample size is calculated according to the following sample size calculation formula:

$$n = \frac{2\bar{p}\bar{q}\left(z_\alpha + z_\beta\right)^2}{(p_1 - p_2)^2}$$

N =2000 nodules are calculated, that is, 2000 nodules are required for each diagnostic test. Meanwhile, the two diagnostic methods examine the same sample separately. Considering15%~30% loss to follow-up and rejection, at least 2351~ 2426 cases are included.

2.Statistical analyses

- **Demographic and background characteristics**

This project consists of two parts: a retrospective study and a prospective multicenter study. Data from three centers are merged and analyzed as a single prospective dataset. Demographic baseline characteristics will be summarized by a retrospective cohort and a prospective cohort. Descriptive summary statistics will be provided for continuous demographic and clinical variables. The descriptive summary statistics will include the following: number of patients, means and standard deviation for

quantitative variables, and percentages for qualitative data.

o Populations

PTC patients underwent surgery from Hangzhou Traditional Chinese Medical Hospital, Affiliated to Zhejiang Chinese Medical University. The inclusion and exclusion criteria were strictly defined as follows: Inclusion Criteria: (1) pathological confirmation of PTC. (2) Patients who underwent primary surgery accompanied by central neck lymph node dissection. (3) Tumors measuring less than 2 cm in diameter. (4) Postoperative pathological reports including detailed information on the number of lymph nodes dissected and the number of metastatic lymph nodes. (5) Availability of comprehensive preoperative thyroid ultrasound images for analysis. Exclusion Criteria: (1) Postoperative pathological diagnosis indicating sub-types of PTC. (2) Tumor invasion into adjacent anatomic structures such as the sternothyroid muscle, surrounding soft tissues, trachea, esophagus, or laryngeal nerve. (3) History of neck trauma, previous tumor surgery, or adjuvant chemoradiotherapy. (4) Fewer than three lymph nodes dissected during surgery. (5) Concurrent acute inflammatory conditions or other hematologic disorders. Preoperative plasma samples and ultrasound data were systematically collected from all participants. In the discovery set, fresh frozen tumor tissues were obtained during surgical procedures. .

o Primary efficacy analysis

The statistical analyses were conducted using R software (version 4.3.1), with the utilization of various visual representation methods such as heatmaps, volcano plots, and receiver operating characteristic (ROC) curve plots. PSM analysis was conducted to minimize the effects of potential confounders on selection bias. And PSM was performed as a 1:1 matching with a caliper value of 0.05. Differentially expressed metabolites (DEMs) and differentially expressed proteins (DEPs) were assessed utilizing the R package, with the significance threshold set at a *P*-value less than 0.05. The least absolute shrinkage and selection operator (LASSO) regression model was used on the discovery dataset training dataset for signature construction. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were computed following standardized procedures, each accompanied by a 95% Wilson confidence interval.

- **Secondary outcomes analysis**

Sensitivity and specificity will be compared between groups using a chi-square test. The larger the κ coefficient, the higher the consistency of the test method with the gold standard. Specifically, a κ coefficient ≥0.75 indicated high consistency; a κ coefficient in the range of 0.40-0.75 indicated intermediate consistency, and a κ coefficient <0.40 suggested low consistency. Ninety-five percent confidence intervals (95%CIs) are reported for κ coefficient values. Confidence intervals were generated by simulating 100 spatially random distributions with the same average molecular density as the data regions. Data were compared using an independent samples t-test. A DeLong test was used to compare the AUC between groups of diagnostic methods.