Retrospective Analysis of Cancer-Specific Gene Expression Panel for Thyroid Fine Needle Aspiration Specimens.

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Research Article

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Abstract

Background: While molecular testing is a promising strategy for preoperative assessment of cytologically indeterminate thyroid nodules, thyroid fine needle aspiration biopsy (FNA) presents unique challenges for molecular assays, including contaminating peripheral blood mononuclear cells (PBMC) and variable numbers of evaluable epithelial thyroid cells. Moreover, the newly recognized entity, noninvasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP), has added an additional challenge to the currently available molecular diagnostic platforms. New diagnostic tools are still needed to correctly distinguish benign and malignant thyroid nodules preoperatively.

Methods: Twenty-two transcript splice variants from 12 genes we previously identified as discriminating benign from malignant thyroid nodules were characterized in 80 frozen thyroid tumors from 8 histological subtypes. Isoforms detectable in PBMC were excluded, and the 5 most discriminating isoforms were further validated by real-time quantitative PCR (qPCR) on intraoperative FNA samples from 59 malignant tumors, 55 benign nodules, and 23 NIFTP samples. The qPCR threshold cycle values for each transcript were normalized to the thyrocyte-specific thyroid peroxidase isoform 1 (TPO1) and z-transformed. Receiver operating characteristic (ROC) analyses of the composite transcript scores were used to evaluate classification of thyroid FNAs by the 5-gene isoform expression panel.

Results: A molecular signature was developed by combining expression levels of specific isoforms of CDH3, FNDC4, HMGA2, KLK7, and PLAG1. FNAs containing at least 12-36 thyrocytes were sufficient for this assay. The 5-gene composite score achieved an area under the ROC curve (AUC) of 0.86 for distinguishing malignant from benign nodules, with a specificity of 91%, sensitivity of 75%, negative predictive value of 91% and positive predictive value of 74%.

Conclusion: Our newly developed 5-gene isoform expression panel distinguishes benign from malignant thyroid tumors and, may help distinguish benign from malignant thyroid nodules in the context of the new NIFTP subtype.

Background

Thyroid cancer incidence is rapidly increasing worldwide. In the United States, its prevalence has nearly quadrupled over the past 15 years, predominantly due to the increased incidence of papillary thyroid carcinoma (PTC) (1). Thyroid nodules are detectable by ultrasound in over 50% of the adult population, and fine needle aspiration (FNA) cytopathology is the most accurate means of pre-operative diagnosis. However, up to 30% of the FNA samples result in indeterminate cytology: atypia of undetermined significance/follicular lesion of undetermined significance (Bethesda III), follicular neoplasm/suspicious for a follicular neoplasm (Bethesda IV), and suspicious for malignancy (Bethesda V) (2), demonstrating inherent limitations of visual microscopic diagnosis. The situation has been further complicated by the recent reclassification in the American Thyroid Association management guidelines for thyroid tumors of a previously considered malignant subgroup of follicular variant of papillary thyroid carcinoma (FVPTC)

to the noninvasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP), an indolent neoplasm with questionable malignant potential (3). NIFTP cannot be differentiated from invasive FVPTC by cytopathology, however, and requires histopathologic evaluation for diagnosis (2, 4).

In the last decade, molecular testing has emerged as a promising strategy for the preoperative assessment of thyroid nodules to enable clinicians to better tailor surgical interventions and avoid overtreatment of benign disease. ThyroSeq v3 next generation sequencing (CBL Path, Rye Brook, NY) (5) Afirma gene expression with recently upgraded gene sequencing classifiers (Veracyte, South San Francisco, CA)(6), and ThygenX/ThyraMIR gene mutation and miRNA analysis (Interpace Diagnostics, Parsippany, NJ) (7) are major molecular tests currently in clinical use in the United States. The DNA-based tests, however, are limited by the prevalence of several of the assessed mutations in benign thyroid lesions (8, 9), and all tests suffer from a lack of specificity after the introduction of the newly defined NIFTP subtype (10–13). Indeed, both ThyroSeq and Afirma tests currently report NIFTP as “positive” or “suspicious” for malignant disease. In a recent study, the positive predictive value (PPV) of ThyroSeq mutational analysis panels (including 7-gene ThyroSeq and ThyroSeq V2) decreased from 43–14% and Afirma gene expression classifier from 30–25% (14), if NIFTP was classified as non-malignant.

Before the introduction of the NIFTP subtype, our group performed a transcriptome microarray analysis of 125 tumor samples representing the most common epithelial thyroid tumor diagnoses: adenomatoid nodules (AN), follicular adenomas (FA), Hürthle cell adenomas (HA), follicular carcinomas (FC), Hürthle cell carcinomas (HC), FVPTC, and PTC, and identified over 75 transcripts that were differentially expressed between benign and malignant tumors (15). In a follow-up study, we further characterized 14 of these transcripts by a combination of immunohistochemical and quantitative reverse transcription-PCR assays, and identified a candidate 3-gene panel as a potential preoperative diagnostic tool for FNA samples (16).

In the current study, we selected 12 of the 14 genes based on their diagnostic performance in a receiver operating characteristic (ROC) analysis, to determine if newly characterized specific isoforms further improved their diagnostic performance, including cases of the pathologically confirmed NIFTP tumor subtype. We also investigated isoforms of thyroglobulin and thyroid peroxidase (TPO) to identify a thyrocyte-specific load control, since, in contrast to qualitative markers such as mutations, quantitative molecular assessments are complicated by highly variable samples and admixture of non-thyroid derived cells like peripheral blood mononuclear cells (PBMC). Finally, we tested our 5 best performing candidate isoforms directly in intra-operatively obtained FNA samples.

Materials And Methods

Clinical samples

Under Institutional Review Board approval, thyroid tumor tissue, intraoperative FNA, and blood specimens were collected from patients undergoing thyroid surgery at Johns Hopkins Hospital. Intraoperative FNAs

were collected from the tumor with a 25-gauge needle syringe immediately prior to resection and preserved in 95% Ethanol at -20°C, or in RNALater at -80°C. The FNA site was marked intraoperatively to ensure correlation with the final pathological diagnosis. During pathological prosection, an aliquot of tumor tissue was snap frozen in liquid nitrogen and stored at -80°C. Tumor tissue was identified by hematoxylin and eosin staining of frozen tissue sections, and final surgical pathological diagnoses of samples were confirmed by a pathologist. All FVPTCs were re-reviewed to allow reclassification of NIFTPs from FVPTCs where indicated. PBMCs were isolated from patient blood drawn intraoperatively with Ficoll-Paque Plus (GE Healthcare) and stored at -80°C.

RNA isolation and reverse transcription

Total RNA from 80 frozen thyroid tumors (15 ANs, 14 FAs, 10 HAs, 5 NIFTPs, 7 FVPTCs, 7 HCs, 7 FCs, and 15 PTCs) and PBMCs from 31 thyroid patients were isolated with Trizol (Invitrogen) and RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. cDNA was synthesized by reverse transcription with 500 ng of total RNA using SuperScript III reverse transcriptase (Invitrogen). Total RNA from single pass FNA samples was isolated using GenElute Single Cell RNA Purification Kit (Sigma-Aldrich) following the manufacturer's instructions. Because of the variable number of thyroid cells in individual FNA samples, the entire total RNA elution volume (11 µl) was used for cDNA synthesis.

PCR of thyroid tumor tissue and PBMC samples

Each PCR assay was performed with 5% of total cDNA using Platinum *Taq* DNA polymerase (Invitrogen) following the manufacturer's instructions. Amplified DNA was analyzed by agarose gel electrophoresis (see below) for 22 isoforms from 12 genes, CEACAM6, CDH3, DIRAS3, DPP4, FNDC4, HMGA2, KLK7, MRC2, SFN, c-KIT, PRSS3, and PLAG1. Additionally, 4 isoforms of thyroglobulin and thyroid peroxidase (TPO) were assessed as potential thyrocyte-specific load control on solid thyroid tumor and PBMC samples. Glyceraldehyde-3-phosphate dehydrogenase (GapDH, NM_002046) served as a total RNA load control.

Real-time quantitative PCR (qPCR) on FNA samples

Selected target and reference gene isoforms were tested by real-time qPCR on 159 FNA samples from 6 confirmed surgical histological subtypes of thyroid tumors; AN, FA, HA, NIFTP, FVPTC, and PTC. (See supplemental Table S1 for PCR primer sequences.) Gene expression was quantitated in duplicate using 5% of total cDNA in each assay and Power SYBR Green Master Mix (Applied Biosystems) on a Bio-Rad iQ5 thermal cycler for 40 cycles. The Ct values of the duplicates were averaged. Serial dilutions of frozen tumor RNA were used to establish the threshold of detectability of the selected thyrocyte-specific reference gene in the assay, and an amplification threshold cycle (Ct) value > 30 was chosen to exclude sample from further analysis for lack of sufficient thyrocytes

Data analysis

For the initial selection of candidate gene isoforms on frozen tumor tissue samples, semi-quantitative densitometry using BioRad Quantity One image analysis software was used to select the isoforms

showing the highest levels of differential expression between cancer and non-cancer samples on agarose gel electrophoresis images. Expression levels of each isoform were assessed by normalizing to the RNA load control gene GapDH and then z-transformed, so all genes could be assessed on the same scale.

For the subsequent qPCR analysis of FNA samples, the relative expression of each target gene was calculated with respect to the reference load control gene TPO1 [$\Delta Ct = Ct\ (target) - Ct\ (reference)$] and then z-transformed for assessment. When a target gene was undetectable after 40 cycles, the sample was assigned the maximum observed ΔCt value + 5% of the standard deviation of the ΔCt values observed for that gene across all samples.

The ability of the candidate genes to distinguish between malignant and benign thyroid tumor subtypes was evaluated using ROC analysis. Overall performance was measured as the AUC.

We then applied Bayes Rule to estimate the positive predictive value (PPV) and negative predictive value (NPV) from our observed sensitivity and specificity using the tumor prevalence rates reported by Steward *et al* (17).

The overall workflow of the study in shown in supplemental Figure S1.

Results

Candidate gene isoform selection by reverse transcription-PCR using tumor tissues and PBMCs

The 22 selected transcript isoforms were characterized on frozen tumor tissue samples from 15 ANs, 14 FAs, 10 HAs, 5 NIFTPs, 7 FVPTCs, 7 HCs, 7 FCs, and 15 PTCs, using semi-quantitative PCR. Five transcript isoforms from the following genes: CDH3, FNDC4, HMGA2, KLK7, PLAG1 showed the most differential expression among thyroid cancers, NIFTPs, and benign tumors (Table 1, Fig. 1), as determined by image analysis of agarose gel electrophoresis of the PCR products. Importantly, none of the isoforms were detectable in PBMCs from 31 patients (Figure S2). Among the candidate thyrocyte load-control isoforms of TPO and thyroglobulin tested, TPO1 was the only isoform not detectable in PBMCs (Figure S3) and stably expressed across the well-differentiated thyroid tumor subtypes (Fig. 1a). Therefore, TPO1 was selected as the load control for thyroid cell content of FNA samples.

Table 1
Gene transcript variants selected

Symbol	Reference	Gene name	Isoform
CDH3	NM_001793.5	Cadherin 3	transcript variant 1
FNDC4	NM_022823.2	Fibronectin type III domain containing 4	transcript variant 1
HMGA2	NM_003483.4	High mobility group AT-hook 2	transcript variant 1
KLK7	NM_005046.3	Kallikrein related peptidase 7	transcript variant 1
PLAG1	NM_002655.2	PLAG1 zinc finger	transcript variant 1
TPO1	NM_000547.5	Thyroid peroxidase	transcript variant 1

One recurrent metastatic HC showed markedly reduced TPO1 levels (Fig. 1a). No FNA samples of this case were available for testing, however.

Validation of the 5 candidate gene isoforms on an independent cohort of FNA samples

Seven (4.4%) of the 159 FNA samples tested had no detectable TPO1 expression with 40 qPCR cycles and, were therefore excluded from the study. Serial dilutions of thyroid tissue-derived RNA revealed that TPO1 was detectable at 30 qPCR cycles using a minimum of 370 pg of total RNA, which corresponds to the total RNA of approximately 12–36 cells (18), lower than the cytopathological threshold of minimal thyrocyte content in FNA samples (6 clusters of at least 10 follicular epithelial cells on 2 or more slides) (19). We therefore selected a TPO1 threshold Ct value of 30 to include FNA samples for our study, a criterion met by a total of 137 of 159 (86.2%) FNA samples tested. There was no significant difference in the fail rates across the diagnostic subgroups tested. Table 2 summarizes the patient information for the FNAs used.

Table 2
Patient information of the FNA study cohort

	AN	FA	HA	NIFTP	FVPTC	PTC
Sample size, *n*	25	16	14	23	34	25
Sex, M/F	8/17	4/12	3/11	7/16	5/29	9/16
Age, years	48.1 (27–68)	46.1 (18–74)	53.6 (29–80)	50.1 (27–72)	44.4 (19–76)	41.5 (18–59)
Nodule size, cm	3.2 (0.8–7.8)	2.8 (1.0-7.5)	3.1 (1.1-7.0)	2.9 (0.9-8.0)	2.5 (0.6-6.0)	2.4 (0.6-5.0)
Bethesda I, *n*	1					
Bethesda II, *n*	11	3		3	4	
Bethesda III, *n*	5	1		12	7	1
Bethesda IV, *n*	7	10	13	2	8	1
Bethesda V, *n*	1			4	9	5
Bethesda VI, *n*		1		2	6	18
Indeterminate cytology*, *n*		1	1			

AN, adenomatoid nodule; FA, follicular adenomas; HA, Hürthle cell adenoma; NIFTP, noninvasive follicular thyroid neoplasm with papillary-like nuclear features; FVPTC, follicular variant of papillary thyroid carcinoma; PTC, papillary thyroid carcinoma.

*Clinical FNA obtained before current Bethesda classification available.

The TPO1-normalized ΔCt data were z-transformed to create a z-ΔCt score for the expression of each target gene. Figure 2 shows the expression profiles of the selected CDH3, FNDC4, HMGA2, KLK7, PLAG1 isoforms and the composite z-ΔCt score in the 6 thyroid tumor subtypes tested (AN, FA, HA, NIFTP, FVPTC, and PTC). Each individual isoform showed higher expression in malignant thyroid tumors, than in benign. When summing the 5 transcripts, the composite z-ΔCt score exhibited a differential profile among thyroid tumors (Fig. 2f).

An ROC analysis (Fig. 3a) was used to evaluate the ability of our 5-gene isoform expression panel to differentiate benign tumors (AN, FA, HA, and NIFTP, $n = 78$) from malignant tumors (FVPTC and PTC, $n = 59$). Overall performance was measured as the AUC, which was 0.86. Several combinations of sensitivity and specificity, representing points along the ROC curve, are shown in Table 3 as well. One of these combinations is highlighted on the ROC curve and on the strip-plots in Fig. 2. Corresponding to a threshold for the composite expression score (z-ΔCt) of -1, this value was chosen to maximize sensitivity (75%) while controlling specificity above 90% (actual value = 91%). Forty-four of 59 (74.6%) malignant

thyroid tumors had a composite score > -1, (23/25, 92.0% of PTCs; 21/34, 61.8% of FVPTCs), while only 7 out of 78 (9.0%) benign and NIFTP nodules had a score > -1 (6/23, 26.1% of NIFTPs; 1/14, 7.1% of HAs; 0/16 of FAs; and 0/25, 0% of ANs; $p < 0.0001$).

Table 3
Performance of the 5-transcript panel in benign *vs.* malignant FNAs

Sensitivity, %	Specificity, %	PPV, %	NPV, %
78	76	53	91
75	81	58	90
75	86	65	91
75	91	74	91
61	96	85	88
19	100	100	78

Table 4

Performance of the 5-transcript panel in differentiating benign *vs.* malignant follicular lesions or NIFTP *vs.* malignant FNAs

NIFTP, HA, and FA *vs.* FVPTC		NIFTP *vs.* FVPTC and PTC	
Sensitivity, %	Specificity, %	Sensitivity, %	Specificity, %
59	75	69	78
56	81	68	83
53	87	63	87
44	91	58	91
29	96	46	96
9	100	19	100

We used the prevalence rates from a previous large multicenter study (17) to apply Bayes Rule to estimate the NPV and PPV from our observed sensitivity and specificity, resulting in an NPV of 91% and a PPV of 74% (Table 3). Variations of this calculation, assuming malignant sample prevalence rates ranging from 20–30%, are shown in Supplemental Table S2.

The panel also significantly differentiated the NIFTPs from the malignant PTCs and FVPTCs (26.1% of NIFTPs versus 74.6% of cancers with scores > -1, $p = 0.0002$). Further, the comparison between NIFTP

versus invasive FVPTC showed a statistically significant separation ($p < 0.05$).

Discussion

In the past decade, molecular testing has emerged as a promising method to increase the accuracy of the preoperative diagnosis of malignant thyroid tumors. Several molecular diagnostic tests, including RNA based gene expression and multi-panel mutation genotyping analysis are commercially available for clinical use (17, 20). Available tests remain, however, limited by relatively low specificity and PPV (21, 22). Furthermore, the recent reclassification of a subgroup of malignant FVPTC to clinically “benign” NIFTP has further complicated the situation, since prior publications assessing the performance of commercially available molecular tests were based upon NIFTP being categorized as malignant. Indeed, most NIFTPs were reported as suspicious/malignant by expression based Afirma or mutation and gene fusion based ThyroSeq in several studies (14, 21, 23, 24).

Our group previously carried out a series of studies to identify genetic markers for distinguishing cancer from benign thyroid tumors using genome-wide gene expression arrays (15, 16). In this study, we have developed a molecular test for evaluating preoperative thyroid FNAs taking NIFTP lesions into consideration, by further characterizing isoforms of our previously profiled gene candidates.

Taking advantage of the improved annotation of the human genome over the last decade, we first explored expression of a broad range of splice variants of our 12 gene set in frozen thyroid tumor samples to select candidates which were differentially expressed in benign and malignant neoplasms. In this study, the 5 isoforms of CDH3, FNDC4, HMGA2, KLK7, and PLAG1 we identified showed potential in differentiating different thyroid tumor subtypes, and importantly, were chosen because they were not expressed in PBMCs. In FNA samples, quantitative molecular tests must address the contribution from PBMCs. Positive or negative selection using antibody-coated magnetic beads can minimize their contribution, but their use decreases overall assay sensitivity (data not shown). In the absence of selection, the load control reference gene needs to reflect the number of thyrocytes in FNA samples rather than a standard total RNA content measure. In our study, we tested two isoforms of TPO and two of thyroglobulin. Only TPO1 was found to have constant levels of expression across differentiated thyroid tumor subtypes (Fig. 1a) and importantly, was undetectable by PCR after 40 cycles in any of the 31 PBMC samples obtained perioperatively (Figure S3). We did observe, however, a decrease in TPO1 expression in one large HC metastasis, possibly a consequence of loss of differentiation in this recurrent advanced tumor.

The 5-isoform panel was tested using our intraoperative FNA samples. In this study, only a single pass of needle aspirate from each tumor was used for our analysis. To ensure the reliability of the assay, only samples reaching a TPO1 threshold of detectability of 30 cycles or less were included in this study. Only 7 (4.4%) of FNA samples showed no detectable TPO1, and 137 (86.2%) of 159 FNA samples met our threshold and produced reliable gene expression profiles. This compares favorably with reported 2–20% of cases yielding cytopathologically non-diagnostic results with two to five FNA passes (25).

In our study, quantitative PCR data generated from 137 qualified FNA samples demonstrated a differential expression among benign and malignant thyroid tumors. The ROC analysis shows our 5-isoform panel has an 86% ability to distinguish benign thyroid tumors (AN, FA, and HA) and NIFTP from malignant tumors (FVPTC and PTC), with a specificity of 91% at a sensitivity of 75%, resulting in an NPV of 91% and a PPV of 74%.

The thyroid follicular-patterned lesions, FA, NIFTP, and FVPTC, contribute the most to the indeterminate cytologies. Molecularly, they all are frequently associated with RAS mutations (26). Currently, histologic evaluation of capsule and vascular invasion is necessary for diagnosis of NIFTP. Thus, an accurate diagnosis of NIFTP is impossible by preoperative cytology or mutation based molecular tests. Nevertheless, NIFTP is an indolent lesion with < 1% risk of recurrence (27), and should not be treated as thyroid cancer, although it may warrant resection as potential premalignant lesion.

The NIFTP classification is new. Currently, the reclassified indolent NIFTP is still considered as a surgical disease by most endocrinologists. Its separation from malignant FVPTC may significantly impact clinical treatment decisions, leading to lesser surgical and other ablative procedures, and potentially simple observation as therapeutic options, which are currently under active investigation. More studies, especially prospective long-term follow-up studies, are needed to evaluate its behavior, progression, and optimal management. Finding tools for accurate preoperative identification of NIFTP will promote the study and management for this lesion. The data presented here show our newly developed 5-isoform panel may reduce cytologically and molecularly indeterminate diagnoses.

Our study also has a number of limitations, foremost the number of available intraoperative FNAs, which also do not exactly replicate the standard preoperative diagnostic FNAs typically obtained percutaneously in a clinic setting. We limited ourselves to epithelial thyroid tumors, and were unable to obtain FNA samples from the infrequent FC and HC cases encountered in the timespan samples were collected for this study. Therefore, although the available FC and HC tissue samples had high scores, suggesting the selected isoform panel may do well diagnostically, the performance of the panel in these cases remains unknown for FNAs.

Conclusions

In conclusion, we have developed a 5-transcript model combining specific splice variants of HMGA2, PLAG1, KLK7, FNDC4, and CDH3 to better characterize thyroid nodules using the technically challenging but clinically relevant diagnostic FNA samples. Further validation trials will be needed to develop this panel as a diagnostic assay to guide preoperative surgical decision making for thyroid tumors.

Abbreviations

FNA: fine needle aspiration biopsy

PBMC: peripheral blood mononuclear cells

AN: adenomatoid nodules

FA: follicular adenomas

HA: Hürthle cell adenomas

NIFTP: noninvasive follicular thyroid neoplasm with papillary-like nuclear features

FVPTC: follicular variant of papillary thyroid carcinoma

PTC: papillary thyroid carcinoma

FC: follicular carcinomas

HC: Hürthle cell carcinomas

qPCR: real-time quantitative PCR

Ct: threshold cycle

ROC: receiver operating characteristic

AUC: area under the ROC curve

PPV: positive predictive value

NPV: negative predictive value

Declarations

Ethics Approval and consent to participate.

Under Johns Hopkins Institutional Review Board approval, thyroid tumor tissue, intraoperative FNA, and blood specimens were collected from patients undergoing thyroid surgery at Johns Hopkins Hospital.

This manuscript does not contain individual patient data.

Consent for publication.

Not applicable.

Availability of Data and Material.

The datasets used during the current study are available from the corresponding author on reasonable request.

Competing Interests.

None of the authors declare any conflicts of interest or competing financial interests.

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Author contributions.

MZ and CU planned and designed the study. YW, BM, and ZL performed and analyzed the experiments. LR was the study pathologist and LC the study bioinformatician. All participated in writing and editing the manuscript.

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Figures

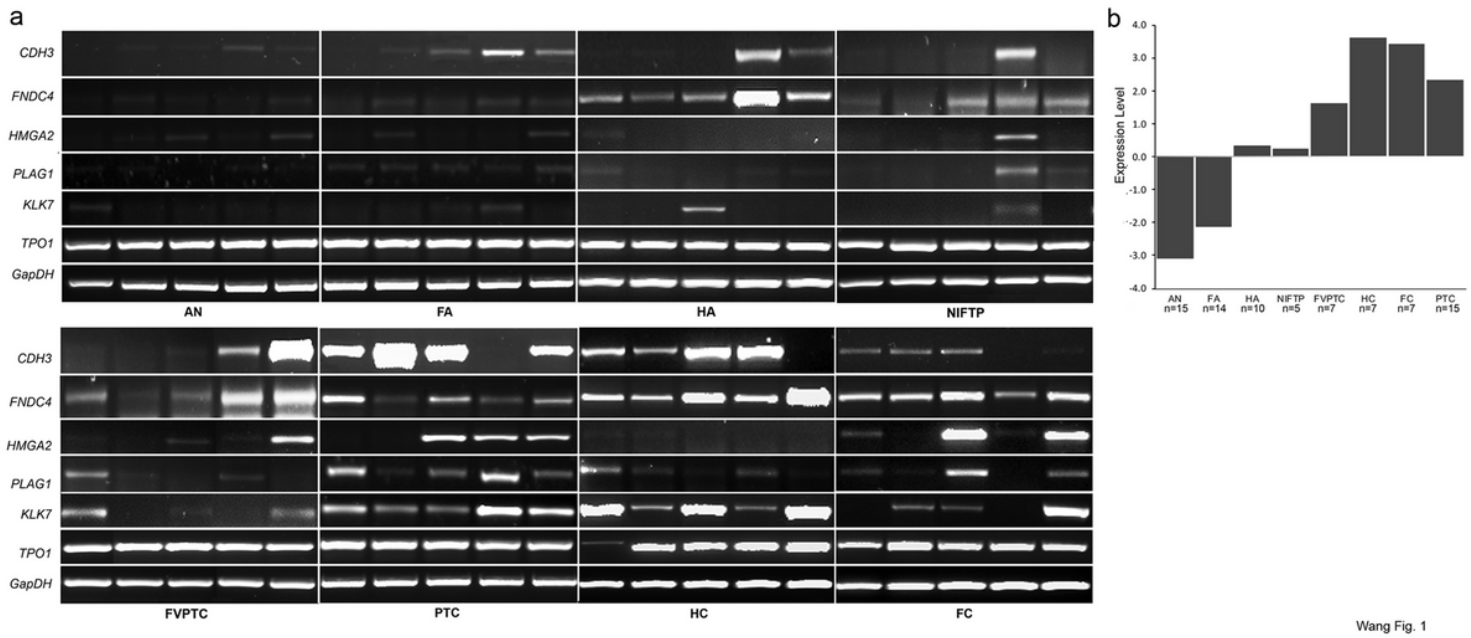

Figure 1

Expression of the 5 gene transcripts and internal controls (TPO1 & GapDH) in solid thyroid tumor frozen samples. a Representative gel image showing each of the 5 isoforms and thyrocyte-specific TPO1 in 8 thyroid tumor subtypes. b Bar plot of the sum of expression levels of the 5 isoforms (AN, n = 15; FA, n = 14; HA, n = 10; NIFTP, n = 5; FVPTC, n = 7; HC, n = 7; FC, n = 7; PTC, n = 15). Semi-quantitative densitometry of each isoform was normalized to GapDH and z-transformed so that all genes would be on the same scale.

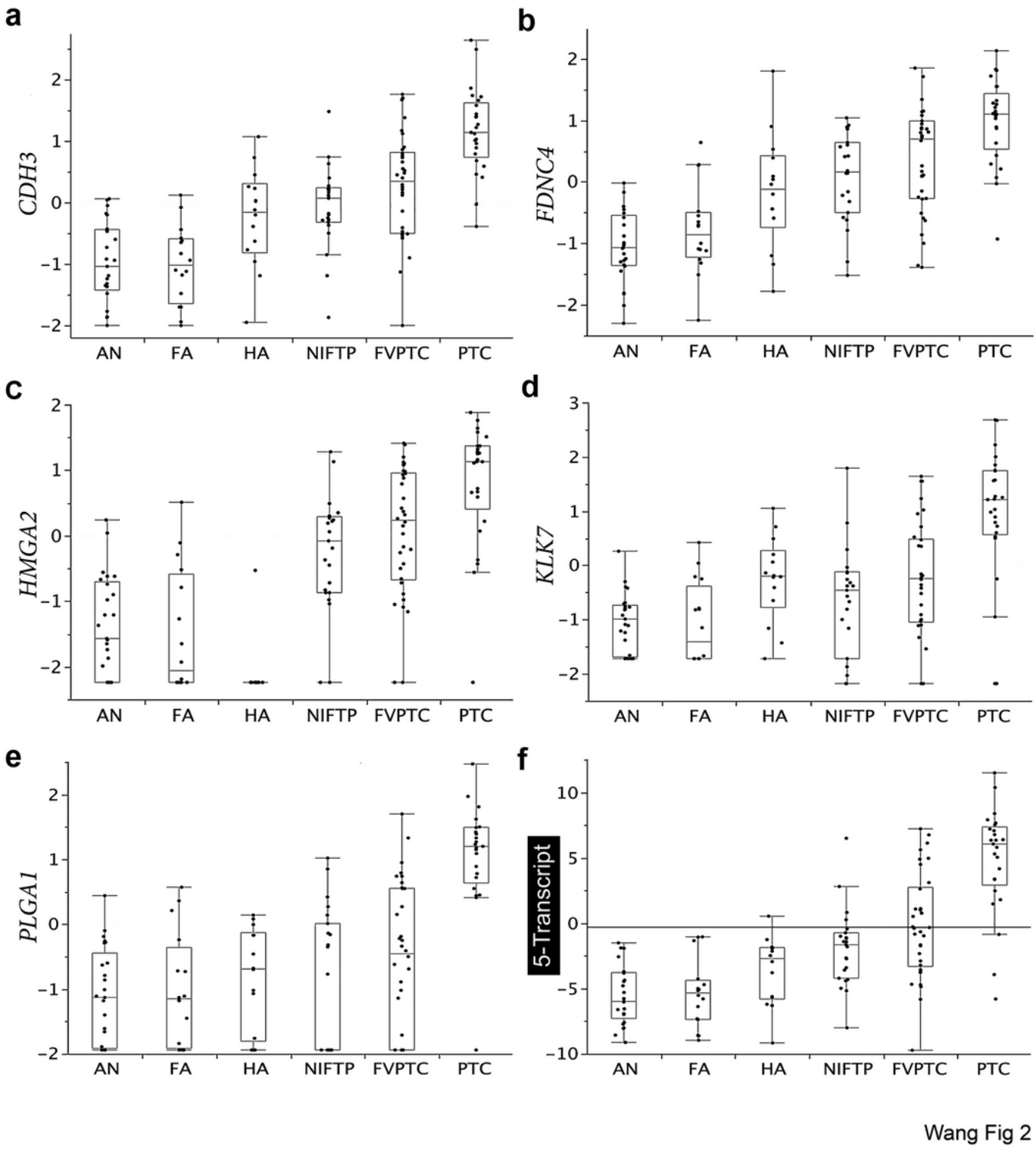

Figure 2

The expression profiles of the 5 gene expression isoforms in FNAs. The box plots show the 5th, 25th, 50th, 75th and 95th percentiles of the sample expression z-ΔCt scores in each thyroid tumor subgroup. Panel f shows the sum of 5-transcript composite z-ΔCt score. The expression reference line is the z-ΔCt score of -1, the threshold corresponding to the sensitivity of 75% at the specificity of 91% . AN, n = 25; FA, n = 16; HA, n = 14; NIFTP, n = 23; FVPTC, n = 34; PTC, n = 25.

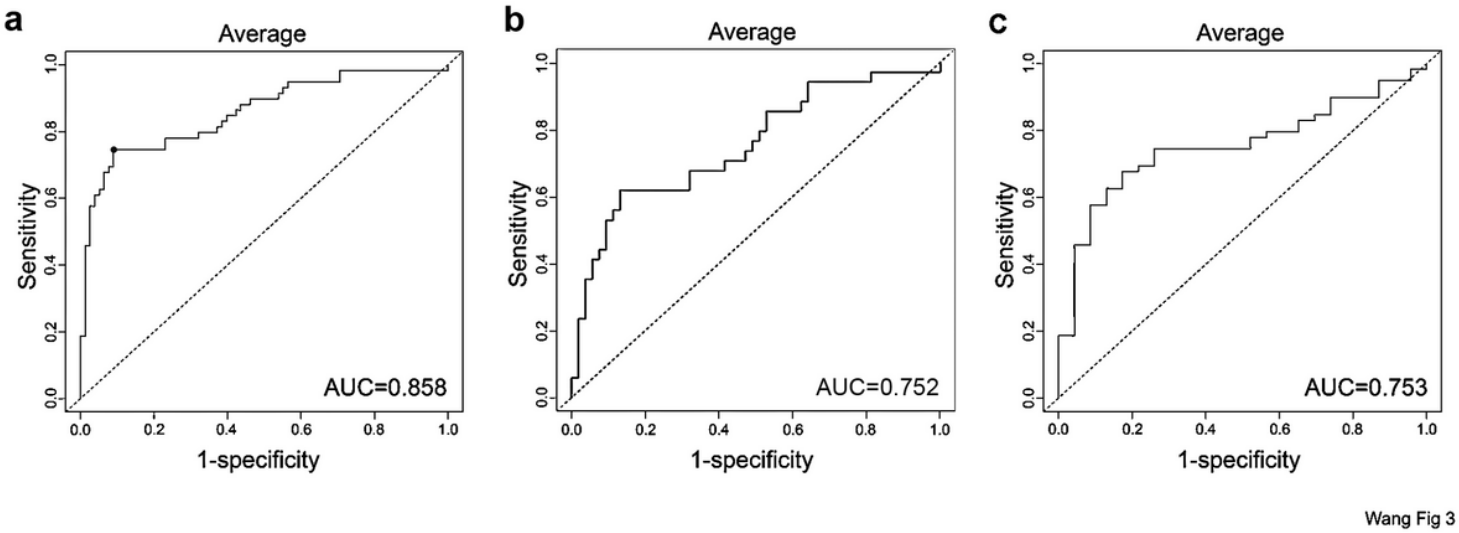

Figure 3

ROC Curves of the diagnostic power of the 5-transcript panel in FNAs. a Benign (AN, FA, HA, and NIFTP, n = 78) vs. malignant (FVPTC and PTC, n = 59) thyroid tumors. The dot on the ROC curve is the threshold for the composite expression z-ΔCt score of -1, corresponding to the sensitivity (75%) at specificity 91%. b Thyroid follicular neoplasms, malignant FVPTC (n = 34) vs. FA (n = 16) and NIFTP (n = 23). c NIFTP (n = 23) vs. malignant thyroid tumors, FVPTC and PTC (n = 59).

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