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Carbonic anhydrase 4 and crystallin alpha-B immunoreactivity may distinguish benign from malignant thyroid nodules in patients with indeterminate thyroid cytology

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Abstract

Background—Thyroid nodules are present in 19%–67% of the population and carry a 5%–10% risk of malignancy. Unfortunately, fine-needle aspiration biopsies are indeterminate in 20%–30% of patients, often necessitating thyroid surgery for diagnosis. Numerous DNA microarray studies including a recently commercialized molecular classifier have helped to better distinguish benign from malignant thyroid nodules. Unfortunately, these assays often require probes for >100 genes, are expensive, and only available at a few laboratories. We sought to validate these DNA microarray assays at the protein level and determine whether simple and widely available immunohistochemical biomarkers alone could distinguish benign from malignant thyroid nodules.

Methods—A tissue microarray (TMA) composed of 26 follicular thyroid carcinomas (FTCs) and 53 follicular adenomas (FAs) from patients with indeterminate thyroid nodules was stained with 17 immunohistochemical biomarkers selected based on prior DNA microarray studies. Antibodies used included galectin 3, growth and differentiation factor 15, protein convertase 2, cluster of differentiation 44 (CD44), glutamic oxaloacetic transaminase 1 (GOT1), trefoil factor 3 (TFF3), Friedreich Ataxia gene (X123), fibroblast growth factor 13 (FGF13), carbonic anhydrase 4 (CA4), crystallin alpha-B (CRYAB), peptidylprolyl isomerase F (PPIF), asparagine synthase (ASNS), sodium channel, non-voltage gated, 1 alpha subunit (SCNN1A), frizzled homolog 1 (FZD1), tyrosine related protein 1 (TYRP1), E cadherin, type 1 (ECAD), and thyroid hormone receptor

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Disclosure

The authors reported no proprietary or commercial interest in any product mentioned or concept discussed in this article.

associated protein 220 (TRAP220). Of note, two of these biomarkers (GOT1 and CD44) are now used in the Afirma classifier assay. We chose to compare specifically FTC *versus* FA rather than include all histologic categories to create a more uniform immunohistochemical comparison. In addition, we have found that most papillary thyroid carcinoma could often be reasonably distinguished from benign disease by morphological cytology findings alone.

Results—Increased immunoreactivity of CRYAB was associated with thyroid malignancy (c-statistic, 0.644; negative predictive value [NPV], 0.90) and loss of immunoreactivity of CA4 was also associated with malignancy (c-statistic, 0.715; NPV, 0.90) in indeterminate thyroid specimens. The combination of CA4 and CRYAB for discriminating FTC from FA resulted in a better c-statistic of 0.75, sensitivity of 0.76, specificity of 0.59, positive predictive value (PPV) of 0.32, and NPV of 0.91. When comparing widely angioinvasive FTC from FA, the resultant c-statistic improved to 0.84, sensitivity of 0.75, specificity of 0.76, PPV of 0.11, and NPV of 0.99.

Conclusions—Loss of CA4 and increase in CRYAB immunoreactivity distinguish FTC from FA in indeterminate thyroid nodules on a thyroid TMA with an NPV of 91%. Further studies in preoperative patient fine needle aspiration (FNAs) are needed to validate these results.

Keywords

Thyroid; Indeterminate cytology; Molecular markers; Follicular neoplasm; Follicular thyroid carcinoma; CA4; CRYAB

1. Introduction

The National Cancer Institute estimates 60,220 new cases of thyroid cancer in 2012 with 1850 deaths [1]. The rate of thyroid cancer is increasing in the United States [2]. New Jersey has the highest rate of thyroid cancer rate and mortality per capita in the nation [3]. Thyroid cancer usually presents as a thyroid nodule. Thyroid nodules, however, are very common, detectable in 19%–67% of the US population [4,5]. Five percent to 10% of thyroid nodules are malignant [6]. Current guideline recommend fine-needle aspiration biopsy (FNAB) for most solid thyroid nodules >1 cm (or complex nodules >1.5–2.0 cm) [4]. Unfortunately, 15%–35% of thyroid FNABs are indeterminate (follicular or hurthle cell neoplasm, or follicular or hurthle cell lesion; Bethesda IV and Bethesda III cytology, respectively) and often require thyroid surgery for diagnosis, although only 10%–30% of indeterminate nodules will harbor malignancy [4]. Although FNAB is most accurate for identifying nodules that contain papillary thyroid carcinoma, it sometimes cannot reliably distinguish benign from malignant cells and is especially unreliable for distinguishing follicular thyroid carcinoma (FTC) and its variant and hurthle cell carcinoma, which accounts for 10%–15% of all thyroid cancer, from the benign follicular adenoma (FA) and from other benign follicular neoplasia [7]. This is largely because the diagnosis of follicular carcinoma requires evidence of capsular or vascular invasion, necessitating tissue architecture and not directly seen with cytology [8].

Despite the limitations of fine needle aspiration (FNA) cytology for the diagnosis of FTC, secondary cytology features on thyroid FNA can raise the suspicion for FTC. These features include a hypercellular monomorphic specimen with little to no colloid [8]. Such features

suggest the diagnosis of follicular (or hurthle cell) neoplasm (Bethesda IV cytology), a diagnosis which carries a 20%–30% risk of malignancy [9]. Patients with follicular (or hurthle cell) neoplasm often require thyroid lobectomy for diagnosis [4]. This practice leaves most of the patients with Bethesda IV cytology undergoing unnecessary surgery for benign disease.

Several DNA microarray studies have already demonstrated change in the expression of nearly 200 genes in benign from malignancy thyroid nodules [10–14]. Some have focused on distinguishing FTC from benign follicular proliferations, as this distinction is most difficult to distinguish by cytology alone. Recently, a commercial assay called the Afirma classifier (Veracyte, San Francisco, CA) has become available for clinical use that helps to distinguish benign from malignant thyroid nodules specifically in those patients with indeterminate thyroid nodules [15]. Unfortunately, this assay is expensive, exhibits low specificity, carries significant false negatives, and requires send-out analysis at the company laboratory, rather than in-house laboratories. A simple immunohistochemical (IHC) biomarker would therefore be potentially useful as this technique is widely available. The aim of this study was to screen 17 potential IHC biomarkers of thyroid cancer on a tissue microarray composed of thyroid tissue from cytologically indeterminate thyroid nodules.

2. Methods

A tissue microarray (TMA) was created from 79 thyroid FNA specimens over a 4-year period that were found to have a Bethesda IV cytology (follicular or hurthle cell neoplasm) diagnosis of which 26 were found to have FTC and 53 found to have FA on final surgical pathology [34]. We chose to compare specifically FTC *versus* FA rather than including all histologic categories to create a more uniform IHC comparison. In addition, we have found that papillary thyroid carcinoma could often be reasonably distinguished from benign disease on morphological cytology findings alone. Of the 26 specimens with FTC, 16 were of the hurthle cell type. Of the 26 specimens, 14 had vascular invasion of which nine were widely angioinvasive. Of the 53 specimens with FA, 13 were of the hurthle cell type.

The tissue microarray was created by embedding 0.6 mm cores of thyroid tissue from formalin-fixed, paraffin-embedded archived tissue specimens into a paraffin block. Two cores of each specimen were embedded in duplicate paraffin blocks such that each specimen had a total of four representative cores. Additionally, normal adjacent cores were obtained as controls for each specimen, resulting in four cores for each normal adjacent tissue. The slides were reviewed by a member of the Department of Pathology so that suitable donor blocks and an appropriate tumor focus on the paraffin wax block were correctly identified and marked. This was done to be sure background goiterous or thyroiditis tissue was not inadvertently sampled. The Beecher Instruments automated tissue arrayer (Sun Prairie, WI) was used. The coordinates of each core were recorded along with a linked deidentified patient number. These paraffin blocks were sectioned 5-μm thick. Primary antibodies for each of 17 potential thyroid cancer biomarkers were used according to the manufacturer's instructions (Table 1).

The 17 antibodies used were selected based on a review of the existing DNA microarray literature and included galectin 3 (GAL3), growth and differentiation factor 15 (GDF15), protein convertase 2 (PCSK2), cluster of differentiation 44 (CD44), glutamic oxaloacetic transaminase 1 (GOT1), trefoil factor 3 (TFF3), Friedreich Ataxia gene (X123), fibroblast growth factor 13 (FGF13), carbonic anhydrase 4 (CA4), crystallin alpha-B (CRYAB), peptidylprolyl isomerase F (PPIF), asparagine synthase (ASNS), sodium channel, non-voltage gated, 1 alpha subunit (SCNN1A), frizzled homolog 1 (FZD1), tyrosine related protein 1 (TYRP1), E cadherin, type 1 (ECAD), and thyroid hormone receptor associated protein 220 (TRAP220) (Table 1) [36]. The TMA slides were deparaffinized, and antigen retrieval was achieved with the Ventana cell conditioning solution (Ventana Medical Systems, Tuscon, AZ). Primary antibodies were optimized on a thyroid mini-TMA using Ventana Medical Systems Discovery XT automated immunostainer. Positive tissue controls as recommended by the manufacturer were created on a multi-tissue array. The primary antibodies were applied at manufacturer's dilution and incubated at 37 °C for 1 h. The Ventana Universal secondary antibody kit was used along with chromogenic detection using the Ventana DABMap kit. Slides were counterstained with hematoxylin.

Analysis of the immunostaining patterns was performed by the same pathologist. The pathologist was blinded to the array design. Scoring was performed on a standard IHC 4-point scale (0 = none; 1 = mild; 2 = moderate; and 3 = strong). The Shapiro test was used to evaluate whether staining was normally distributed. Mann–Whitney (two-sample Wilcoxon) test was used to compare the median values of the different groups. Receiver operator curves (ROC) were created for each biomarker and biomarker combinations. A comparison between FTC and FA was performed. In addition, a comparison between angioinvasive and widely angioinvasive FTC *versus* FA was performed. Moreover, an analysis of the best two-biomarker and the best three-biomarker combinations for distinguishing benign from malignant nodules was performed (Tables 2 and 3). We chose to establish cutoffs for each biomarker by creating a plot of each biomarker's staining distribution and determining a cutoff that results in best discrimination for that particular immunostain. To calculate negative predictive value (NPV) and positive predictive value (PPV), the following prevalence assumptions were made based on existing epidemiological data [25]: prevalence rate for FTC was set at 0.2, angioinvasive at 0.05, and widely angioinvasive at 0.04. NPV was calculated as (1 − prevalence rate) × specificity/[(1 − sensitivity) × prevalence rate + specificity × (1 − prevalence rate)]. Likewise, PPV was calculated as prevalence rate × sensitivity/[sensitivity × prevalence rate + (1 − specificity) × (1 − prevalence rate)].

To help maintain consistent IHC scoring, the pathologist compared immunostaining on each tissue core to the control we created for each stain. We chose to make determination of stain positivity by focusing on the staining on the cellular areas of the core. For example, focally strong staining in a hypercellular cluster of follicular cells would have been deemed strongly positive. By contrast, weak background staining over the entire core including in the colloid follicles would have been deemed weak or absent.

3. Results

Patient age and gender did not differ significantly between the FTC and FA groups. The seven biomarkers with the best discriminating power to distinguish benign from malignant thyroid tissue as measured by c-statistic were CRYAB, CA4, PPIF, TRAP22, ASNS, GDF15, and X123. Similar results were obtained when comparing the widely angioinvasive FTCs *versus* benign FA. Figure 1 shows the ROC curves for the top three individual biomarkers. The top two IHC biomarkers were CRYAB and CA4, with an increased immunoreactivity of CRYAB associated with malignancy (c-statistic, 0.64; sensitivity, 0.80; specificity, 0.43; NPV, 0.90; PPV, 0.26) and a loss of immunoreactivity of CA4 also associated with malignancy (c-statistic, 0.72; sensitivity, 0.72; specificity, 0.61; NPV, 0.90; PPV, 0.32). Immunoreactivity of CRYAB was even stronger in widely angioinvasive FTC (c-statistic, 0.67; sensitivity, 0.75; specificity, 0.45; NPV, 0.99; PPV, 0.05), and loss of immunoreactivity of CA4 was more pronounced with CA4 (c-statistic, 0.80; sensitivity, 0.75; specificity, 0.78; NPV, 0.99; PPV, 0.12).

Analysis of the best-combined two IHC biomarkers revealed that the top five two-biomarker combinations that carried the best discriminating power were ASNS and TYRP2, CA4 and CRYAB, CA4 and PPIF, CA4 and CD44H, and CA4 and GAL3. Figure 2 shows the ROC curves for the top three two-biomaker combinations. The combination of CA4 and CRYAB had the highest discriminating power and resulted in a better c-statistic of 0.75, sensitivity of 0.76, specificity of 0.59, PPV of 0.32, and NPV of 0.91. When comparing widely angioinvasive FTC from FA, the resultant c-statistic improved to 0.84, sensitivity of 0.75, specificity of 0.76, PPV of 0.11, and NPV of 0.99.

Analysis of the best three IHC biomarkers revealed that the top 3 three-biomarker combinations were ASNS, CRYAB, and TYRP1; ASNS, CA4, and TYRP1; and ASNS TRAP22, and TYRP1. Figure 3 shows the ROC curves for the trio of ASNS, CA4, and CRYAB. This three-biomarker combination showed good discriminating ability in predicting malignancy (c-statistic, 0.82; sensitivity, 0.72; specificity, 0.86; NPV, 0.92; and PPV, 0.56).

4. Discussion

According to the Bethesda System for reporting thyroid cytology, there are six categories for thyroid FNA results: (1) nondiagnostic, (2) benign, (3) atypia of undetermined significance and/or follicular lesion of undetermined significance, (4) follicular neoplasm, (5) suspicious for malignancy, and (6) malignant [9]. Patients with follicular lesion of undetermined significance and FN carry a 5%–15% and 15%–30% risk of malignancy, respectively. Repeat FNA or diagnostic surgery is typically recommended [4]. The 2009 American Thyroid Association (ATA) guidelines indicate that molecular markers such as serine/threonine RAF oncogene B1 (BRAF), rat sarcoma oncogene (RAS), RET/PTC, paired box gene 8 / peroxisome proliferation activated receptor gamma (PAX8/PPARγ), or GAL3 may be considered for indeterminate cytology [4,35]. Because these guidelines were published, there has been significant growth in the study of molecular biomarkers for use with indeterminate thyroid nodules.

Biomarkers for thyroid cancer may be in the form of a mutational assay (e.g., BRAF V600E mutation), DNA microarray-based assay (e.g., the Afirma classifier), or an IHC stain (e.g., GAL3). There is extensive literature on the utility of mutational assays for diagnostic and prognostic purposes in thyroid cancer. The BRAF V600E gene mutation has been well studied and is thought to be associated more aggressive forms of PTC [24]. It may provide prognostic information and assist clinical management [26]. For diagnostic purposes, the BRAF mutation along with several other well-described mutations in patients with PTC, including the RAS mutation, RET/PTC translocation, and the PAX8/PPARγ rearrangement, has been combined for use in commercially available assays (e.g., Asuragen's MiRInform thyroid mutational panel, Asuragen, Austin, TX) to help risk-stratify patients with indeterminate and suspicious thyroid cytology [28,29]. Subsequent studies have shown that these mutational assays in practice are more useful for the patient with a suspicious (Bethesda V cytology) FNA or when cytologic nuclear feature are suggestive but not diagnostic of papillary thyroid carcinoma [25]. Its discriminating ability is more limited for patients specifically with follicular neoplasm diagnosis alone.

There are >30 published DNA microarray studies comparing thyroid cancer with benign thyroid conditions. Of these, only 10 studies specifically compared gene profiles of FTC from benign follicular neoplasia, and of these, only six studies had at least 10 patients on a broad reproducible platform (e.g., the Affymetrix GeneChips, Affymetrix, Santa Clara, CA) [10–15].

The largest study specifically addressing the problem of FTC diagnosis using a genome-wide DNA microarray platform was composed of 114 patient samples [14]. Using 12 frozen FTC and 12 FA specimens (the initial or training set) hybridized on the Affymetrix U133A GeneChip (representing 14,500 gene transcripts), the authors showed 80 genes with the largest differential expression in FTC compared with FA using a training and validation set. Supervised hierarchical cluster analysis showed three genes that demonstrated the greatest fold change and significance (*P* values and *t* statistics). These were cyclin D2 (a cell cycle regulator involved in Wnt signaling pathway), PCSK2 (an enzyme involved in activating precursor proteins), and prostate differentiation factor (a member of the TGF-β superfamily known to prevent apoptosis by activating the Akt pathway, today better known as GDF15). None of these three genetic markers alone could reliably distinguish FTC from FA. However, by combining these top three genetic markers and analyzing the expression of these three genes in another 12 FTC *versus* 19 benign thyroid lesions (validation set), they suggested a 96.7% accuracy in differentiating FTC from benign disease. The authors further validated the gene expression differences at the protein level by demonstrating that differences in IHC staining of cyclin D2 and PCSK2 in a series of 57 paraffin-embedded FTC *versus* 26 benign thyroid nodules had an accuracy of 87% in distinguishing these lesions [14].

Another group used the Affymetrix U95 GeneChip platform to compare nine follicular carcinomas with 26 benign tumors and found 627 gene expression differences, of which 106 were most significant [10]. These genes included GAL3, CA4, X123, and DNA damage inducible transcript 3 [10]. Their data were consistent and built on their earlier DNA microarray work on seven FTC and 10 FA specimens [11]. Genes that were consistently

differentially expressed included GAL3, CA4, ASNS, X123 (Freidrich ataxia gene), and DNA damage inducible transcript 3.

Validation studies using quantitative polymerase chain reaction and immunohistochemistry in attempt to validate genomic difference in FTC have been performed [16–23,30–33]. Several authors have consistently shown that GAL3, TFF3, CA4, CD44, and GDF15 show increased expression using various techniques including quantitative polymerase chain reaction, immunohistochemistry, as well as nylon macroarrays, and serial analysis of gene expression. GAL3 has been especially well studied and repeatedly found to be overexpressed in patients with malignant thyroid nodules. In fact, it is the only IHC biomarker to have been prospectively studied in patient's FNA specimens [39]. In a study of 465 patients with indeterminate thyroid FNAs, GAL3 or LGALS3 immunostaining had a sensitivity of 78%, specificity of 93%, PPV of 82%, and NPV of 91% for distinguishing benign from malignant disease [39]. Unfortunately, 29 of 130 cancers (22%) were missed by this biomarker alone in indeterminate specimens. For this reason, GAL3 has not entered clinical practice.

The new Afirma molecular classifier assay has been commercialized and is increasingly being used clinically [37]. In a study of 265 indeterminate thyroid nodules, the Afirma classifier exhibited a sensitivity of 92%, specificity of 52%, and an NPV of 85%–95%. However, seven of 85 malignant nodules were missed. The authors did note that six of these seven false negative specimens were paucicellular. A new molecular classifier, KNN249, is in the process of becoming commercialized [38]. Other investigators continue to work on micro-RNA based or serum-based diagnostic assays [40].

In our study, we found that CA4, CRAYB, and ASNS immunostaining were most differentially expressed when comparing FTC and FA specimens. These genes code for three well-described proteins that have been associated with certain illnesses and malignancies. CRYAB is a ubiquitous protein that acts as a molecular chaperone. It may be oncogenic, with elevated levels of CRYAB having been associated with breast cancer, hepatocellular carcinoma, and squamous cell carcinoma of the head and neck, as well as neurodegenerative disease [41,42]. CA4 is a zinc metalloenzyme that catalyzes the reversible hydration of carbon dioxide. It has important roles in retinal physiology, acid–base balance, renal function, pulmonary function, and gastric pH. A reduction in CA4 from autoantibodies has been associated with rheumatoid arthritis and lupus [43]. ASNS is an enzyme involved in the synthesis of the asparagine. It also may have oncogenic properties, with studies showing that its over-expression has been associated with hepatocellular carcinoma, pancreatic carcinoma, and acute lymphoblastic leukemia [44,45].

A review of our statistical data set and the ROC curves show that CA4, CRYAB, and ASNS are biomarkers that are consistently differentially expressed between follicular carcinoma tissue and FA tissue in patients with a follicular neoplasm diagnosis. CRYAB and ASNS show marked increase in expression, and CA4 shows marked decrease in expression. These differences were also consistently more pronounced when comparing widely angioinvasive FTC with FA. One possible explanation might be that the cores of widely invasive FTC would be more likely to uniformly represent malignant tissue, whereas a tissue core of

minimally invasive FTC might not exclusively represent malignant tissue and might be present alongside benign tissue. An increased immunoreactivity of CRYAB was associated with thyroid malignancy (c-statistic, 0.644; NPV, 0.90) and the loss of immunoreactivity of CA4 was also associated with malignancy (c-statistic, 0.715; NPV, 0.90) in indeterminate thyroid specimens. The combination of CA4 and CRYAB for discriminating FTC from FA resulted in a reasonable c-statistic of 0.75, sensitivity of 0.76, specificity of 0.59, PPV of 0.32, and NPV of 0.91. This compares favorably to the Afirma molecular classifier that shows an NPV of 85%–95%, with a 167-gene set compared with only two immunhistochemical biomarkers.

Despite these promising results, there are several important limitations. First, because the study was performed on samples with known pathology, a validation set is needed, which has yet to be performed. Second, ultimately an IHC biomarker would need to be effective on a cell block of preoperative patient FNA rather than in surgical specimens. This has yet to be performed. It is certainly possible that cell blocks from FNAs may not yield adequate tissue to make IHC feasible. Third, although the data are promising, a sensitivity of 72%–80% for a single IHC biomarker would be considered inadequate as a clinical screening tool to avoid unnecessary diagnostic surgery. By comparison, the Afirma assay has a sensitivity of 92%, in which many would consider adequate. Indeed, the prospective 2008 Italian study on GAL3 IHC also showed a sensitivity of only 78%, explaining why this immunostain is not used clinically. Fourth, although there is improvement in discriminating ability of three biomarkers, we believe it is unlikely that cytologists will embrace the use of multiple mediocre IHC biomarkers, with sensitivities under 90%. This study was meant to rapidly screen and identify the best single biomarker using high-throughput thyroid tissue microarray methodology. We successfully screened 17 biomarkers in 79 specimens using 17 IHC runs (instead of 1343 runs without a TMA). Unfortunately, none of these biomarkers achieved sensitivity >90% alone. We hope to continue this work by validating the results of the most promising biomarkers in this study and other IHC biomarkers from new DNA microarray assays on cell blocks of thyroid fine needle aspirates.

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T.D. wrote the article and obtained the funding. M.N. and M.K. created tissue microarray from cell blocks. M.C. scored all the tissue microarray's immunohistochemical. C.C., S.L., and Y.L. contributed to statistical analysis and interpretation. V.C., L.P., and D.A. collected data. N.B. and S.T. contributed to the conception and design.

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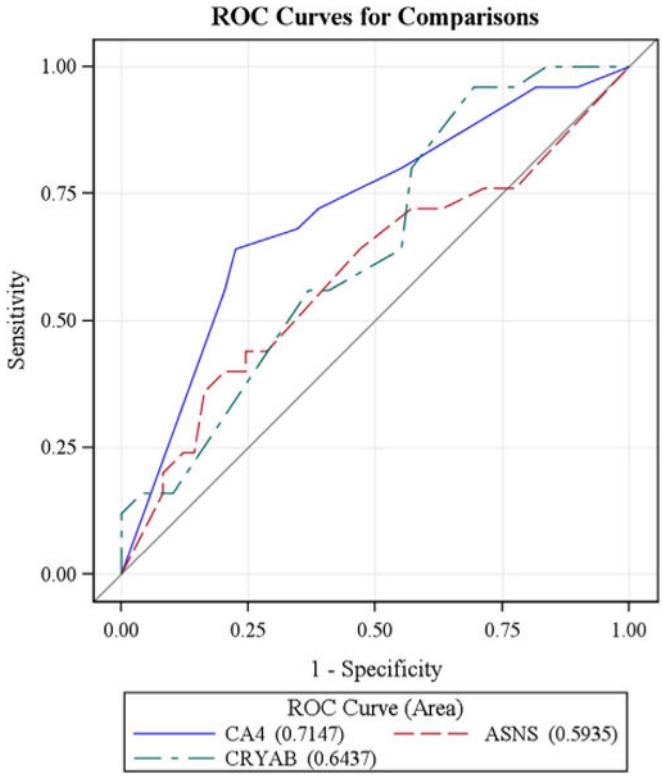

Fig. 1.

ROC curves for the top three individual IHC biomarkers for distinguishing malignant from benign thyroid tissue: CA4, CRYAB, and ASNS. (Color version of figure is available online.)

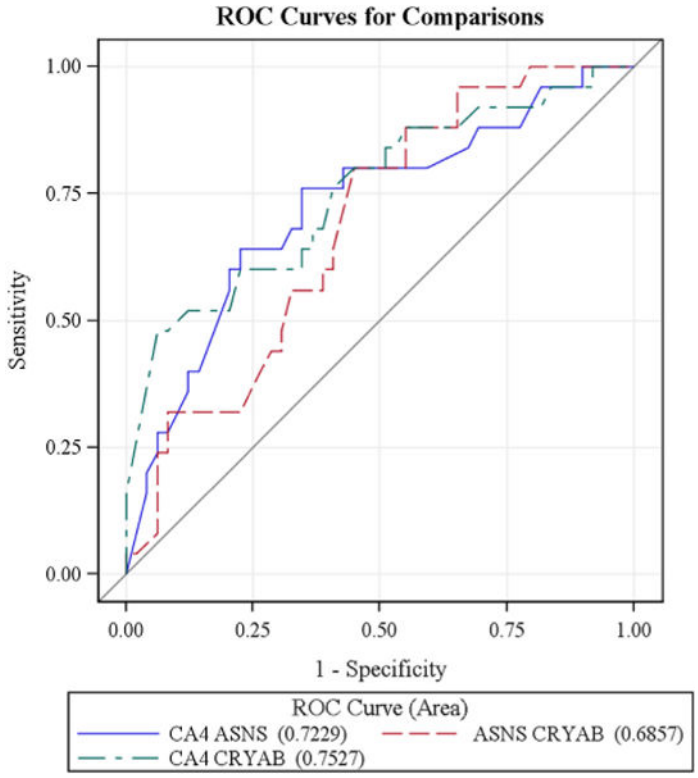

Fig. 2.

ROC curve for top three two-biomarker combination for distinguishing malignant from benign thyroid tissue: CA4 and ASNS; CA4 and CRYAB; ASNS and CRYAB. (Color version of figure is available online.)

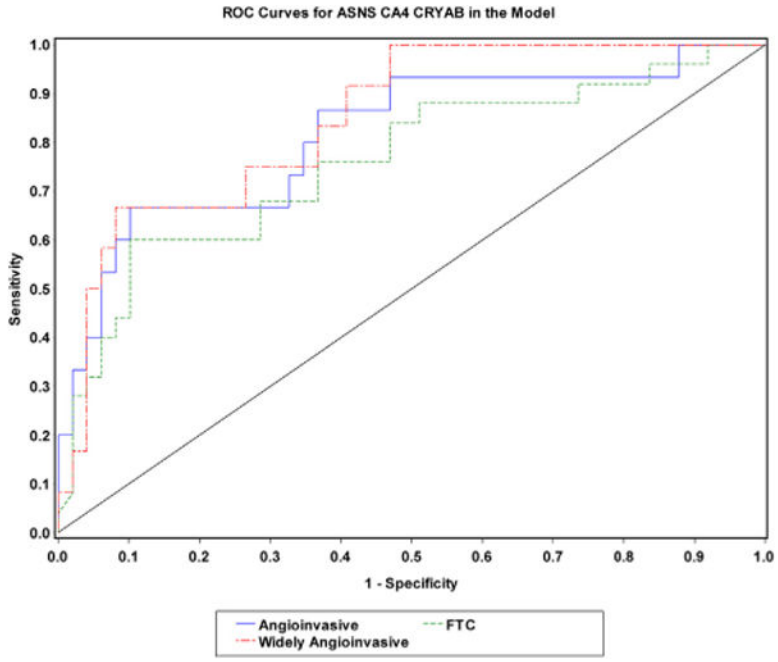

Fig. 3.

ROC curves for the three-biomarker combination of ASNS, CA4, and CRYAB comparing ability to distinguish FTC from FA; angioinvasive FTC from FA; and widely angioinvasive FTC from FA. (Color version of figure is available online.)

Table 1

Discriminating ability of a panel of IHC biomarkers for distinguishing FTC from FA.

Biomarker	c-value	Immunostaining intensity cutoff for FTC detection	Sensitivity	Specificity	NPV	PPV
FTC *versus* FA						
CA4	0.715	<0.67	0.72	0.61	0.90	0.32
CRYAB	0.644	<1.75	0.80	0.43	0.90	0.26
PPIF	0.615	>2.5	0.80	0.35	0.87	0.23
TRAP22	0.604	<2.5	0.92	0.29	0.93	0.24
ASNS	0.593	>0.75	0.72	0.43	0.86	0.24
GDF15	0.569	<1	1.00	0.04	1.00	0.21
X123	0.561	>0	1.00	0	—	0.20
CD44H	0.545	<1.75	0.72	0.41	0.85	0.23
PCSK2	0.540	<2	0.80	0.29	0.85	0.22
GOT1	0.532	>1	0.92	0.06	0.75	0.20
SCNN1A	0.527	>0.5	0.80	0.24	0.83	0.21
TFF3	0.525	<1.33	0.72	0.35	0.83	0.22
FGF13	0.521	>0	1.00	0	—	0.20
GAL3	0.511	>0.25	0.80	0.27	0.84	0.21
TYRP1	0.511	<1.5	0.80	0.20	0.80	0.20
ECAD	0.504	<3	1.00	0	—	0.20
FZD1	0.493	>1	0.71	0.44	0.86	0.24
Angioinvasive FTC *versus* FA						
CA4	0.760	<0.25	0.73	0.78	0.98	0.15
CRYAB	0.702	<1.5	0.73	0.45	0.97	0.07
GDF15	0.621	<1	1.00	0.04	1.00	0.05
TFF3	0.610	<1	0.80	0.37	0.97	0.06
X123	0.604	>0	1.00	0	—	0.05
ASNS	0.603	>0.75	0.73	0.43	0.97	0.06
ECAD	0.589	<3	1.00	0	—	0.05
PCSK2	0.574	<1.5	0.73	0.47	0.97	0.07
PPIF	0.560	>2.5	0.73	0.35	0.96	0.06

Biomarker	c-value	Immunostaining intensity cutoff for FTC detection	Sensitivity	Specificity	NPV	PPV
TYRP1	0.556	<1.33	0.73	0.29	0.95	0.05
TRAP22	0.555	<2.5	0.93	0.29	0.99	0.06
GAL3	0.543	>0.25	0.87	0.27	0.97	0.06
CD44H	0.532	>0.75	0.73	0.37	0.96	0.06
SCNN1A	0.527	>0.5	0.80	0.24	0.96	0.05
GOT1	0.521	<1.75	0.73	0.39	0.97	0.06
FGF13	0.518	>0	1.00	0	—	0.05
FZD1	0.507	<1.5	0.93	0.27	0.99	0.06
Widely angioinvasive FTC *versus* FA						
CA4	0.796	<0.25	0.75	0.78	0.99	0.12
CRYAB	0.674	<1.5	0.75	0.45	0.98	0.05
ASNS	0.668	>1	0.75	0.53	0.98	0.06
X123	0.645	>0	1.00	0	—	0.04
ECAD	0.644	<3	1.00	0	—	0.04
PPIF	0.600	>2.5	0.75	0.35	0.97	0.05
TFF3	0.591	<1	0.83	0.37	0.98	0.05
GDF15	0.587	<1	1.00	0.04	1.00	0.04
FZD1	0.580	>1	0.82	0.44	0.98	0.06
SCNN1A	0.576	>0.75	0.75	0.35	0.97	0.05
GOT1	0.563	>1.5	0.75	0.55	0.98	0.07
FGF13	0.543	>0	1.00	0	—	0.04
CD44H	0.541	>0.75	0.75	0.37	0.97	0.05
TRAP22	0.533	<2.5	0.92	0.29	0.99	0.05
TYRP1	0.513	<1.5	0.92	0.20	0.98	0.05
GAL3	0.509	<0.5	0.83	0.22	0.97	0.04
PCSK2	0.509	<2	0.83	0.29	0.98	0.05

GOT = glutamic oxaloacetic transaminase 1; TFF3 = trefoil factor 3; FGF13 = fibroblast growth factor 13; SCNN1A = sodium channel, non-voltage gated, 1 alpha subunit; ECAD = E cadherin, type 1; FZD1 = frizzled homolog 1.

Table 2

Analysis of two-biomarker combinations for distinguishing FTC from FA.

Biomarker	c-value	Immunostaining intensity cutoff for FTC detection	Sensitivity	Specificity	NPV	PPV
FTC *versus* FA						
ASNS and TYRP1	0.793	>−0.622	0.84	0.67	0.94	0.39
CA4 and CRYAB	0.753	>−0.925	0.76	0.59	0.91	0.32
CA4 and TRAP22	0.740	>−0.696	0.72	0.67	0.91	0.36
CA4 and PPIF	0.739	>−0.672	0.72	0.63	0.90	0.33
CA4 and CD44H	0.730	>−0.782	0.72	0.61	0.90	0.32
CA4 and PCSK2	0.729	>−0.707	0.72	0.61	0.90	0.32
CA4 and GAL3	0.729	>−0.840	0.72	0.61	0.90	0.32
ASNS and CA4	0.723	>−0.724	0.76	0.65	0.92	0.35
CA4 and TYRP1	0.722	>−0.701	0.72	0.67	0.91	0.36
CA4 and FGF13	0.721	>−0.500	0.72	0.73	0.91	0.40
CA4 and TFF3	0.719	>−0.832	0.72	0.61	0.90	0.32
CRYAB and PPIF	0.715	>−0.834	0.80	0.59	0.92	0.33
CA4 and GOT1	0.715	>−0.808	0.72	0.61	0.90	0.32
CA4 and GDF15	0.714	>−0.838	0.72	0.63	0.90	0.33
CA4 and ECAD	0.713	>−0.702	0.71	0.63	0.90	0.32
CA4 and FZD1	0.709	>−0.762	0.71	0.63	0.90	0.32
CA4 and SCNN1A	0.707	>−0.846	0.72	0.63	0.90	0.33
CA4 and X123	0.705	>−0.763	0.71	0.63	0.90	0.32
ASNS and CRYAB	0.685	>−0.718	0.80	0.55	0.92	0.31
CD44H and CRYAB	0.678	>−0.794	0.76	0.51	0.89	0.28
Angioinvasive FTC *versus* FA						
ASNS and TYRP1	0.878	>−1.173	0.73	0.80	0.98	0.16
CA4 and CRYAB	0.801	>−1.415	0.73	0.61	0.98	0.09
CA4 and TFF3	0.783	>−0.86	0.73	0.78	0.98	0.15
CA4 and ECAD	0.779	>−0.653	0.71	0.77	0.98	0.14
CRYAB and ECAD	0.778	>−1.149	0.79	0.58	0.98	0.09
CA4 and ASNS	0.772	>−0.725	0.73	0.80	0.98	0.16

Biomarker	c-value	Immunostaining intensity cutoff for FTC detection	Sensitivity	Specificity	NPV	PPV
ASNS and GOT1	0.758	>−0.743	0.73	0.78	0.98	0.15
CRYAB and X123	0.753	>−1.108	0.71	0.58	0.97	0.08
ECAD and TFF3	0.743	>−0.943	0.71	0.71	0.98	0.11
TYRP1 and X123	0.741	>−0.984	0.71	0.73	0.98	0.12
ASNS and CRYAB	0.735	>−1.195	0.73	0.57	0.98	0.08
CRYAB and GDF15	0.734	>−1.542	0.73	0.47	0.97	0.07
CRYAB and PPIF	0.729	>−1.142	0.73	0.55	0.98	0.08
ASNS and GDF15	0.722	>−1.293	0.73	0.61	0.98	0.09
CRYAB and TYRP1	0.718	>−1.507	0.73	0.49	0.97	0.07
CRYAB and PCSK2	0.715	>−1.356	0.73	0.53	0.97	0.08
CRYAB and SCNN1A	0.713	>−1.096	0.73	0.63	0.98	0.10
CRYAB and FGF13	0.707	>−1.412	0.73	0.45	0.97	0.07
CD44H and CRYAB	0.705	>−1.325	0.73	0.53	0.97	0.08
CRYAB and TFF3	0.701	>−1.478	0.73	0.47	0.97	0.07
CRYAB and GOT1	0.694	>−1.463	0.73	0.45	0.97	0.07
Widely angioinvasive FTC *versus* FA						
ASNS and TYRP1	0.896	>−0.771	0.75	0.86	0.99	0.18
CA4 and CRYAB	0.840	>−1.455	0.75	0.76	0.99	0.11
CA4 and PPIF	0.832	>−1.209	0.75	0.78	0.99	0.12
CA4 and ECAD	0.831	>−0.753	0.73	0.77	0.99	0.12
ASNS and CA4	0.820	>−0.935	0.75	0.80	0.99	0.13
CA4 and TFF3	0.817	>−0.893	0.75	0.82	0.99	0.15
CA4 and FGF13	0.813	>−1.019	0.75	0.76	0.99	0.11
CA4 and TRAP22	0.813	>−1.052	0.75	0.78	0.99	0.12
CA4 and X123	0.810	>−1.199	0.73	0.77	0.99	0.12
CA4 and CD44H	0.810	>−0.715	0.75	0.78	0.99	0.12
CA4 and SCNN1A	0.802	>−0.894	0.75	0.80	0.99	0.13
CA4 and FZD1	0.796	>−0.952	0.73	0.79	0.99	0.13
CRYAB and ECAD	0.779	>−1.507	0.82	0.58	0.99	0.08
ECAD and TFF3	0.771	>−1.327	0.73	0.71	0.98	0.09
ECAD and PPIF	0.741	>−1.531	0.91	0.48	0.99	0.07

Biomarker	c-value	Immunostaining intensity cutoff for FTC detection	Sensitivity	Specificity	NPV	PPV
ASNS and CRYAB	0.722	>−1.377	0.75	0.61	0.98	0.07
ECAD and X123	0.707	>−1.648	0.73	0.63	0.98	0.07
ECAD and FGF13	0.688	>−2.109	1.00	0	—	0.04
ASNS and ECAD	0.684	>−2.008	0.73	0.44	0.97	0.05
ECAD and TRAP22	0.655	>−1.836	0.82	0.38	0.98	0.05
ECAD and FZD1	0.636	>−1.821	0.73	0.44	0.97	0.05

GOT = glutamic oxaloacetic transaminase 1; TFF3 = trefoil factor 3; FGF13 = fibroblast growth factor 13; SCNN1A = sodium channel, non-voltage gated, 1 alpha subunit; ECAD = E cadherin, type 1; FZD1 = frizzled homolog 1.

Table 3

Analysis of three-biomarker combinations for distinguishing FTC from FA.

Biomarker	c-value	Immunostaining intensity cutoff for FTC detection	Sensitivity	Specificity	NPV	PPV
FTC *versus* FA						
ASNS, CRYAB, and TYRP1	0.824	>−0.286	0.72	0.86	0.92	0.56
ASNS, CA4, and TYRP1	0.815	>−0.011	0.72	0.88	0.93	0.60
ASNS, TRAP22, and TYRP1	0.812	>−0.414	0.72	0.78	0.92	0.45
ASNS, CD44H, and TYRP1	0.804	>−0.309	0.72	0.80	0.92	0.47
ASNS, PPIF, and TYRP1	0.800	>−0.431	0.72	0.73	0.91	0.40
ASNS, GDF15, and TYRP1	0.794	>−0.192	0.72	0.80	0.92	0.47
ASNS, PCSK2, and TYRP1	0.793	>−0.595	0.76	0.69	0.92	0.38
ASNS, FGF13, and TYRP1	0.789	>−0.653	0.80	0.65	0.93	0.37
ASNS, CA4, and PCSK2	0.782	>−0.556	0.72	0.71	0.91	0.39
CA4, CRYAB, and PPIF	0.773	>−0.553	0.72	0.76	0.92	0.42
ASNS, CA4, and CRYAB	0.758	>−0.844	0.72	0.63	0.90	0.33
Angioinvasive FTC *versus* FA						
ASNS, CRYAB, and TYRP1	0.941	>−0.315	0.73	0.90	0.98	0.27
ASNS, CA4, and TYRP1	0.921	>−0.352	0.87	0.88	0.99	0.27
ASNS, GDF15, and TYRP1	0.898	>−0.391	0.73	0.86	0.98	0.21
ASNS, TFF3, and TYRP1	0.895	>−0.391	0.73	0.84	0.98	0.19
ASNS, GAL3, and TYRP1	0.891	>−0.369	0.73	0.90	0.98	0.27
ASNS, PCSK2, and TYRP1	0.890	>−0.601	0.73	0.86	0.98	0.21
ASNS, GOT1, and TYRP1	0.890	>−0.624	0.73	0.84	0.98	0.19
ASNS, ECAD, and TYRP1	0.888	>−0.635	0.71	0.83	0.98	0.18
ASNS, TRAP22, and TYRP1	0.884	>−0.712	0.73	0.82	0.98	0.17
ASNS, TYRP1, and X123	0.877	>−0.763	0.71	0.83	0.98	0.18
ASNS, CA4, and CRYAB	0.815	>−1.372	0.73	0.67	0.98	0.11
Widely angioinvasive FTC *versus* FA						
ASNS, CA4, and TYRP1	0.943	>−0.538	0.75	0.94	0.99	0.34
ASNS, CRYAB, and TYRP1	0.926	>−0.857	0.75	0.86	0.99	0.18
ASNS, TFF3, and TYRP1	0.915	>−0.878	0.75	0.86	0.99	0.18

Biomarker	c-value	Immunostaining intensity cutoff for FTC detection	Sensitivity	Specificity	NPV	PPV
ASNS, PPIF, and TYRP1	0.914	>−0.690	0.75	0.90	0.99	0.23
ASNS, PCSK2, and TYRP1	0.908	>−0.847	0.75	0.88	0.99	0.20
ASNS, FGF13, and TYRP1	0.905	>−0.675	0.75	0.90	0.99	0.23
ASNS, GDF15, and TYRP1	0.904	>−0.739	0.75	0.90	0.99	0.23
ASNS, TRAP22, and TYRP1	0.902	>−0.964	0.75	0.88	0.99	0.20
ASNS, ECAD, and TYRP1	0.900	>−0.903	0.73	0.88	0.99	0.20
ASNS, TYRP1, and X123	0.891	>−0.751	0.73	0.88	0.99	0.20
ASNS, CA4, and CRYAB	0.847	>−1.419	0.75	0.73	0.99	0.11

GOT = glutamic oxaloacetic transaminase 1; TFF3 = trefoil factor 3; FGF13 = fibroblast growth factor 13; SCNN1A = sodium channel, non-voltage gated, 1 alpha subunit; ECAD = E cadherin, type 1.