Original Article

Stepwise analysis of thyroid diagnostic modalities with genomic imprinting detection

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Background: The current preoperative malignancy risk evaluation for thyroid nodules involves stepwise diagnostic modalities including ultrasonography, thyroid function serology and fine-needle aspiration (FNA) cytopathology, respectively. We aimed to substantiate the stepwise contributions of each diagnostic step and additionally investigate the diagnostic significance of quantitative chromogenic imprinted gene in-situ hybridization (QCIGISH)—an adjunctive molecular test based on epigenetic imprinting alterations.

Methods: A total of 114 cytopathologically-diagnosed and histopathologically-confirmed thyroid nodules with complete ultrasonographic and serological examination records were evaluated using QCIGISH in the study. Logistic regression models for thyroid malignancy prediction were developed with the stepwise addition of each diagnostic modality and the contribution of each step evaluated in terms of discrimination performance and goodness-of-fit.

Results: From the baseline model using ultrasonography [area under the receiver operating characteristics curve (AUROC): 0.79; 95% confidence interval (CI): 0.71–0.86], significant improvements in thyroid malignancy discrimination were observed with the stepwise addition of thyroid function serology (AUROC: 0.82; 95% CI: 0.74–0.90; P=0.23) and FNA cytopathology (AUROC: 0.88; 95% CI: 0.81–0.94; P=0.02), respectively. The inclusion of QCIGISH as an adjunctive molecular test further advanced the preceding model's diagnostic performance (AUROC: 0.95; 95% CI: 0.91–1.00, P=0.007).

Conclusions: Our study demonstrated the significant stepwise diagnostic contributions of standard clinical

assessments in the malignancy risk stratification of thyroid nodules. However, the addition of molecular imprinting detection further enabled a more accurate and definitive preoperative evaluation especially for morphologically indeterminate thyroid nodules and cases with potentially discordant results among standard modalities.

Keywords: Indeterminate thyroid nodule; molecular test; epigenetics; genomic imprinting; quantitative chromogenic imprinted gene in-situ hybridization (QCIGISH)

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Introduction

Thyroid nodules are prevalent in the general adult population (1), but the vast majority of these nodules represent benign lesions with only 5% clinically diagnosed as malignant (2). Although most thyroid cancers tend to behave more indolently with a relatively slower development, certain cases will subsequently become invasive and metastasize (3). Therefore, an accurate preoperative diagnosis of biologically significant cancers to enable timely clinical intervention for high-risk patients, while avoiding unnecessary diagnostic testing and potential thyroid surgery in low-risk individuals with benign diseases, is particularly essential. To manage the risk assessment for thyroid nodules, a combination of different post-clinical evaluation diagnostic modalities including ultrasonography, thyroid function serology, fine-needle aspiration (FNA) cytopathology and supplementary molecular tests are recommended by current clinical guidelines (4-6).

As a non-invasive approach for the initial clinical assessment of thyroid nodules, several ultrasonographic reporting systems have been proposed, among which, the American College of Radiology Thyroid Imaging, Reporting and Data System (ACR TI-RADS) is the most widely used (7). According to certain morphological characteristics including solid composition, hypoechogenicity, microcalcification, nonparallel orientation and irregular margins, ACR TI-RADS classify thyroid nodules into five categories with an increasing risk of malignancy: 1, benign; 2, not suspicious; 3, mildly suspicious; 4, moderately suspicious; and 5, highly suspicious (7). Suspicious nodules, with sizes above the specific criteria defined individually for categories 3 to 5, are recommended for FNA for a more definitive diagnosis (4,5).

As an endocrine gland, the aberrant serum levels of thyroid secreted hormones such as tri-iodothyronine (T3) and thyroxine (T4) can provide clinical insights into diagnosing thyroid disorders (8,9). The relationships between elevated levels of serum free thyroxine (FT4) with increased risks of thyroid malignancy have been well documented (10-12). Inconsistent free T3 (FT3) levels have also been reported in thyroid cancers (13-16). Although, certain studies have shown that increased FT4/FT3 ratio could be a more reliable malignancy predictor for thyroid nodules (17-19). Despite being important risk factors for malignancy, the FT4/FT3 ratio results do not provide a conclusive diagnosis for thyroid cancer.

Highlight box

Key findings

- Given the preliminary thyroid malignancy discrimination obtained from ultrasonography [area under the receiver operating characteristics curve (AUROC): 0.79], improvements in diagnostic contributions were significant when thyroid function serology (AUROC: 0.82) and FNA cytopathology (AUROC: 0.88) were stepwise added. Including quantitative chromogenic imprinted gene in-situ hybridization (QCIGISH) as an adjunctive molecular test for the standard clinical assessments further enhanced the preceding model's diagnostic performance (AUROC: 0.95).

What is known and what is new?

- Recent studies have highlighted how genomic imprinting biomarkers through QCIGISH significantly advanced early thyroid cancer detection by identifying subtle molecular changes associated with carcinogenesis.
- This research demonstrated the stepwise diagnostic contribution of QCIGISH to standard modalities in malignancy risk stratification of thyroid nodules which has not been previously explored.

What is the implication, and what should change now?

- A collective clinical malignancy risk assessment using QCIGISH in addition to standard diagnostic modalities may prove useful for improving the overall preoperative evaluation of thyroid nodules, and their subsequent therapeutic management.

FNA cytopathology uses morphological evidences for the preoperative diagnosis of thyroid nodules. Under the Bethesda classification system, thyroid nodules are classified into six categories with an increasing risk of malignancy: Bethesda I, non-diagnostic or unsatisfactory; Bethesda II, benign; Bethesda III, atypia of undetermined significance or follicular lesion of undetermined significance; Bethesda IV, follicular neoplasm or suspicious for a follicular neoplasm; Bethesda V, suspicious for malignancy; and Bethesda VI, malignant (20). The Bethesda III to V categories, comprising approximately 20–30% of thyroid nodules, are considered indeterminate predominantly due to unclear morphology (21,22). The clinical dilemma from inconclusive cytopathological diagnoses can be further aggravated by the potentially inconsistent results from earlier diagnostic steps including suspicious findings from prior ultrasound or thyroid function tests. For these atypical and suspicious results from cytopathology, patients with truly benign thyroid nodules are potentially subjected to unwarranted surgical interventions with lifelong repercussions, while patients with truly malignant thyroid nodules might remain undiagnosed thereby increasing the risk for distant metastasis.

To address such clinical predicament, the American Thyroid Association has suggested the molecular profiling of thyroid nodules with indeterminate cytopathology as supplement diagnostic methods (23), including BRAF V600E mutation (24,25), Thyroseq™ gene sequencing (26,27), Afirma GEC or GSC™ gene expression classifier (28) and mir-THYtype™ microRNA (miRNA) classifier (29). In addition to these molecular tests, the quantitative chromogenic imprinted gene in-situ hybridization (QCIGISH) methodology has previously been reported to directly visualize and quantitatively assess epigenetic imprinting alterations in clinical specimens obtained from ten cancer types, including thyroid cancer (30). Genomic imprinting is an epigenetic regulatory mechanism that controls the single-allelic expression of imprinted genes via allele-specific methylation and long non-coding RNAs (lncRNAs) (31,32). Regular genomic imprinting plays an important role in mammalian embryo development, while aberrant expressions of imprinted genes are related to tumorigenesis (32,33). Through this *in-situ* hybridization (ISH)-based approach, the expression sites of imprinted genes have been visualized and the aberrant expressions quantified including their biallelic, multiallelic and total expressions. The previous research demonstrated the differential epigenetic imprinting signatures for cancers, predominantly observed with elevated aberrant allelic expressions, which were significantly different as compared with those obtained from benign lesions and normal controls. As a molecular profiling test based on epigenetic imprinting alterations which precede morphological changes, QCIGISH could provide clearer malignancy differentiation for thyroid nodules (30,34,35). However, validation studies are necessary to independently evaluate the diagnostic contribution of QCIGISH as an adjunctive molecular test in further improving the capabilities of existing clinical assessments for stratifying malignancy risks in thyroid nodules (36).

In this study, we aimed to substantiate the stepwise contributions of each diagnostic modality, namely ultrasonography, thyroid function serology and FNA cytopathology in thyroid malignancy prediction as similarly explored in literature (37,38). However, as molecular testing of FNA biopsy specimens continue to gain acceptance as a substantial advancement in the accurate clinical diagnosis of indeterminate thyroid nodules (39-41), we also investigated the potential diagnostic significance of QCIGISH, a molecular test based on epigenetic imprinting alterations previously reported as efficient cancer biomarkers for the diagnosis of morphologically indeterminate thyroid nodules, adjunctive to standard clinical diagnostic procedures. Using a cohort of surgical patients, we formulated a univariate baseline model using the ultrasonographic results as predictor, being the first diagnostic step for risk-stratifying thyroid malignancy. All other subsequent diagnostic factors were individually and sequentially considered—thyroid function serology, FNA cytopathology and molecular imprinting detection through QCIGISH, respectively, into the multivariate logistic regression model with the objective of evaluating the additive contribution of each diagnostic step in the ability to accurately predict thyroid cancer. We present this article in accordance with the STROBE reporting checklist (available at https://cco.amegroups.com/article/view/10.21037/cco-23-89/rc).

Methods

Patients

Patients with thyroid nodules detected from ultrasound examination who were recommended for fine-needle aspiration biopsy (FNAB), driven by suspicious findings from ultrasonography, nodule growth, elevated clinical risk factors or patient preference, were consecutively

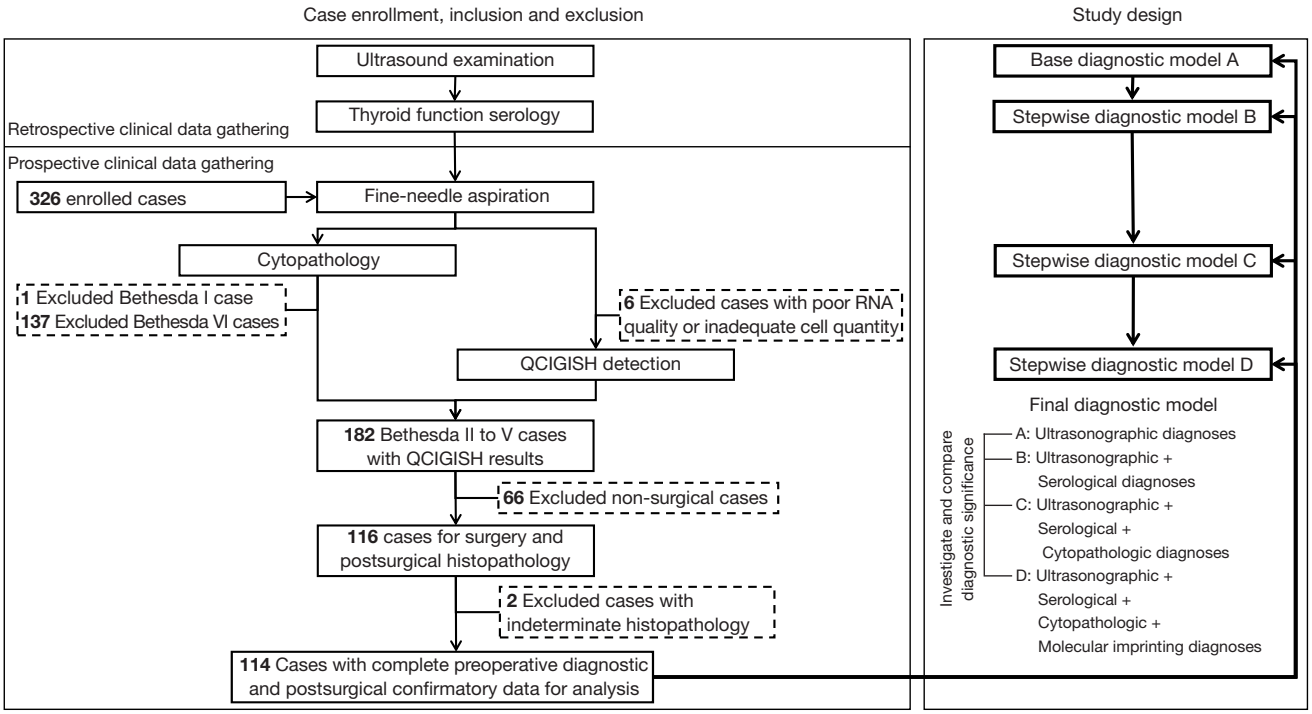

Figure 1 Study design and workflow diagram. QCIGISH, quantitative chromogenic imprinted gene in-situ hybridization.

recruited from Taizhou People's Hospital, Taizhou Third People's Hospital, Shanghai Tenth People's Hospital of Tongji University School of Medicine and Jiangyuan Hospital Affiliated to Jiangsu Institute of Nuclear Medicine between May 21, 2019 and Jan 5, 2021 following a defined list of study inclusion and exclusion criteria (Appendix 1, *Figure 1*). Patients with complete clinical, ultrasonographic and serological diagnostic information gathered retrospectively; and preoperative cytopathologic, QCIGISH detection and postsurgical histopathologic evaluation results obtained prospectively following the procedures described under Appendix 1 and Figure S1 were included in the analysis. Histopathological diagnoses were reviewed by three independent pathologists (Rulong Shen, Wenbin Huang and Hongyu Yu). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committees of Taizhou People's Hospital and Taizhou Third People's Hospital (approval No. TZ20190520), Shanghai Tenth People's Hospital (approval No. SHSY-IEC-4.1/19-6/01) and Jiangyuan Hospital Affiliated to Jiangsu Institute of Nuclear Medicine (approval No. YL201811). Informed consent was obtained from all individual participants.

Sample size calculation applied the equation used for evaluating diagnostic accuracy studies involving binormal receiver operating characteristics (ROC) curve indices with ordinal discrete categories as response criterion (42). Under an assumption of a 75% ratio between the negative and positive groups, a minimum sample size of 35 from the positive group and 26 from the negative group achieves 81% power to detect a difference of at least 0.25, 0.20 and 0.15 between the AUROC values under the null hypothesis of 0.70, 0.75 and 0.80 (hypothesized for the ultrasonographic, serological or cytopathologic assessments considered as existing standards) and an AUROC under the alternative hypothesis of 0.95 (hypothesized for the imprinting assessment considered as the new test under evaluation) using a two-sided Z-test at a significance level of 0.05. Accounting for a 20% dropout rate, at least 75 cases will be gathered for the study. The sample size calculation was estimated using PASS V.21.0.3 (NCSS, Kaysville, Utah, USA).

Stepwise diagnostic model development and validation

Factors representing the diagnostic steps identified in the study, namely ultrasonography, thyroid function serology, FNA cytopathology and molecular imprinting detection

through QCIGISH were preprocessed and transformed accordingly, as described under Supplement: Materials and Methods. Serving as model predictors, all were initially entered into a univariate analysis to assess their potential association towards thyroid malignancy *a priori*. The endpoint of interest was the postsurgically determined histopathological diagnosis for each nodule. Using a baseline model involving the ultrasonographic factor, other diagnostic factors including thyroid function serology, FNA cytopathology and molecular imprinting detection were then sequentially included in subsequent multivariate logistic regression models to evaluate the odds of developing thyroid malignancy. The area under the receiver operating characteristics curve (AUROC) and various goodness-of-fit assessment measures were determined to evaluate if the addition of each diagnostic step to the prior model resulted in a statistically significant improvement in thyroid malignancy discrimination and model fit, respectively.

Statistical analysis

Continuous variables such as age and thyroid function serological test measurements were reported as medians with interquartile ranges (IQR). Frequencies and proportions were used to describe categorical variables including sex and the various malignancy risk categories for ultrasonography, thyroid function serology, FNA cytopathology and molecular imprinting detection. Driven by the non-normal distributions determined using the Shapiro-Wilk test (43), differences between the benign and malignant groups for continuous clinical variables were evaluated using the Mann-Whitney *U* test. Categorical clinical variables for the benign and malignant cases were analyzed and compared using Chi-squared or Fisher exact tests, as applicable.

Odds ratios and their 95% confidence intervals (CIs) were reported for the univariate and multivariate logistic regression models. In analyzing the diagnostic contributions of each individual predictive factor in the stepwise regression models, likelihood ratio test was performed to measure the drop in residual deviance for the current model against the prior model, with the hypothesized significance evaluated using Chi-squared test. Akaike information criterion (AIC) and McFadden's R^2 (44) were used as additional model goodness-of-fit measures. The variance inflation factor (VIF) metric (45) was used to assess for potential multicollinearity among the predictor variables in the diagnostic models.

The discrimination performance of the diagnostic models was assessed using the AUROC metric with 95% CI determined using the DeLong method. Optimism-adjusted AUROC values were estimated to correct against potential overfitting using a 500-cycle internal bootstrap validation method (46).

All hypothesis tests conducted were two-sided, with computed $P<0.05$ considered to be statistically significant. All statistical analyses and visualizations were performed using R software (version 3.5.0) (47).

Results

Patient characteristics

A total of 326 consecutive cases of thyroid nodules with complete medical records for clinical, ultrasonographic and thyroid function serological examinations which were assessed for FNA cytopathology were recruited for the study. Each thyroid FNA specimen was divided for simultaneous cytopathology evaluation and blinded QCIGISH testing. From this initial number, 212 cases were subsequently excluded including 1 Bethesda I case, 137 Bethesda VI cases, 6 with poor RNA quality or inadequate cell quantity for QCIGISH testing, 66 non-surgical cases and 2 with indeterminate cytopathology—with details illustrated under *Figure 1*. The remaining 114 thyroid nodules (34.97%) which had valid and complete results in FNA cytopathology, molecular imprinting detection and thyroid postsurgical histopathology were considered eligible for the analysis. 69.29% (79/114) of the cases were evaluated as cytopathologically indeterminate which were classified under Bethesda III to V, with the remaining 30.70% (35/114) having been risk-stratified as benign under Bethesda II. The clinicopathological characteristics of the patient cohort are summarized in *Tables 1*,*2* with supplementary exploratory analysis results provided in Figures S2,S3.

The median age at diagnosis was 50 years (IQR, 36–62 years) with female predominance (71.05% *vs.* 28.95%; female to male ratio, 2.5:1). Overall, 50 patients were diagnosed with histopathologically benign nodules [including 27 nodular goiter (NG), 7 follicular thyroid adenoma (FTA), 6 adenomatous goiter, 5 Hashimoto's thyroiditis (HT), 2 adenoma, 2 thyroiditis and 1 goiter with adenoma], and 64 patients were diagnosed with thyroid cancer [including 47 papillary thyroid carcinoma (PTC), 12 papillary thyroid microcarcinoma (PTMC), 3 follicular thyroid carcinoma (FTC), 1 medullary thyroid carcinoma (MTC) and

Table 1 Case information

Patient information	Overall (n=114)	Benign (n=50)	Malignant (n=64)	P
Clinical characteristics				
Sex, n (%)				0.30†
Male	33 (28.95)	12 (24.00)	21 (32.81)	
Female	81 (71.05)	38 (76.00)	43 (67.19)	
Age (years), median [IQR]	50 [36–62]	58 [47–64]	41 [33–52]	<0.001‡
Ultrasonographic assessment				
ACR TI-RADS, n (%)				<0.001†
Category 2	14 (12.28)	13 (26.00)	1 (1.56)	
Category 3	20 (17.54)	18 (36.00)	2 (3.12)	
Category 4	22 (19.30)	12 (24.00)	10 (15.63)	
Category 5	58 (50.88)	7 (14.00)	51 (79.69)	
Serological assessment				
FT4 (pmol/L), median [IQR]	16.7 [15.4–18.6]	15.9 [14.7–17.2]	17.3 [16.2–19.7]	<0.001‡
FT3 (pmol/L), median [IQR]	5.1 [4.6–5.5]	5.0 [4.6–5.4]	5.1 [4.6–5.6]	0.40‡
FT4/FT3 ratio, median [IQR]	3.3 [3.0–3.7]	3.2 [2.9–3.5]	3.5 [3.1–3.8]	0.01‡
Cytopathologic assessment				
Bethesda classification, n (%)				<0.001†
Category II	35 (30.70)	33 (66.00)	2 (3.12)	
Category III	24 (21.05)	10 (20.00)	14 (21.88)	
Category IV	11 (9.65)	3 (6.00)	8 (12.50)	
Category V	44 (38.60)	4 (8.00)	40 (62.50)	
Imprinting assessment				
QCIGISH classification, n (%)				<0.001†
Grade 0	20 (17.54)	20 (40.00)	0	
Grade I	24 (21.05)	23 (46.00)	1 (1.56)	
Grade II	8 (7.02)	2 (4.00)	6 (9.38)	
Grade III	40 (35.09)	5 (10.00)	35 (54.69)	
Grade IV	22 (19.30)	0	22 (34.38)	
Postsurgical histopathologic diagnosis				<0.001†
Benign, n (%)				
Nodular goiter	27 (23.68)	27 (54.00)		
Follicular thyroid adenoma	7 (6.14)	7 (14.00)		
Adenomatous goiter	6 (5.26)	6 (12.00)		
Hashimoto's thyroiditis	5 (4.39)	5 (10.00)		
Adenoma	2 (1.75)	2 (4.00)		

Table 1 (*continued*)

Table 1 (*continued*)

Patient information	Overall (n=114)	Benign (n=50)	Malignant (n=64)	P
Thyroiditis	2 (1.75)	2 (4.00)		
Goiter with adenoma	1 (0.88)	1 (2.00)		
Malignant, n (%)				
Papillary thyroid carcinoma	47 (41.23)		47 (73.44)	
Papillary thyroid microcarcinoma	12 (10.53)		12 (18.75)	
Follicular thyroid carcinoma	3 (2.63)		3 (4.69)	
Medullary thyroid carcinoma	1 (0.88)		1 (1.56)	
Papillary/follicular thyroid carcinoma	1 (0.88)		1 (1.56)	

Baseline clinical characteristics and diagnostic information of the patient cohort (n=114). †, categorical variables were compared using Chi-squared or Fisher's exact tests, as applicable. ‡, continuous variables were compared between the benign and malignant cases using the Mann-Whitney *U* test. All hypothesis tests applied were two-tailed. Computed P values of less than 0.05 were considered as significant. IQR, interquartile ranges; FT4, free thyroxine; FT3, free triiodothyronine; ACR TI-RADS, American College of Radiology Thyroid Imaging, Reporting and Data System; QCIGISH, quantitative chromogenic imprinted gene in-situ hybridization.

Table 2 Malignancy rates by diagnostic modality

Diagnostic modality	Overall (n=114)	Benign (n=50)	Malignant (n=64)	P
Ultrasonographic assessment				
ACR TI-RADS, n (%)				<0.001†,‡
Categories 2, 3	34 (29.82)	31 (62.00)	3 (4.69)	
Categories 4, 5	80 (70.18)	19 (38.00)	61 (95.31)	
Serological assessment				
FT4/FT3 level, n (%)				0.02†,§
Low	54 (47.37)	30 (60.00)	24 (37.50)	
High	60 (52.63)	20 (40.00)	40 (62.50)	
Cytopathologic assessment				
Bethesda classification, n (%)				<0.001†,¶
Categories II, III, IV	70 (61.40)	46 (92.00)	24 (37.50)	
Category V	44 (38.60)	4 (8.00)	40 (62.50)	
Imprinting assessment				
QCIGISH classification, n (%)				<0.001†,††
Grades 0, I	44 (38.60)	43 (86.00)	1 (1.56)	
Grades II, III, IV	70 (61.40)	7 (14.00)	63 (98.44)	

Proportions of benign and malignant cases across the different diagnostic modalities evaluated for the patient cohort (n=114). †, categorical variables were compared using Chi-squared or Fisher's exact tests, as applicable. Computed P values of less than 0.05 were considered as significant. ‡, ACR TI-RADS categories were combined prior to hypothesis testing (combined Categories 2 and 3 were tested against combined Categories 4 and 5). §, FT4/FT3 ratio values were dichotomized into low (≤3.3) and high (>3.3) levels. ¶, Bethesda categories were combined prior to hypothesis testing (combined Categories II, III and IV were tested against Category V). ††, QCIGISH grades were combined prior to hypothesis testing (combined Grades 0 and I were tested against combined Grades III, IV and V). FT4, free thyroxine; FT3, free triiodothyronine; ACR TI-RADS, American College of Radiology Thyroid Imaging, Reporting and Data System; QCIGISH, quantitative chromogenic imprinted gene in-situ hybridization.

1 combined PTC and FTC]. A malignancy rate of 56.14% was noted from the study.

Malignancy rates across ultrasonographic, serological, cytopathologic and imprinting assessments for thyroid malignancy risk evaluation

Increasing malignancy rates were observed across the transformed binary categorical predictors for the ultrasonographic, serological, cytopathologic and imprinting factors prior to logistic regression model development (*Table 1*, Figure S2). 8.82% (3/34) of the combined ACR TI-RADS categories 2 and 3 were confirmed malignant, while that determined for the combined categories 4 and 5 was 76.25% (61/80). Malignancy rates for the low and high FT4/FT3 ratio levels were 44.44% (24/54) and 66.67% (40/60), respectively. Increasing proportions of histopathologically malignant cases were similarly observed for the combined Bethesda categories II, III and IV at 34.28% (24/70) and Bethesda category V at 90.91% (40/44). QCIGISH classification demonstrated malignancy rates of 2.27% (1/44) for combined Grades 0 and I, and 90.00% (63/70) for combined Grades II, III and IV. Since these grades already represent the actual QCIGISH-positive and QCIGISH-negative categories, diagnostic performance estimates were determined as follows: 98.44% sensitivity (95% CI: 95.40–100.00%), 86.00% specificity (95% CI: 76.38–95.62%), 90.00% positive predictive value (PPV) (95% CI: 82.97–97.03%) and 97.72% negative predictive value (NPV) (95% CI: 93.32–100.00%).

Additive stepwise contributions of ultrasonographic, serological, cytopathologic and imprinting assessments for thyroid malignancy risk evaluation

Univariate logistic regression applied on the clinical diagnostic evaluation factors showed that imprinting (AUROC: 0.92; 95% CI: 0.87–0.97; optimism-adjusted AUROC: 0.91), cytopathologic (AUROC: 0.77; 95% CI: 0.70–0.84); optimism-adjusted AUROC: 0.76) and ultrasonographic (AUROC: 0.79; 95% CI: 0.71–0.86; optimism-adjusted AUROC: 0.78) factors demonstrated superior discrimination performance as compared to the serological (AUROC: 0.61; 95% CI: 0.52–0.70; optimism-adjusted AUROC: 0.60) factor (P<0.001 to P=0.003) (*Table 2* and Table S1, *Figure 2*). All modalities were however independent predictors of thyroid malignancy with statistically significant odds ratios as determined for the ultrasonographic [odds ratio (OR): 33.18; 95% CI: 10.42–149.44; P<0.001], serological (OR: 2.50; 95% CI: 1.18–5.41; P=0.02), cytopathologic (OR: 19.17; 95% CI: 6.76–69.58; P<0.001) and imprinting (OR: 387.00; 95% CI: 68.66–7,413.33; P<0.001) factors (*Table 3*).

With the ultrasonographic factor used as the baseline model [AUROC: 0.79 (95% CI: 0.71–0.86), optimism-adjusted AUROC: 0.78], multivariate logistic regression for predicting thyroid malignancy was formulated and stepwise regressed on the serological, cytopathologic and imprinting factors, respectively, reflecting the actual sequential clinical utility of these diagnostic evaluations (*Table 3*). Each added factor improved the stepwise model's AUROC to 0.82 (95% CI: 0.74–0.90; P=0.23; optimism-adjusted AUROC: 0.80), 0.88 (95% CI: 0.81–0.94; P=0.02; optimism-adjusted AUROC: 0.86) and 0.95 (95% CI: 0.91–1.00; P=0.007; optimism-adjusted AUROC: 0.93), respectively (Table S1, *Figure 2*).

Different sets of factors were determined as independent predictors of thyroid malignancy for each stepwise regression implemented in the study as shown on *Table 3*. However, for the final model which combined all four factors simultaneously into the model, only imprinting remained statistically significant (OR: 195.45; 95% CI: 29.63–4,098.60; P<0.001).

Goodness-of-fit measures applied on all four stepwise models showed generally decreasing AIC (112.00, 112.69, 103.63 and 58.54, respectively) and increasing McFadden's R^2 (0.31, 0.32, 0.39 and 0.69, respectively) with the best fit identified for the final model (*Table 4*).

These findings demonstrated the significant stepwise diagnostic contributions of standard clinical assessments in the malignancy risk stratification of thyroid nodules. However, the addition of molecular imprinting detection may further enable a more accurate and definitive preoperative evaluation to improve clinical management.

Adoption of molecular imprinting detection findings into ultrasonographic, serological and cytopathologic results collectively improved thyroid malignancy risk assessment

To illustrate, four representative cases were presented on *Figure 3*. Case 1 involved moderately suspicious findings for thyroid cancer from ultrasound examination (ACR TI-RADS category 4) with normal serum FT4/FT3 ratio level and benign FNA cytopathology (Bethesda II)—representing discordant assessments without molecular imprinting detection. However, thyroid malignancy risk was identified

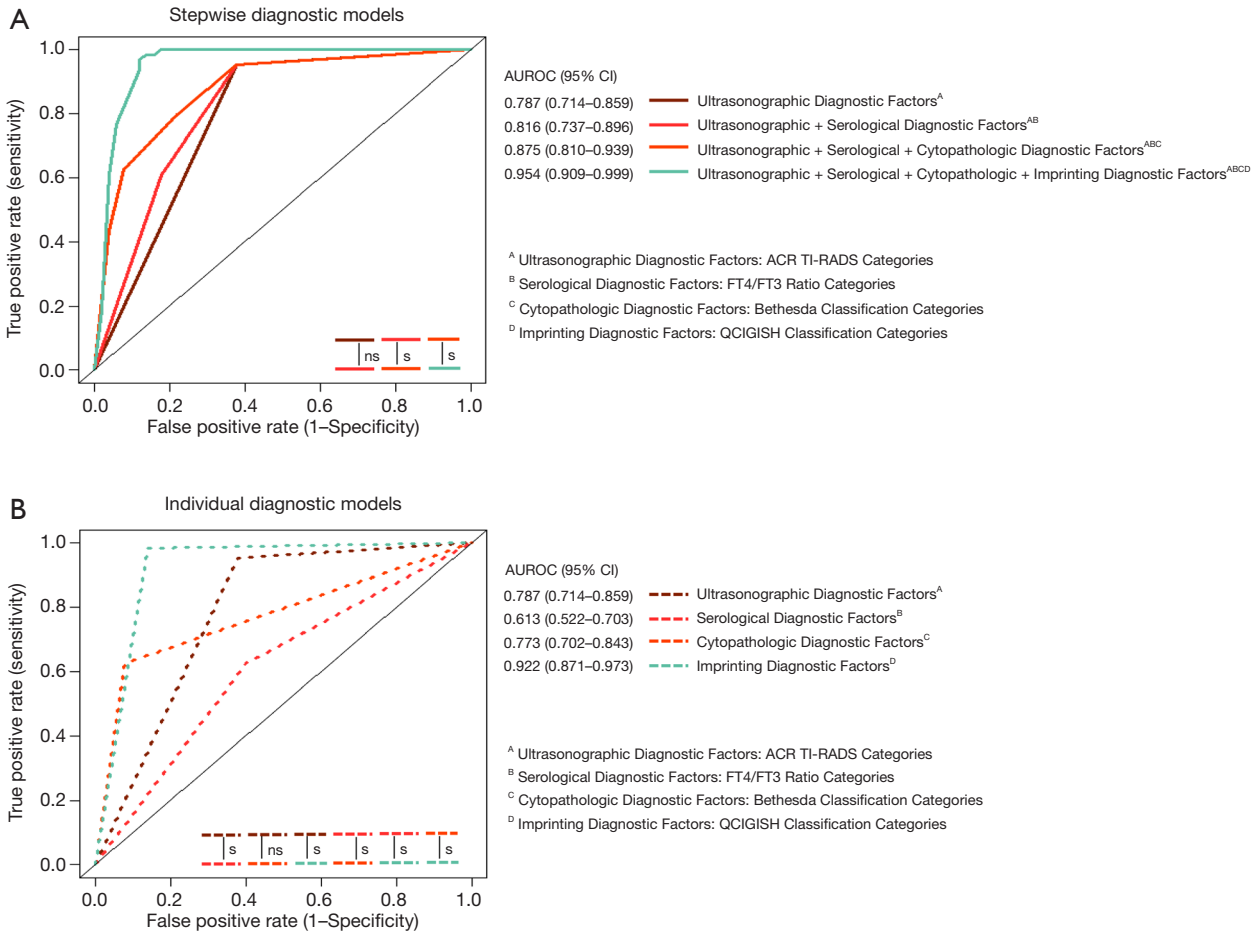

Figure 2 Comparison of the AUROC between the respective stepwise and individual diagnostic models. (A) ROC curves of stepwise diagnostic models. (B) ROC curves of individual diagnostic models. s, statistically significant difference in AUROC observed between the given pair of diagnostic models; ns, no statistically significant difference in AUROC observed between the given pair of diagnostic models. AUROC, area under the receiver operating characteristics curve; CI, confidence interval; ACR TI-RADS, American College of Radiology Thyroid Imaging, Reporting and Data System; FT4, free thyroxine; FT3, free tri-iodothyronine; QCIGISH, quantitative chromogenic imprinted gene in-situ hybridization; ROC, receiver operating characteristic.

for this patient based on imprinting signatures (QCIGISH-positive). With the imprinting diagnostic information added, this case had an increased malignancy risk, which was confirmed as PTC from postsurgical histopathology. Considering the preceding modalities which involved all diagnostic factors except imprinting might have indicated a lower malignancy risk.

Case 2 was examined as moderately suspicious for thyroid cancer (ACR TI-RADS category 4) with normal serum FT4/FT3 ratio level but presurgically diagnosed as suspicious for malignancy (Bethesda V)—resulting in a high malignancy risk evaluation using only these factors in the multivariate logistic regression model. This patient was QCIGISH-negative who was identified with low malignancy risk based from molecular imprinting detection. Combining all these factors including the imprinting findings resulted to a low-risk assessment which was confirmed by postsurgical histopathology as HT. In the absence of the imprinting factor in the model, this particular case would have been mistakenly evaluated as potentially malignant.

Case 3's findings were isolated which involved a patient with ACR TI-RADS category V under ultrasound examination (highly suspicious for thyroid cancer), elevated FT4/FT3 ratio levels and Bethesda V under FNA cytopathology (highly suspicious for thyroid cancer).

Table 3 Malignancy odds of individual diagnostic modalities for thyroid nodules

Diagnostic modality	Reference category	Univariate analysis (n=114)		Stepwise multivariate analysis (n=114)							
		Model 1‡		Model A§		Model B¶		Model C††		Model D‡‡	
		OR (95% CI)	P†	OR (95% CI)	P†	OR (95% CI)	P†	OR (95% CI)	P†	OR (95% CI)	P†
Ultrasonographic assessment											
ACR TI-RADS											
Categories 4, 5	Categories 2, 3	33.18 (10.42–149.44)	<0.001	33.18 (10.42–149.44)	<0.001	30.37 (9.45–137.43)	<0.001	13.97 (4.03–66.23)	<0.001	2.45 (0.25–20.21)	0.40
Serological assessment											
FT4/FT3 ratio											
High (>3.3)	Low (≤3.3)	2.50 (1.18–5.41)	0.02	–	–	1.75 (0.67–4.57)	0.25	1.38 (0.48–3.83)	0.54	0.74 (0.13–3.50)	0.72
Cytopathologic assessment											
Bethesda classification											
Category V	Categories II, III, IV	19.17 (6.76–69.58)	<0.001	–	–	–	–	6.79 (2.13–26.43)	0.002	6.13 (1.09–52.24)	0.06
Imprinting assessment											
QCIGISH classification											
Grades II, III, IV	Grades 0, I	387.00 (68.66–7,413.33)	<0.001	–	–	–	–	–	–	195.45 (29.63–4,098.60)	<0.001

Univariate and stepwise multivariate logistic regression of malignancies in thyroid nodules using ultrasonographic, serological, cytopathologic and imprinting assessments. †, Wald's test was applied to test the significance of the individual regression coefficients. ‡, univariate logistic regression was developed separately using ultrasonographic, serological, cytopathologic, and imprinting assessments. §, base univariate logistic regression was developed using ultrasonographic assessment. ¶, stepwise multivariate logistic regression was developed using ultrasonographic and serological assessments. ††, stepwise multivariate logistic regression was developed using ultrasonographic, serological, and cytopathologic assessments. ‡‡, stepwise multivariate logistic regression was developed using ultrasonographic, serological, cytopathologic, and imprinting assessments. OR, odds ratio; CI, confidence interval; FT4, free thyroxine; FT3, free triiodothyronine; ACR TI-RADS, American College of Radiology Thyroid Imaging, Reporting and Data System; QCIGISH, quantitative chromogenic imprinted gene in-situ hybridization.

Table 4 Model fit assessment of the stepwise diagnostic models

Diagnostic model	AIC††	McFadden's $R^{2‡‡}$	Residual deviance	LRT§§	P¶¶
Ultrasonographic†	112.00	0.31	108.00	NA	NA
Ultrasonographic† + serological‡	112.69	0.32	106.69	1.31	0.25
Ultrasonographic† + serological‡ + cytopathologic§	103.63	0.39	95.63	11.06	<0.001
Ultrasonographic† + serological‡ + cytopathologic§ + imprinting¶	58.54	0.69	48.54	47.09	<0.001

Evaluation of the goodness of fit for the stepwise logistic regression models involving ultrasonographic, serological, cytopathologic and imprinting diagnoses. †, ACR TI-RADS categories used as ultrasonographic diagnostic factors. ‡, FT4/FT3 ratio categories used as serological diagnostic factors. §, Bethesda classification categories used as cytopathologic diagnostic factors. ¶, QCIGISH classification categories used as imprinting diagnostic factors. ††, lower computed AIC values indicate a better model fit. ‡‡, higher computed McFadden's R^2 values indicate a better model fit. §§, LRT values represent the drop in residual deviance for the current model with respect to the prior model. ¶¶, Chi-squared test was used to test the significance between the likelihood under the current model against the prior model. AIC, Akaike information criterion; LRT, likelihood ratio test; FT4, free thyroxine; FT3, free triiodothyronine; ACR TI-RADS, American College of Radiology Thyroid Imaging, Reporting and Data System; QCIGISH, quantitative chromogenic imprinted gene in-situ hybridization.

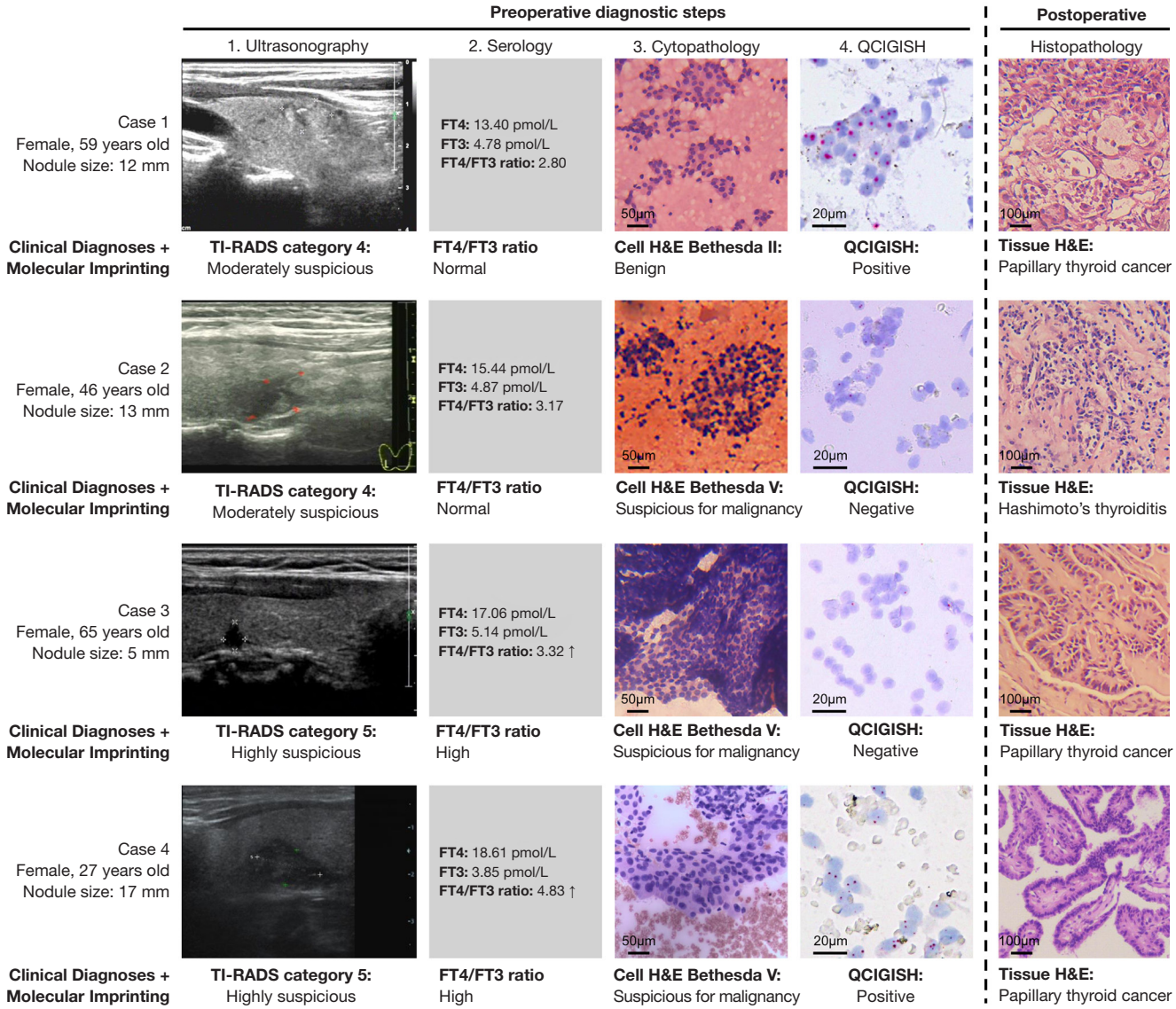

Figure 3 Case scenarios comparing sonographic, serologic, cytopathologic and imprinting modalities for thyroid cancer risk assessment. Cellular structures and tissue morphology for the preoperative cytopathology and post-operative histopathology specimens were visualized using H&E staining. QCIGISH, quantitative chromogenic imprinted gene in-situ hybridization; H&E, hematoxylin and eosin staining; TI-RADS, Thyroid Imaging, Reporting and Data System; FT4, free thyroxine; FT3, free tri-iodothyronine.

QCIGISH detection was however negative. This case was confirmed PTC by surgical histopathology. Despite the discordant results from QCIGISH, the physicians were able to give a definitive malignant diagnosis based on the consistently high malignancy risk findings from ultrasonography, serology and cytopathology. For instance, when preoperative assessments using standard modalities remain coherent, the malignancy risk evaluation becomes more reliable making adjunctive molecular tests like QCIGISH optional.

Case 4 showed a patient with ACR TI-RADS category V under ultrasound examination (highly suspicious for thyroid cancer), elevated FT4/FT3 ratio levels, Bethesda V under FNA cytopathology (highly suspicious for thyroid cancer) and positive QCIGISH detection. Despite the concordant results from QCIGISH to the assessments made using

ultrasonography, serology and cytopathology, the consistent preoperative evaluation using standard modalities were sufficient to stratify the malignancy risk for this case which may not require further confirmation from an adjunctive molecular test based on clinical guidelines.

Discussion

A thorough and accurate preoperative assessment of thyroid nodules which enables the ability to effectively distinguish between malignancy and benignity is critical in achieving the best therapeutic implications for patients. Despite the collective risk information gathered from standard thyroid diagnostic tests including ultrasonography, serology and FNA biopsy, the management of approximately 20–30% of thyroid nodules which are cytopathologically indeterminate with highly varying malignancy risks ranging from 5% to 75%, or cases with discordant preoperative assessment using standard diagnostic modalities, remains a clinical challenge (21,22). Avoiding overtreatment in low-risk patients while informing effective diagnostic interventions for high-risk patients often require incorporating additional risk factors into clinical decision-making including a more definitive guidance from molecular markers, among others (4,5). Molecular profiling, such as imprinting detection in particular, therefore has the potential to drive better clinical decisions in the setting of indeterminate thyroid nodules by guiding both the need for and extent of thyroid surgery.

In the present study, we particularly investigated a set of predominantly cytopathologically indeterminate thyroid nodules which continue to be a diagnostic challenge in terms of interpretation and clinical management. FNAB examination, which is solely based on morphological features, may be unable to optimally provide definitive diagnoses in most of these cases. We consolidated all related diagnostic information predictive of thyroid malignancy using the ultrasonographic, thyroid function serological, FNA cytopathologic and imprinting detection factors, which were all sequentially applied based in the particular order of clinical practice. With postsurgical histopathology applied as the diagnostic gold standard, multivariate regression models were formulated from a stepwise combination of all these predictive factors. Our results showed that each diagnostic step has additively contributed in the accurate prediction of thyroid malignancy. The final diagnostic model incorporating all factors demonstrated high discrimination power with an AUROC of 0.95. Although the inclusion of the ultrasonographic, serological and cytopathologic assessments have already significantly improved model performance, QCIGISH—an imprinting-based molecular test further elevated malignancy discrimination demonstrating its clinical value in effectively differentiating benign lesions from thyroid cancers, especially in indeterminate cases.

Thyroid cancer, being an indolent and slow progressing type of cancer, poses a different set of challenges during clinical management as both under-treatment and over-treatment have implications (4). Although a considerable number of protein biomarkers have been discovered to further improve the accuracy of diagnosing thyroid nodules (48-52), these markers are usually only detected through immunohistochemistry on tissue resections obtained postsurgically. For preoperative risk assessment, several molecular tests on FNA cytology samples have been developed, including BRAF V600E mutation which is specific for papillary thyroid cancer (24); RNA sequencing-based Afirma GSC^{TM} gene expression classifier as a good rule-out test (28); and Thyroseq^{TM} genomic classifier and mir-THYtype^{TM} miRNA classifiers for both rule-in and rule-out testing (27,29), despite several noted limitations. In PTCs which account for 80–85% of all thyroid cancers, the frequency of BRAF V600E mutation varies from 29% to 83% among different cohorts, sample sizes and detection methods (24,53-56). Considering other subtypes of thyroid cancer with rare BRAF V600E mutation, more than 30% of thyroid cancer patients who may not harbor BRAF V600E mutation will not be effectively diagnosed with malignancy using this particular molecular test. In addition, next generation sequencing methods including Afirma GSC^{TM}, Thyroseq^{TM} and mir-THYtype^{TM}, have yet to improve their technical feasibility (29) to optimize their continued clinical application.

Conceptually different from these aforementioned molecular tests, QCIGISH uses an epigenetics-based approach. Instead of detecting methylation and lncRNAs which regulate the expression of imprinted genes, a previously reported ISH-based methodology targeting the intronic regions of nascent RNAs was applied to detect the gene expression sites in the nuclei (30). Particularly using this experimental method, the allelic expression status of imprinted genes in individual cells can be visually assessed and quantified. Based from the imprinting signatures, a predictive model which effectively differentiated thyroid malignancy from benign lesions using five grades (Grades 0, I, II, III and IV) was previously developed with increasing malignancy risks (30). The QCIGISH grades

were subsequently grouped into two categories to facilitate diagnostic performance assessment—QCIGISH-negative (Grades 0 and I) and QCIGISH-positive (Grades II, III and IV), representing benign and malignant predictions, respectively. As epigenetic alterations usually occur at early stages of carcinogenesis and precede cellular morphological changes (57), QCIGISH showed excellent diagnostic sensitivity and specificity from a previous study (35). In an independent prospective validation, QCIGISH detection demonstrated 100.00% sensitivity, 91.45% specificity, 100.00% NPV and 96.52% PPV for presurgical FNA specimens (35). Particularly for the Bethesda III, IV and V cases with indeterminate FNA cytopathology, QCIGISH also showed high NPV of 100.00%, PPV of 96.55% and overall accuracy 97.53% (35). With high NPV and PPV, QCIGISH can be used as an excellent rule-in and rule-out diagnostic tool and could help the diagnosis of the indeterminate nodules.

From the stepwise model developed in this study, we accumulatively added the QCIGISH classification categories with the results of prior standard clinical diagnostic evaluations for thyroid nodules. QCIGISH can provide an additional risk stratification method with high diagnostic accuracy to supplement standard diagnostic modalities in thyroid clinical assessment. As an adjunctive molecular test, QCIGISH will be most helpful in further evaluating the likelihood of malignancy particularly for cases with either an indeterminate cytopathology or had discordant assessment results among different modalities which commonly cause a diagnostic dilemma for physicians during clinical management. A collective clinical malignancy risk assessment using QCIGISH in addition to standard diagnostic modalities including ultrasonography, serology and cytopathology may prove useful for improving the overall preoperative evaluation of thyroid nodules, and their subsequent therapeutic management. Powered by ISH-based technology, QCIGISH also presents an accurate, functional and immediately feasible thyroid molecular diagnostic solution for clinical applications as compared to sequencing-based methods.

We recognize that this study had several limitations. The study was limited by a small sampling of patients who have chosen to undergo thyroid surgery in a major referral cancer center. The inclusion of a purely surgical cohort introduces a selection bias as reflected in the high malignancy rate of 56.14% in the study. This likely limits the use of our results within a community setting with a potentially lesser proportion of malignant cases. Additional insights and a more conclusive validation on the utility of the study could also be obtained from a prospective large-scale evaluation involving more medical centers and higher patient case numbers with more diverse clinical characteristics and disease subtypes.

Conclusions

Our study demonstrated the significant stepwise diagnostic contributions of ultrasonography, thyroid function serology and FNA cytopathology in the malignancy risk assessment of thyroid nodules. However, the addition of molecular imprinting detection through QCIGISH complemented and further improved the collective diagnostic contributions obtained from standard clinical risk-assessment procedures, further enabling a more accurate and definitive preoperative evaluation and medical management, especially for morphologically indeterminate thyroid nodules and cases with potentially discordant results among standard modalities.

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Footnote

Reporting Checklist: The authors have completed the STROBE reporting checklist. Available at https://cco.amegroups.com/article/view/10.21037/cco-23-89/rc

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://cco.amegroups.com/article/view/10.21037/cco-23-89/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all

aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committees of Taizhou People's Hospital and Taizhou Third People's Hospital (approval No. TZ20190520), Shanghai Tenth People's Hospital (approval No. SHSY-IEC-4.1/19-6/01) and Jiangyuan Hospital Affiliated to Jiangsu Institute of Nuclear Medicine (approval No. YL201811). Informed consent was obtained from all individual participants.

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Appendix 1 Materials and methods

Study inclusion and exclusion criteria

The inclusion criteria defined for the study cohort included the following: (I) cases clinically advised for fine-needle aspiration (FNA) with complete medical records from ultrasound examination and thyroid function serology for free thyroxine (FT4) and free tri-iodothyronine (FT3); (II) patients with nodules classified under Bethesda III to V who received surgery owing to local compressive symptoms due to large nodule size, substernal goiter, nodule growth or patient preference; and (III) patients who have not previously received partial or total thyroidectomy. Due to the considerable proportion of malignant histopathology findings observed from patients who opted for surgical intervention despite the benign diagnoses from FNA cytopathology (Bethesda II), these cases were also included as part of the study cohort.

Cases with the following characteristics were excluded from the study: (I) specimen evaluated with poor RNA quality or inadequate number of cells for quantitative chromogenic imprinted gene *in-situ* hybridization (QCIGISH) detection; (II) non-diagnostic cytopathology (Bethesda) I or determinate cytopathology assessed with 97–99% malignancy risk (Bethesda VI); (III) cases not recommended for surgery or refusal to undergo surgical treatment; and (IV) indeterminate postsurgical histopathology.

Ultrasound examination

All ultrasound examinations were performed using a 9–15 MHz linear-array probe (LOGIQ E9, GE Healthcare, Wauwatosa, WI, USA; EPIQ7, Philips Healthcare, Bothell, WA, USA; Aplio 500, Canon Medical Systems, Tokyo, Japan; iU22, Philips Healthcare, Bothell, WA, USA) by experienced radiologists in thyroid imaging and reviewed by two of the authors (Y. Zhang and H. Wu). The main ultrasound features which predicted the probability of thyroid malignancy, including echogenicity, composition, margin, shape and echogenic foci, as outlined by American College of Radiology Thyroid Imaging, Reporting and Data System (ACR TI-RADS) were recorded (7). Points were subsequently assigned for each ultrasound feature for the individual nodule.

Thyroid function serology test

The FT3 and FT4 measurements were obtained using Beckman Coulter UniCel DxI 800 Access Immunoassay System (Beckman Coulter, Boston, MA, USA). The reference normal ranges applied were 2.8–6.3 pmol/L for FT3 and 10.5–24.4 pmol/L for FT4.

Fine-needle aspiration cytopathology

FNA was performed on nodules with relatively high-grade ACR TI-RADS categories and other clinical risk indications. All FNAs were conducted by experienced radiologists under ultrasound guidance. The samples were obtained and smeared onto glass slides with 95% alcohol. The biopsy samples were immediately analyzed by pathologists and reported according to the Bethesda system (20). Patients with Bethesda II thyroid nodules were treated following the American Association of Endocrine Surgeons Guidelines stating that these cases can be safely observed, and that surgery might be considered for cases associated with significant local compressive symptoms due to large nodule size (>3 cm), or per the preference of the patient (5).

QCIGISH detection

For each patient, the same thyroid FNA specimen was divided into two parts for simultaneous cytopathology evaluation and blinded QCIGISH testing. *In-situ* hybridization (ISH) was performed following the procedure previously described (30). Briefly, samples were fixed immediately after sampling in 10% neutral buffered formalin (NBF) for 48 hours at room temperature. The dissociated cells were directly mounted onto positively charged slides. After sample pretreatment, the ISH was performed using probes targeting the non-coding intronic regions of nascent RNAs from small nuclear ribonucleoprotein polypeptide N (SNRPN) and minor histocompatibility antigen H13 (HM13) following the manual instruction of RNAscope 2.5 HD Assay kit (Advanced Cell Diagnostics, Newark, CA, USA) (58). After signal amplification, the detected gene-expressing site appeared as a distinct red or brown dot under common bright field microscope (*Figure S1A*). Data collected from

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microscopic images were used to determine the biallelic expression (BAE), multiallelic expression (MAE) and total expression (TE) according to the equations shown in *Figure S1B*. The QCIGISH detection results were classified into five grades (Grades 0, I, II, III and IV) with the diagnostic grading model development process detailed from a previous thyroid diagnosis study (35) using various sensitivity targets representative of progressive thyroid malignancy risks. Grade 0 indicated a benign result while grade I suggested a possible but low malignancy potential, with both being classified as QCIGISH-negative. Grades II, III and IV were all considered QCIGISH-positive, indicating low, moderate and high malignancy risks, respectively. QCIGISH-negative and QCIGISH-positive classifications represent minimal and elevated aberrant allelic expressions corresponding to low and high malignancy risks, respectively.

Model predictor variable pre-modeling transformation

Predictor variables used in the study to model thyroid malignancy include relevant factors, namely ultrasonography, thyroid function serology, FNA cytopathology and molecular imprinting detection through QCIGISH, which directly represent the diagnostic procedures in the order these are implemented in the clinic. To simulate the process of clinical diagnosis, these factors were transformed into binary categories, as applicable, and modeled against postsurgically confirmed benign and malignant thyroid cases, both individually and collectively, in sequential combination depending on how these diagnostic steps are clinically administered (*Figures S2*,*S3*).

For the ultrasonographic factor, the risk-stratification categories determined using ACR TI-RADS involving categories 2 (not suspicious), 3 (mildly suspicious), 4 (moderately suspicious) and 5 (highly suspicious) were applied. Since the malignancy risks for ACR TI-RADS categories 2 and 3 were relatively low (<2% and 5%, respectively) as compared to categories 4 and 5 (5–20% and >20%, respectively) (1), these categories were aggregated into two levels consisting of category 2 and 3 (assigned as the reference category) against categories 4 and 5 combined.

The serological factors identified for the study, which included the biochemical serum markers FT4 and FT3 for thyroid hormone status were similarly transformed prior to inclusion as predictor variables for model development. The ratios of the serum FT4 and FT3 measurements were determined, as this factor has been similarly reported as an effective indicator for thyroid cancer (17). The range of values for the computed FT4/FT3 ratio was dichotomized into high and low categories using a threshold equal to 3.3 based from a related study (17). As the risk for thyroid malignancy has been associated with higher FT4/FT3 ratio, a low FT4/FT3 level was used as the reference category for the model.

The FNA cytopathology examination results categorized under the Bethesda system consisting of Bethesda II (benign cytopathology), Bethesda III (atypia of undetermined significance or follicular lesions of undetermined significance), Bethesda IV (follicular neoplasm or suspicious for a follicular neoplasm) and Bethesda V (suspicious for malignancy) were used to represent the cytopathologic factor for the model. The categories were transformed from four to two levels. Combined Bethesda II, III and IV categories (relatively low malignancy risks) were used as the reference category and evaluated against the Bethesda V classification (relatively high malignancy risk).

For the imprinting factor, the QCIGISH measurements were stratified into QCIGISH-negative (Grades 0 and I) and QCIGISH-positive (Grades II, III and IV) categories as described from a previous study (35), representing low and high malignancy risks, respectively. The QCIGISH-negative category was assigned as the reference category.

References

58. Wang F, Flanagan J, Su N, *et al.* RNAscope: a novel *in situ* RNA analysis platform for formalin-fixed, paraffin-embedded tissues. J Mol Diagn 2012;14:22-9.

https://dx.doi.org/10.21037/cco-23-89

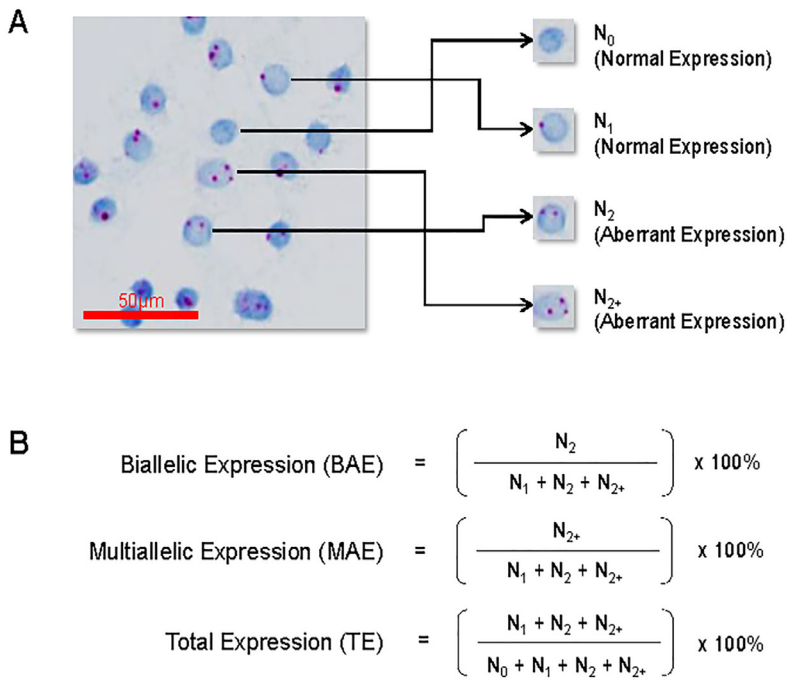

Figure S1 QCIGISH visualization and quantification of the allelic expression status for imprinted genes. (A) QCIGISH staining showing different imprinted gene expression status in cell nuclei; (B) formulas for calculating BAE, MAE and TE measurements. QCIGISH, quantitative chromogenic imprinted gene in-situ hybridization; BAE, biallelic expression; MAE, multiallelic expression; TE, total expression.

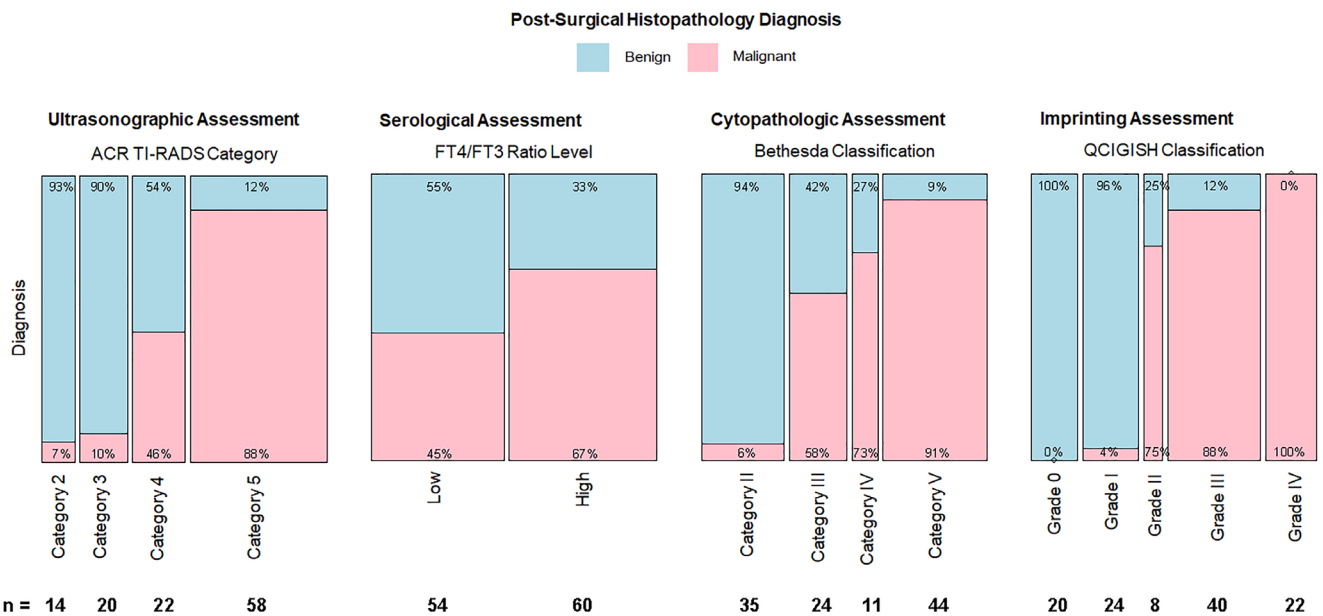

Figure S2 Raw categorical predictors for the ultrasonographic, serological, cytopathologic and imprinting factors prior to logistic regression model development. ACR TI-RADS, American College of Radiology Thyroid Imaging, Reporting and Data System; FT4, free thyroxine; FT3, free tri-iodothyronine; QCIGISH, quantitative chromogenic imprinted gene in-situ hybridization.

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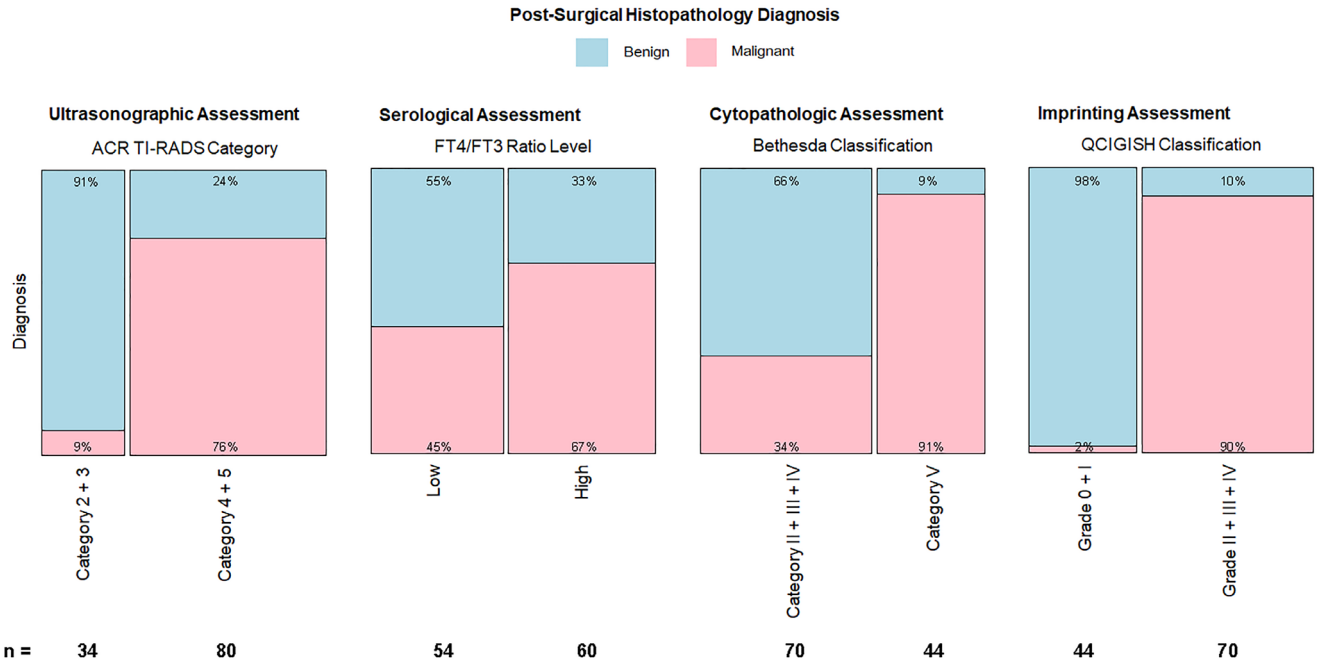

Figure S3 Transformed binary categorical predictors for the ultrasonographic, serological, cytopathologic and imprinting factors prior to logistic regression model development. ACR TI-RADS, American College of Radiology Thyroid Imaging, Reporting and Data System; FT4, free thyroxine; FT3, free tri-iodothyronine; QCIGISH, quantitative chromogenic imprinted gene in-situ hybridization.

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Table S1 AUROC comparison of the stepwise and individual diagnostic models

Diagnostic model assessment	AUROC (95% CI)
Individual diagnostic model	
Utrasonographic[†] (A)	0.787 (0.714 to 0.859)
Serological[‡] (B)	0.613 (0.522 to 0.703)
Cytopathologic[§] (C)	0.773 (0.702 to 0.843)
Imprinting[¶] (D)	0.922 (0.871 to 0.973)
P values[††]	
A *vs.* B	0.001
A *vs.* C	0.75
A *vs.* D	<0.001
B *vs.* C	0.003
B *vs.* D	<0.001
C *vs.* D	<0.001
Stepwise diagnostic model	
Ultrasonographic[†] (E)	0.787 (0.714 to 0.859)
Ultrasonographic[†] + serological[‡] (F)	0.816 (0.737 to 0.896)
Ultrasonographic[†] + serological[‡] + cytopathologic[§] (G)	0.875 (0.810 to 0.939)
Ultrasonographic[†] + serological[‡] + cytopathologic[§] + imprinting[¶] (H)	0.954 (0.909 to 0.999)
P values[††]	
E *vs.* F	0.23
F *vs.* G	0.02
G *vs.* H	0.007

AUROC of the different diagnostic models were compared using the [††]DeLong's test for paired ROC curves. [†], ACR TI-RADS categories used as ultrasonographic diagnostic factors. [‡], FT4/FT3 ratio categories used as serological diagnostic factors. [§], Bethesda classification categories used as cytopathologic diagnostic factors. [¶], QCIGISH classification categories used as imprinting diagnostic factors. AUROC, area under the receiver operating characteristics curve; CI, confidence interval; ACR TI-RADS, American College of Radiology Thyroid Imaging, Reporting and Data System; FT4, free thyroxine; FT3, free tri-iodothyronine; QCIGISH, quantitative chromogenic imprinted gene in-situ hybridization.

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