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ORIGINAL ARTICLE

Molecular characterization of tumors meeting diagnostic criteria for the non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP)

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Abstract

"Follicular variant" papillary thyroid carcinomas (FV-PTC) that do not histologically invade have a miniscule risk of metastasis, and thus been reclassified as a tumor of low malignant potential, the non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP). There are few molecular studies of this tumor type. We performed gene expression analysis, by RNA sequencing, on a series of FV-PTCs, NIFTPs, and follicular adenomas. A training set comprised tumors from The Cancer Genome Atlas (TCGA) repository ($n = 46$), digital slides from which were reviewed and classified as invasive or non-invasive FV-PTC. A validation set comprised in-house NIFTPs, invasive FV-PTCs, and follicular adenomas ($n = 26$). In the training set, unsupervised clustering separated tumors into three distinct expression subtypes, which associated with invasion and characteristic molecular alterations. Specifically, the "BRAF-like" subtype was enriched in invasive FV-PTCs and tumors with *BRAF* V600E mutations. The "THADA-like" subtype was enriched in non-invasive tumors and those with rearrangements involving *THADA*. The "RAS-family-like" subtype included many invasive and non-invasive FV-PTCs and was enriched in tumors with mutations in RAS family genes. In the validation set, nearest centroid analysis classified all invasive FV-PTCs as "BRAF-like" and all follicular adenomas as either "RAS-like" or "THADA-like." NIFTPs were the most molecularly diverse histologic type, with cases classified as "BRAF-like," "THADA-like," and "RAS-family-like." In conclusion, tumors fitting criteria for NIFTP are molecularly diverse, making it difficult to diagnose them with molecular studies, likely including matrial from cytopathology samples.

Keywords Thyroid cancer · Follicular variant papillary thyroid carcinoma · Non-invasive follicular thyroid neoplasm with papillary-like nuclear features · BRAF-like · RAS-like

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Introduction

A subset of papillary thyroid carcinomas (PTCs) lacks the histologic papillae of classic PTC and is instead composed entirely of structures recapitulating the thyroid follicle. Such tumors are referred to as follicular variant PTCs (FV-PTCs) [1]. Despite designation as carcinoma, independent studies have shown FV-PTCs that lack invasive histologic architecture have a very low risk of metastasis and recurrence [2–8]. The World Health Organization has thus reclassified these as a tumor of low malignant potential, the non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) [9]. This change reclassifies 10–20% of PTCs into the NIFTP category [9].

Multiple studies have shown that histologic subtypes of thyroid neoplasia associate with specific molecular alterations. Classic PTC is heavily enriched in *BRAF* V600E mutations and *RET*/*PTC* rearrangements [10, 11]. In contrast, follicular neoplasms, such as follicular adenoma and follicular carcinoma, are enriched in *THADA* and *PAX8* rearrangements [12, 13]. Mutations in RAS family genes are unique in thyroid neoplasia, as these are seen in both classic PTC and follicular neoplasms, though mutations are more heavily enriched in follicular neoplasia [14]. Histologic subtypes also associate with gene expression signatures, and expression signatures associate with specific underlying mutations. Specifically, classic PTCs usually express genes associated with high-level ERK signaling, indicating MAP kinase activation, and underexpress genes of thyroid differentiation. This expression signature has been termed "RAF-like," owing to its strong association with classic PTCs harboring *BRAF* V600E mutation [15]. In contrast, follicular adenomas and follicular carcinomas express genes of thyroid differentiation, and underexpress genes associated with ERK signaling [16]. This expression signature has been termed "RAS-like," owing to its strong association with tumors harboring RAS family gene mutations [15–17].

The landmark study by The Cancer Genome Atlas (TCGA) Consortium showed most tumors designated "FV-PTC" have a RAS-like expression signature, though a significant minority have *BRAF* V600E mutation and/or a RAF-like expression signature [15]. FV-PTCs in this study also frequently had mutations in RAS family genes, and a subset harbored rearrangements involving *THADA*. FV-PTC thus appeared unique from a molecular standpoint, because it could have molecular features of either follicular or papillary neoplasia. However, this study did not distinguish NIFTP from invasive FV-PTC, including both of these as "FV-PTC" without further qualification. Additional study has since shown that invasive FV-PTC differs biologically from NIFTP, in that invasive FV-PTC frequently has *BRAF* V600E mutation, similar to classic PTC [5]. We set out to continue this line of research, specifically by comparing gene expression and mutational profiles of NIFTP to invasive FV-PTC and follicular adenoma.

Materials and methods

This study was performed with approval from the Penn State College of Medicine Human Subjects Protection Office (Institutional Review Board). Data analysis was performed using the R programming language, version 3.2.2 [18]. Expression subtypes were identified in a training set derived from the TCGA cohort, and validated on a data set derived from in-house samples.

TCGA cohort (training set)

FV-PTC cases were identified from the TCGA data set (https://portal.gdc.cancer.gov). Cases were excluded if lymph node data were not available (i.e., data blank or assigned pNX). Digital slides on selected cases were identified in the digital slide archive (http://cancer.digitalslidearchive.net/) and reviewed by a pathologist who regularly sees thyroid cases on a subspecialized head and neck pathology service (JIW), blinded to all clinical and molecular data. A single slide with tumor was available for these cases. The lack of evaluable slides made it impossible to confidently diagnose NIFTP, because the WHO has stringent criteria for the diagnosis of NIFTP, which include complete examination of the tumor capsule [9]. To deal with this limitation, cases were classified as invasive or non-invasive FV-PTC, based on evidence of histologic invasion on the available slide. Further, cases were classified as non-invasive FV-PTC in the training set only if they fit the remaining NIFTP criteria, including encapsulation or clear demarcation, follicular growth pattern (no papillae, no psammoma bodies, and < 30% solid, trabecular, or insular growth pattern), nuclear features of papillary carcinoma, no lympho-vascular or capsular invasion, no tumor necrosis, and low mitotic activity (< 3 mitoses per 10 high-powered fields). Cases without adjacent non-neoplastic thyroid tissue were excluded. We considered the non-invasive designation to be a useful, but imperfect, estimate for the diagnosis of NIFTP.

Gene expression analysis was performed on RNA-sequencing (RNA-seq) data taken from the Genomic Data Commons [19]. Expression analysis entailed unsupervised consensus clustering, which classified tumors into subtypes. These subtypes were then used to create subtype-specific centroids for classifying in-house (validation) cases. See Supplemental Methods [20–25] for the complete protocol.

In-house cohort (validation set)

Thyroid tumors from our archives were identified, including 11 cases originally diagnosed as FV-PTC and 15 follicular adenomas. All histology slides were reviewed by a pathologist who regularly sees thyroid cases on a subspecialized head and neck pathology service (JIW) to confirm the diagnosis and

assess for evidence of invasion (Fig. 1). In equivocal cases, a second pathologist on the head and neck pathology service (HC) reviewed the case for consensus opinion. These pathologists have similar thresholds to consider nuclei as papillary. The complete tumor capsule was evaluated on all cases. Cases were classified as NIFTP if, and only if, they fit full NIFTP criteria per the WHO manual [9], including lack of invasive architecture, encapsulation or clear demarcation, follicular growth pattern (no papillae, no psammoma bodies, and < 30% solid, trabecular, or insular growth pattern), nuclear features of papillary carcinoma, no lympho-vascular or capsular invasion, no tumor necrosis, and low mitotic activity (< 3 mitoses per 10 high-powered fields). Clinical data were collected by chart review. To obtain DNA and RNA from thyroid tumors, areas of interest were extracted by punch biopsy from formalin-fixed paraffin-embedded (FFPE) tissue blocks. To insure against contamination with non-neoplastic tissue or other tumor, blocks were selected if, and only if, tissue from flanking blocks contained high-volume tumor. This approach assured selected blocks contained full-thickness tumor, and sampled cores thus contained high percentage of tumor. RNA and DNA were extracted using Qiagen DNA FFPE Tissue Kit (Qiagen, Germantown, MD). RNA sequencing was performed. RNA-sequencing libraries were generated using TruSeq RNA Access Library Prep Kit (Illumina, San Diego, CA), followed by single-end 50-bp sequencing to obtain 25 million sequencing reads per sample on an Illumina HiSeq 2500. Selected mutations common in thyroid neoplasia, including those involving *HRAS*, *KRAS*, *NRAS*, and *BRAF* (Table S1), were interrogated by targeted polymerase chain reaction (see Supplemental Methods for details on RNA and DNA sequencing [26]).

In-house cases were assigned to the expression subtypes identified in the training set using nearest centroid analysis, using Person correlation distance.

Differential expression and pathway analysis

Differential expression analyses were performed in the training cohort using groups defined by the expression subtypes. In brief, the voom transformation was applied to the gene-level read count data [27], and the limma R package was used to identify genes differentially expressed in each subtype versus the others (combined) based on a false discovery rate (FDR) threshold of $q < 0.05$ [28]. The DAVID annotation database was applied to highlight gene ontology (GO) terms and KEGG pathways enriched for sets of differentially expressed genes [29, 30]. These gene sets were manually reviewed, and pathways of interest presented.

Expression subtypes and genes evaluated in commercially available molecular tests

Afirma and Thyroseq are two commercially available molecular tests designed to help distinguish carcinoma from benign tumors sampled by thyroid fine-needle aspiration [31, 32]. We

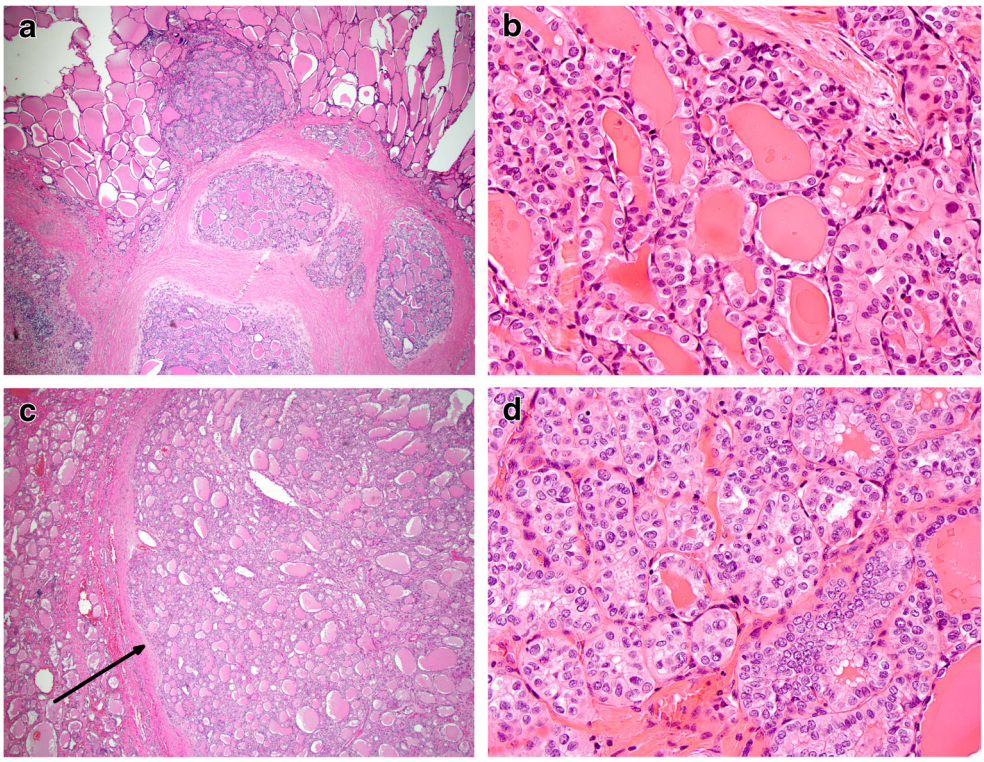

Fig. 1 Histology images. Invasive FV-PTC has follicular architecture and invades thyroid tissue (**a**—H&E, × 100); it has nuclear features of papillary thyroid carcinoma, including nuclear grooves and intranuclear pseudoinclusions (**b**—H&E, × 400). NIFTP has follicular architecture but is circumscribed and encapsulated (arrow) and does not invade (**c**—H&E, × 100); it has nuclear features of papillary thyroid carcinoma, including nuclear grooves and, in this case, intranuclear pseudoinclusions (**d**—H&E, × 400)

investigated the ability of genes used in these tests to distinguish invasive from non-invasive FV-PTC. We did not directly perform either Afirma or Thyroseq on the cases in this study. The Afirma test is a gene expression–based classifier, and uses a complex model incorporating 167 genes to classify cytologically indeterminate fine-needle aspiration samples as "suspicious for malignancy" or "benign," with some additional subtlety [31, 33]. It is difficult to reproduce the Afirma test, because the final model is not available to our knowledge. We thus investigated the Afirma gene list in non-invasive and invasive FV-PTC, by performing hierarchical clustering of the 46 cases taken from the TCGA data set, using the Afirma gene list (see Supplemental Methods [34]). The Thyroseq test (V2) sequences DNA from fine-needle aspiration material to identify somatic mutations and rearrangements common in thyroid neoplasia [32, 35, 36]. Tumors with an identified molecular alteration are considered "positive," and tumors lacking a molecular alteration are considered "negative," with additional subclasses in both positive and negative groups. We specifically looked at common mutational events interrogated by the Thyroseq test (V2) in the 46 cases taken from the TCGA data set, which had comprehensive molecular data available.

Results

Training set

Of the FV-PTC cases in the TCGA data set, 67 had lymph node data. Review of digital pathology slides revealed that 46 of the 67 cases had adjacent non-neoplastic tissue to assess for invasion. These 46 samples comprised the training set. Of these, 18 were classified as invasive FV-PTC and 29 as non-invasive FV-PTC. Assignment as invasive FV-PTC was strongly associated with lymph node metastasis (odds ratio 22.0, $p < 0.001$; Fisher test). One case classified as non-invasive FV-PTC metastasized to a lymph node. This was not unexpected, as only one slide was available for review on each case, and complete evaluation of tumor capsule was thus impossible. This case probably had invasive tumor on another slide, which was unavailable for review. However, we took the strong association between invasive histology and lymph node metastasis as evidence that review of a single slide is a reasonable approximation of true diagnosis.

Unsupervised clustering showed invasive FV-PTC and non-invasive FV-PTC were best classified into 3 distinct groups (Fig. 2A). The first group was highly enriched in mutations in RAS family genes, with 81% (13 of 16 cases) harboring such a mutation. This group was thus named "RAS-family-like" to distinguish it from the "RAS-like" designation previously described. That is, in this study, we use the term "RAS-like" to describe subtype assignments reported in the TCGA data, while we use the term "RAS-family-like" to describe subtype assignments generated by our analysis. The majority of tumors in the RAS-family-like group were non-invasive FV-PTC (75%, 12 of 16 cases), and only 13% (2 of 16 cases) had lymph node metastasis, both invasive FV-PTCs. The second group was composed almost entirely of non-invasive FV-PTC (93%, 14 of 15 cases), and contained all tumors with *THADA* rearrangement. Indeed, rearrangements involving THADA were the most common mutational even in this subtype, seen in 27% (Fig. 2A). The group was thus named "THADA-like." The group also contained the only tumor with an *EIF1AX* mutation, and two tumors with mutation in a RAS gene. This latter observation highlights molecular overlap between THADA-like and RAS-family-like tumors. The third group contained all tumors with *BRAF* mutation or *RET*/*PTC* gene rearrangement, and was thus named "BRAF-like," to distinguish these from the "RAF-like" designation previously described (see "Discussion"). The majority of cases in this group were invasive FV-PTC (75%, 12 of 16 cases), and 38% (6 of 16 cases) had lymph node metastasis, the highest rate of these findings among the three groups. The single non-invasive FV-PTC with lymph node metastasis was BRAF-like. Expression subtype associated with histologic diagnosis (Fig. 2A; $p < 0.001$, Fisher test).

The majority of tumors designated BRAF-like by our analysis had been assigned to the RAF-like group per the TCGA analysis (88%, 14 of 16), and all tumors designated RAS-family-like or THADA-like by our analysis had been assigned to the RAS-like group per TCGA analysis. In keeping with this, tumors in our BRAF-like group had higher ERK signaling than RAS-like and THADA-like tumors (Fig. 3a; $p < 0.001$, Kruskal-Wallis). Tumors in the BRAF-like group expressed lower levels of thyroid differentiation genes than RAS-family-like and THADA-like tumors (Fig. 3b; $p < 0.001$, Kruskal-Wallis).

Validation set

Invasive FV-PTCs, NIFTPs, and follicular adenomas from the validation set were assigned to the BRAF-like, RAS-family-like, or THADA-like group by nearest centroid analysis, as shown in Fig. 2B. The BRAF-like group comprised all invasive FV-PTCs and five NIFTPs. The remaining NIFTPs and all follicular adenomas were split between the RAS-family-like group (2 NIFTPs, 5 adenomas) and the THADA-like cluster (one NIFTP, 10 adenomas). Four cases harbored mutations in RAS family genes; three of these were assigned to the RAS-family-like cluster, and the other to the BRAF-like cluster. No tumor had a BRAF mutation. Expression subtype associated with histologic diagnosis ($p < 0.01$, Fisher). All NIFTPs and follicular adenomas had no evidence of metastasis or recurrence per chart review. Table 1 demonstrates

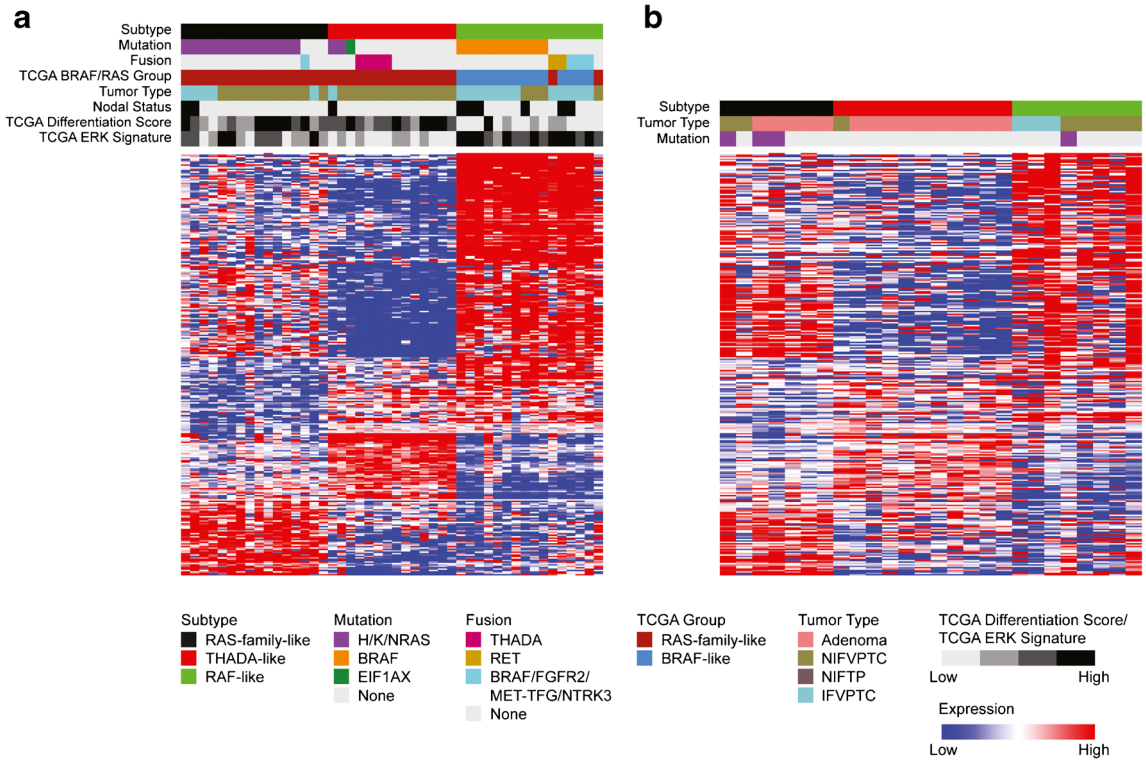

Fig. 2 Gene expression analysis in the training set (A) showed invasive FV-PTCs were primarily BRAF-like, while a smaller portion were RAS-family-like, and a single case was THADA-like. In contrast, non-invasive FV-PTCs were primarily RAS-family-like and THADA-like. Gene expression analysis in the validation set (B) showed follicular adenomas were all RAS-family-like or THADA-like, while NIFTPs fell into all three expression subtypes. IFVPTC, invasive FV-PTC. (IFVPTC, invasive FV-PTC; NIFVPTC, non-invasive FV-PTC)

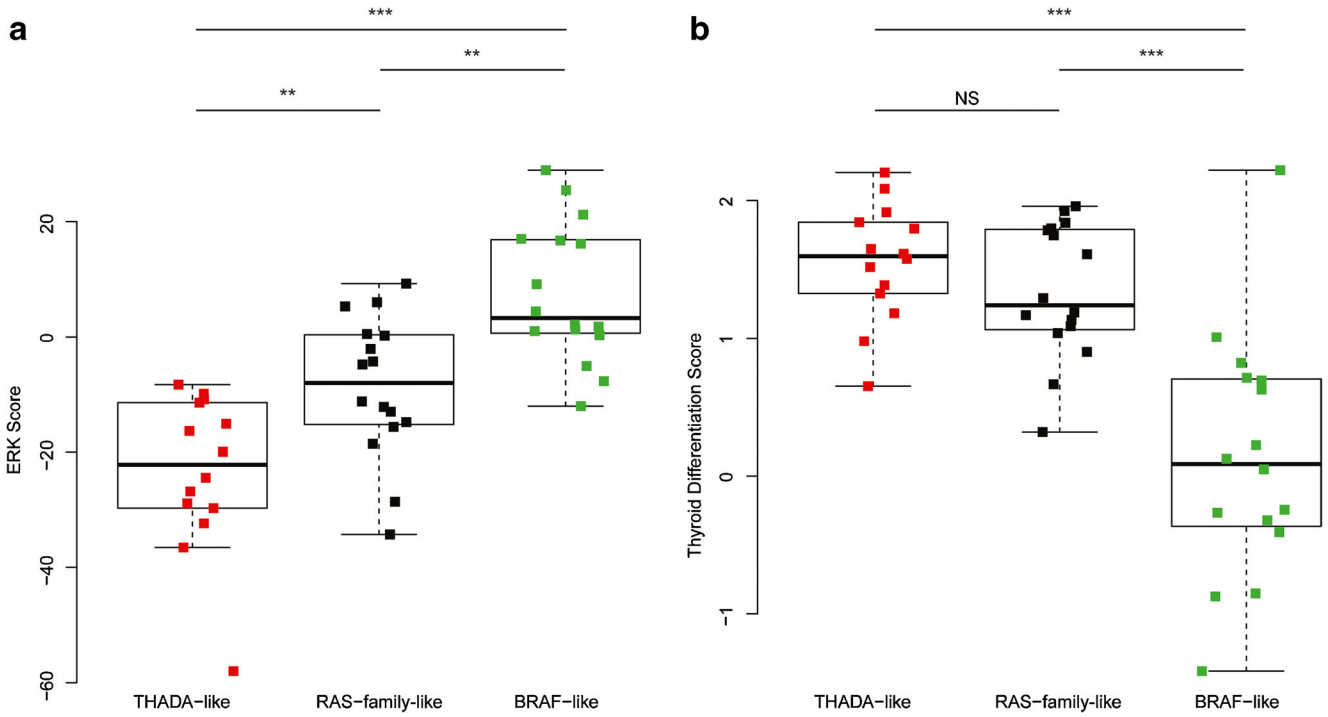

Fig. 3 **a**, **b** ERK and differentiation scores in training set, grouped by gene expression subtype. ERK score was highest in BRAF-like tumors and lowest in THADA-like tumors, while differentiation score was highest in THADA-like tumors and lowest in BRAF-like tumors. $^{**}p < 0.01$, $^{***}p < 0.001$, n.s. = $p > 0.05$ (Wilcoxon rank-sum test)

Table 1 Genetic alterations by expression subtype. BRAF mutations were exclusive to BRAF-like tumors. RAS family mutations were highly enriched in RAS-family-like tumors. THADA-rearrangement was the single most common mutation in THADA-like tumors, though the majority of THADA-like tumors had no identified mutation

Genetic alteration	BRAF-like ($n = 24$)	RAS-family-like ($n = 23$)	THADA-like ($n = 25$)
BRAF point mutation or rearrangement	11	0	0
HRAS point mutation	1	6	0
NRAS point mutation	0	10	1
KRAS point mutation	0	0	1
RET rearrangement	2	0	0
THADA rearrangement	0	0	4
NTRK3 rearrangement	1	0	0
MET-TFG rearrangement	1	0	0
EIF1AX point mutation	0	0	1
FGFR2 rearrangement	0	1	0
No mutation identified	8	6	18

mutations and expression subtypes, combining training and validation sets.

Expression subtypes and the genes evaluated by the Afirma and Thyroseq tests

We clustered the 46 TCGA cases using the Main Classifier genes from the Afirma gene set (there are additional classifiers in this test designed to identify other cancer types, such as medullary thyroid carcinoma, but we restricted the analysis to the Main Classifier genes for the sake of focus) [31], which revealed three main clusters. These clusters were highly concordant with the BRAF-like, RAS-family-like, and THADA-like groups identified above (Fig. 4, $p < 0.001$, Fisher exact test). Regarding the Thyroseq test (V2), several genetic events considered positive by this test were present in non-invasive FV-PTC, including RAS mutation (67%, 10/15 cases), EIF1AX mutation (100%, 1/1 case), and THADA rearrangement (100%, 4/4 cases).

Pathway analysis

Pathway analysis showed differences in the three molecular subtypes. RAS-family-like tumors overexpressed genes associated with hydrogen peroxide biosynthetic and catabolic processes. In particular, these tumors expressed high levels of the NADPH oxidases DUOX1/2, as well as their maturation factors DUOXA1/2. Although increased expression of DUOX1/2 and DUOXA1/2 is associated with tumor differentiation, it has been hypothesized that increased expression of these

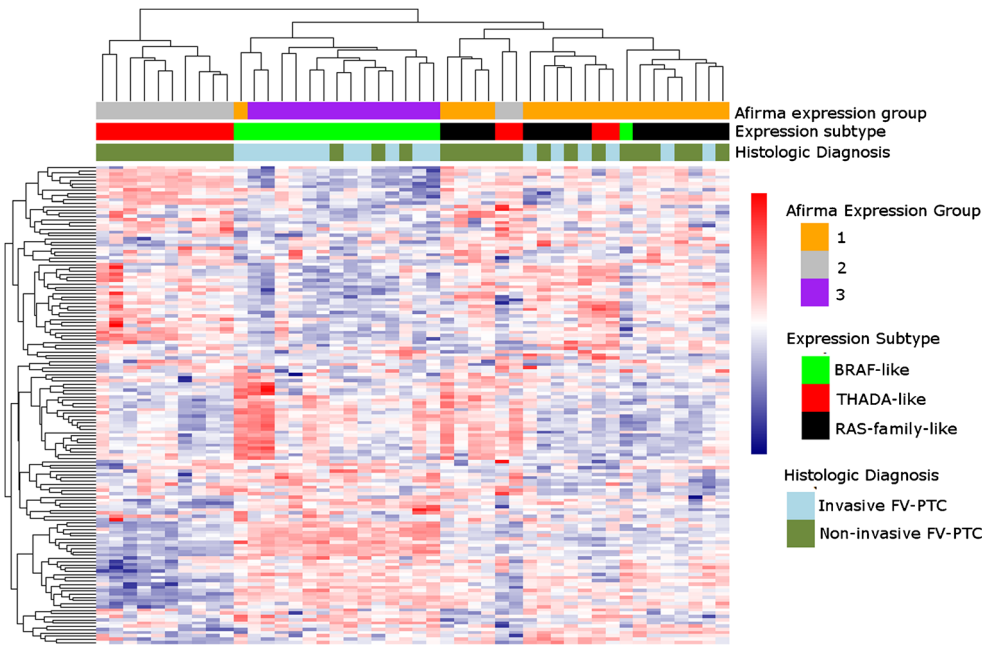

Fig. 4 Gene expression subtypes, determined using the Main Classifier genes from the Afirma gene list. Unsupervised clustering showed these genes separate tumors into three expression subtypes, with striking overlap with the BRAF-like, RAS-like, and THADA-like subtypes. Initial branches show THADA-like tumors separate from BRAF-like and RAS-family-like tumors, possibly because the Afirma gene list was selected to identify aggressive tumors

genes could lead to accumulation of H_2O_2 in the nucleus, and this in turn could increase the likelihood of DNA damage [37]. Elevated expression of DUOX1 has been observed in patients exposed to radiation, which suggests that it may play a role in tumorigenesis [38].

The DAVID analysis showed THADA-like tumors expressed genes involved in PPAR and PI(3)K-AKT signaling. PPAR signaling is notable because PAX8-PPARG gene fusions are common in follicular thyroid neoplasia [39]. Although no THADA-like tumor in our study had a PAX8-PPARG rearrangement, the effect of PPAR signaling in our cases may be similar. Prior studies have shown PI(3)K-Akt signaling may contribute to thyroid cancer tumorigenesis and progression [40], highlighting the importance of this pathway in THADA-like tumors, which expressed several relevant genes, including AKT2, KIT, and RPTOR.

BRAF-like tumors expressed genes associated with chemokine signaling, including many chemokine ligands and receptors, as well as genes associated with extracellular matrix catabolism, including several matrix metalloproteases and collagens. BRAF-like tumors also exhibited increased levels of NFKβ signaling, which may contribute to the elevated immune and inflammatory response observed in this expression subtype, and genes associated with anti-apoptotic signaling, including *BIRC3* and *BCL2A1/L1*. We also observed increased expression of inflammatory receptors and cytokines, including *IL1R2* and *IL6* that may be associated with tumor-promoting inflammation [41]. Elevated expression of pro-proliferative factors such as *TGFA* and *EGFR* were seen, as was increased expression of *STAT3*, which may contribute to immune escape [42].

Discussion

FV-PTC was previously distinguished from follicular adenoma and follicular carcinoma based on the presence of "papillary" nuclear features, namely open chromatin, nuclear grooves, and nuclear pseudoinclusions [1, 43]. All thyroid tumors with papillary nuclear features were considered PTC, and treated similarly, typically total thyroidectomy with possible radioactive iodine administration. However, the diagnostic criteria for FV-PTC had two major problems. First, independent studies found that pathologists cannot reproducibly classify nuclei as papillary, indicating distinction of FV-PTC from follicular lesions is subjective, including distinction of FV-PTC from benign follicular adenoma [44–46]. Second, several studies showed that FV-PTC is capable of metastasis only if it invades the tumor capsule or vascular spaces [2–7]. These findings prompted reclassification of non-invasive FV-PTC as a distinct tumor of low malignant potential, the NIFTP.

In the present study, we have shown that the thyroid neoplasms under study fall into three distinct expression subtypes, termed "BRAF-like," "RAS-family-like," and "THADA-like." The BRAF-like assignment overlapped almost perfectly with the "RAF-like" assignment in the TCGA analysis, showing high ERK signaling and low expression of thyroid differentiation genes. The majority of invasive FV-PTCs were in this category. Several NIFTPs had a BRAF-like signature, while no follicular adenoma had this signature. The THADA-like group was, in many ways, opposite the BRAF-like group, because THADA-like tumors had low ERK signaling and high expression of thyroid differentiation genes. Invasion was uncommon in the THADA-like group, with only a single invasive case. Perhaps tellingly, the *THADA* gene rearrangement enriched in our THADA-like tumors was first reported in follicular adenomas [47], keeping with the more indolent nature of this expression group. The RAS-family-like group was intermediate between BRAF-like tumors and THADA-like tumors regarding ERK signaling and thyroid differentiation signatures. This group included several invasive FV-PTCs, NIFTPs, and follicular adenomas, and was thus the most histologically diverse expression subtype in this study. Notably, all cases from the TCGA set that we classified as THADA-like and RAS-family-like had been classified as RAS-like in the TCGA schema. We thus consider these two categories as more similar to one another than to the BRAF-like group.

Our analysis indicates that NIFTP comprises tumors with a diversity of expression signatures, as the BRAF-like, RAS-family-like, and THADA-like groups were well represented in this histologic tumor type (Fig. 5). It thus appears that a subset of NIFTP is biologically similar to follicular adenomas (i.e., the THADA-like tumors), while another subset is biologically similar to classic PTCs and invasive FV-PTCs (i.e., the BRAF-like tumors). This contrasts with follicular adenomas, which were all RAS-family-like or THADA-like groups, and invasive FV-PTCs, which were mostly BRAF-like or RAS-family-like.

Pathway analysis corroborated biological differences among expression subtypes. In addition to high ERK signaling, our analysis showed BRAF-like tumor genes associated with chemokine signaling, matrix reorganization, and NFKβ signaling. In contrast, THADA-like tumors expressed genes indicative of PPAR signaling. Still different were the RAS-family-like tumors which expressed genes indicative of hydrogen peroxide biosynthetic and catabolic processes, specifically DUOX1/2 and DUOXA1/A2.

The results of the present study help explain recent studies showing that commercially available tests are less valuable in distinguishing benign from malignant thyroid neoplasia, now that NIFTP is recognized as a tumor of low malignant potential. Specifically, we found the Afirma gene list separates tumors into BRAF-like, RAS-family-like, and THADA-like subtypes by clustering methods. Given the Afirma test was designed to have a high negative predictive value [31], and

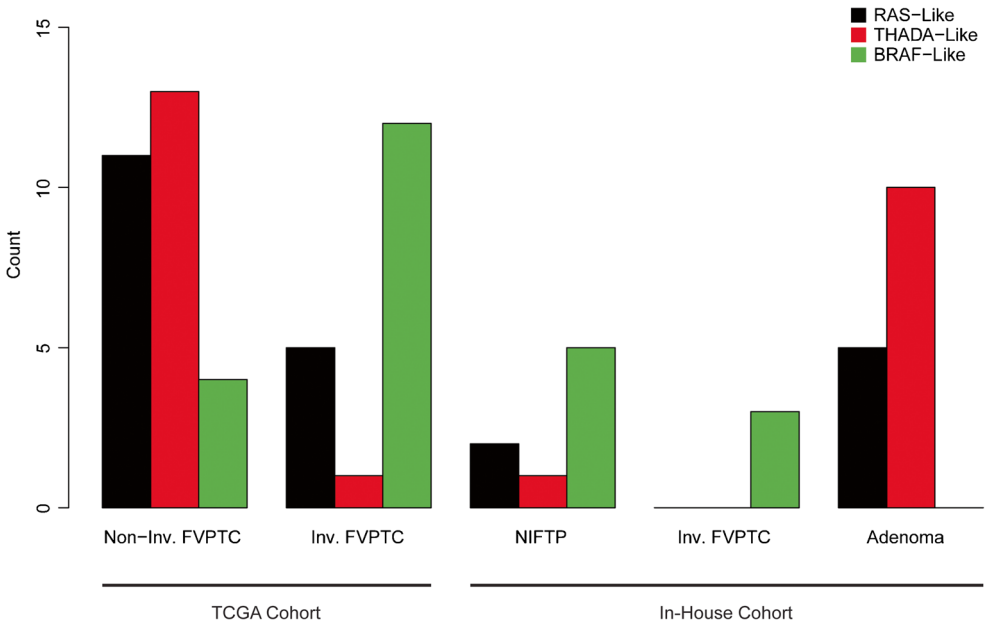

Fig. 5 Histologic tumor type and molecular subtype, both training and validation sets. NIFTP was the most diverse tumor type, with all three molecular subtypes well represented. In contrast, no follicular adenoma had a BRAF-like expression signature, and the great majority of invasive FV-PTCs had a BRAF-like expression signature. (IFVPTC, invasive FV-PTC; NIFVPTC, non-invasive FV-PTC)

invasive FV-PTC was rare in our THADA-like group, we suspect the Afirma test considers THADA-like tumors as "benign," and other tumors as "suspicious for malignancy" [33]. A previous report by Hang et al. [48] showed that recognition of NIFTP as a tumor of low malignant potential reduces the positive predictive value of Afirma testing. Our results may explain this finding, as we showed a large fraction of NIFTPs have BRAF-like or RAS-family-like expression signatures, which Afirma probably considers "suspicious for malignancy." That is, original studies of Afirma likely considered these test-positive/carcinoma-positive, while these would now be considered test-positive/carcinoma-negative.

Regarding the Thyroseq test, the present study shows several mutational events are nonspecific for thyroid carcinoma, particularly in the context of NIFTP versus invasive FV-PTC. For example, the Thyroseq test (V2) considers tumors with *RAS* mutation or *THADA* rearrangement as "positive" [35]. However, we found *RAS* mutations were more common in NIFTP (and non-invasive FV-PTC in the training set) than invasive FV-PTC. We also found *THADA* rearrangements were enriched in non-invasive FV-PTC, without a single example of this event in invasive FV-PTC. These molecular events thus appear less specific for invasive PTC than previously considered [35], an observation keeping with findings from other studies [5, 49, 50]. Recognition of NIFTP as a tumor of low malignant potential thus appears to reduce the positive predictive value of the Thyroseq test (V2), similar to the Afirma test. Notably, a new version of Thyroseq (V3) has recently become available, and utilizes a complex formula including mutational, copy number, and gene expression analysis to classify tumors as high or low cancer risk [51]. We did not attempt to reproduce this version in the present study.

Our training and validation cohorts differed somewhat in molecular classification of NIFTP. Specifically, while the majority of NIFTPs in the validation cohort were BRAF-like, only a minority of non-invasive FV-PTCs in the training cohort were BRAF-like, and 80% of these had *BRAF* V600E mutations. This suggests these were actually invasive FV-PTCs in which the invasive carcinoma was on slides unavailable for review. This highlights a major limitation of our study. That is, only one slide was available for review in the cases in the training set, making it impossible to confidently diagnose NIFTP. In contrast, the complete tumor capsule was available for all cases in the validation set, and no NIFTP from the validation set harbored a *BRAF* V600E mutation or metastasized. We offer two possible explanations for this discrepancy between the TCGA cohort and the in-house cohort. First, our pathology group, including the main pathologist reviewing cases in this study, maintains a high threshold to consider a tumor NIFTP. In keeping with this, many NIFTPs from the in-house cohort had classic papillary nuclear features including nuclear pseudoinclusions, prominent nuclear crowding, and frequent nuclear grooves. In contrast, it appears a lower threshold was employed in classifying TCGA thyroid cancers. Many non-invasive FV-PTCs in the TCGA data set would probably have been diagnosed as follicular adenomas by our pathology group. Thus, if nuclear features and expression signature associate, it is possible we enriched our in-house NIFTPs in BRAF-like tumors by keeping a high threshold for diagnosis. A second possibility relates to the type of samples utilized. The TCGA study used fresh tissue, while our in-house cases were derived from FFPE. It is possible FFPE damage, or another unknown process, biased the results from the in-house cohort, placing a larger number of tumors in the BRAF-like group.

It should be recognized that we are not the first to identify an expression signature distinct from the "RAF-like" and "RAS-like" signatures in thyroid neoplasia. Yoo et al. [16] similarly identified a distinct signature, which they designated "non-RAF, non-RAS," in a study comprising a diverse group of thyroid tumors. In contrast to this study, our analysis focused on molecular characterization of NIFTP, specifically as it differs from invasive FV-PTC and follicular adenoma.

In summary, our results indicate invasive FV-PTC is similar to classic PTC from a molecular standpoint, as the majority had a BRAF-like signature, and *BRAF* V600E mutations were common. This contrasts with the follicular adenomas, all of which were either THADA-like or RAS-family-like. Finally, our results indicate that while NIFTP is morphologically uniform, it is molecularly protean, as tumors with this morphology had THADA-like, BRAF-like, and RAS-family-like signatures. These findings corroborate histologic studies showing that NIFTP is best classified as a distinct tumor type.

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Authors' contributions Christopher Pool, MD—data curation, methodology, writing of the original manuscript

Vonn Walter, PhD—data analysis, visualization, writing of the original manuscript

Darrin Bann, MD PhD—conceptualization, data curation, critical review of the final manuscript

David Goldenberg, MD—supervision, critical review of the final manuscript

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Yuka Imamura, PhD—methodology, supervision, formal analysis, critical review of the final manuscript

Joshua I. Warrick, MD—conceptualization, data curation, formal analysis, writing original manuscript, critical review of the final manuscript

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Compliance with ethical standards

This study was performed with approval from the Penn State College of Medicine Human Subjects Protection Office (Institutional Review Board). The study complies with all ethical standards as stated in the Ethical Responsibilities of Authors on the Virchows Archiv webpage (https://www.springer.com/medicine/pathology/journal/428). This study was entirely funded by departmental funds from the Department of Pathology at Penn State University College of Medicine.

Conflict of interest The authors declare that they have no conflicts of interest.

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