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Molecular Characterization of Sporadic Pediatric Thyroid Carcinoma with the DNA/RNA ThyroSeq v2 Next-Generation Sequencing Assay

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Abstract

The aim of this study was to test the hypothesis that our 60-gene DNA/RNA ThyroSeq v2 next-generation sequence (NGS) assay would identify additional genetic markers, including gene fusions in sporadic pediatric differentiated thyroid carcinomas (DTC) that had no known molecular alterations. Sporadic pediatric DTCs with informative molecular testing ($n = 18$) were studied. We previously tested 15 cases by our standard 7-gene (*BRAF, NRAS, HRAS, KRAS, RET/PTC1, RET/PTC3, PAX8/PPARg*) mutation panel. Three cases were not tested previously. The standard 7-gene panel identified molecular alterations in 9 of 15 tumors (60%). Cases analyzed by ThyroSeq v2 NGS included the six previously negative cases by the standard 7-gene panel and three cases not previously tested. The NGS assay revealed new gene fusions in four of six previously negative cases (67%). These gene fusions included *ETV6/NTRK3* ($n = 3$) and *TPR/NTRK1* ($n = 1$). A point mutation (*BRAF-V600E*) was detected in one of three untested cases. While standard testing could identify only molecular alterations in 60% of cases, with the addition of the ThyroSeq v2 NGS, this increased to 87% ($n = 13/15$). Some cases with chromosomal rearrangements, including *ETV6/NTRK3*, appear to be associated with an aggressive histopathologic phenotype, but had no documented history of radiation exposure. Additional work is needed to investigate if pediatric DTCs could benefit from a reclassification based on molecular subtypes, which may better reflect their underlying biologic potential. Our data support the use of broad gene panels for the molecular diagnostics of pediatric thyroid nodules to aid future classification, treatment, and clinical management recommendations.

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Keywords

ETV6/NTRK3; gene fusions; molecular diagnostics; molecular pathology; pediatric; thyroid neoplasms

INTRODUCTION

The molecular genetics of sporadic differentiated thyroid cancer (DTC) is better characterized in the adult literature when compared to pediatrics. Recent data indicate there is a greater risk of malignancy in pediatric thyroid nodules as opposed to adults [1,2]. Previous reports with pediatric cases have shown a higher prevalence of chromosomal rearrangements, especially *RET/PTC*, and lower prevalence of *BRAF* mutations compared to adults [1,3–6]. Chromosomal rearrangements are more frequent in young patients, particularly in children with a history of ionizing radiation exposure [7–10].

We have shown previously that the overall prevalence of molecular alterations in our pediatric cohort with DTC is lower (26%) [11] compared to adults (70%) [12] when using a standard 7-gene mutation panel which interrogates for point mutations in proto-oncogenes (*BRAF*, *NRAS*, *HRAS, KRAS*) and the most common chromosomal rearrangements (*RET/PTC1*, *RET/PTC3*, and *PAX8/PPARg*) known to occur in DTC. Recently, a novel chromosomal rearrangement, *ETV6/NTRK3*, was detected in pediatric radiation–induced PTC using a RNA-based next generation sequence (NGS) panel [8,10]; however, its overall prevalence and impact on outcome and survival in sporadic pediatric DTC is not known since most clinical molecular platforms do not screen for this novel chromosomal rearrangement.

We hypothesized that current thyroid molecular genetic testing with limited gene mutation and chromosomal rearrangement panels fails to identify potentially important molecular alterations associated with sporadic pediatric DTC. Identifying these alterations in preoperative cytologic material may significantly impact clinical management. Tumors with molecular alterations that are associated with more aggressive behavior could be treated with more comprehensive therapies, such as initial total thyroidectomy, extensive neck dissections, postoperative RAI, and more rigorous long-term surveillance [13], whereas small, indolent nodules without molecular alterations may be approached with more conservative treatment, as recommended by the new American Thyroid Association (ATA) Guidelines Task Force on Pediatric Thyroid Cancer [1]. We applied a new 60-gene ThyroSeq v2 next-generation sequence (NGS) assay to sporadic pediatric DTC to test our hypothesis that this DNA/RNA based assay would identify additional genetic markers in our pediatric DTC.

MATERIAL AND METHODS

Case selection and specimens

Study review and approval was obtained by our institutional review board at the University of Pittsburgh, in accord with the ethical standards established by the University of Pittsburgh. Review of the pathology database for pediatric patients younger than 18 years

with a diagnosis of DTC and surgical resection at Children's Hospital of Pittsburgh (CHP) of the University of Pittsburgh Medical Center (UPMC) from January 2002 to July 2014 was performed; 26 cases were identified (Supplemental Figure; http://dx.doi.org/10.2350/15-07-1667-OA.S1). The final surgical pathology was reviewed by two pediatric pathologists (J.P., J.O.) and an expert in thyroid pathology and molecular biology (Y.N.) to confirm diagnosis. Histopathology, cytopathology, and available clinical information, including a history of radiation exposure and outcome, were collected. Eight cases were excluded. One case was reclassified as a benign hyperplastic nodule after further review and was excluded from further study. One case with a previous history of adrenal neuroblastoma had a *STK11* point mutation in the neuroblastoma and papillary thyroid carcinoma. This point mutation subsequently was a germ-line mutation during clinical work-up and was not considered to be a sporadic DTC [14]. There was a high rate of cases with uninformative ThyroSeq v2 NGS molecular results that were excluded from further study (40%, $n = 6/15$; Supplemental Figure; http://dx.doi.org/10.2350/15-07-1667-OA.S1). This included one case with negative testing by the standard 7-gene mutation panel that did not have enough material to amplify by the ThyroSeq v2 NGS assay (eg, cytology material of a recurrence). Five cases without previous molecular testing had uninformative NGS testing with the available FFPE material (i.e., failed to amplify). These failed FFPE samples were unusual as they were old archival samples (>10 years old) and, thus, the quality of the nucleic acids were poor and were excluded from further analysis. Nine informative cases by ThyroSeq v2 NGS were included in the study (Supplemental Figure; http://dx.doi.org/10.2350/15-07-1667-OA.S1).

Nucleic acids isolation

Extraction of DNA and RNA from either thyroid FNA samples collected in the nucleic acid (DNA/RNA) stabilizing agent, frozen samples, or FFPE samples was performed as described previously [15]. For FFPE samples, manual microdissection was performed from unstained slides under the microscope with H&E guidance. Specimens with a minimum of 50% of tumor cells or at least 300 tumor cells in a microdissection target were accepted for analysis. DNA and RNA quantity and quality was evaluated by spectrophotometric (NanoDrop Instruments, Wilmington, DE, USA) and by fluorometric (Qubit; Life Technologies, Carlsbad, CA, USA) analysis.

Seven-gene mutation panel

The standard 7-gene mutation panel had been in use from 2007 to 2014 at our institution for testing specific point mutations in proto-oncogenes (*BRAF*, *HRAS*, *NRAS*, and *KRAS*) and chromosomal rearrangement (*RET/PTC1, RET/ PTC3, PAX8/PPARg*), as described previously [16,17]. Briefly, fine needle aspiration (FNA) material collected directly in nucleic acid preservation media during the FNA procedure underwent mutational analysis, for all final cytological diagnoses, except negative for malignant cells (benign) and unsatisfactory/nondiagnostic diagnoses. Using this criterion from April 2007, cytologic material from thyroid FNA cases ($n = 11$) and FFPE tissue from a needle-core biopsy ($n = 1$) underwent the standard 7-gene mutation panel testing before surgery. In four additional cases testing was performed on material obtained at the time of or after surgical resection,

including FFPE tissue of the TC specimen ($n = 3$) and cytological material from a recurrence ($n = 1$).

Mutational analysis was performed for *BRAF* (V600E), *NRAS* codon 61, *HRAS* codon 61, and *KRAS* codons 12 and 13 using real time polymerase chain reaction (PCR) and post-PCR fluorescence melting curve analysis on the LightCycler (Roche Life Science, Penzberg, Germany), as described previously [16,17]. Samples that were positive for a mutation on fluorescence melting curve analysis were confirmed by Sanger sequencing. The *RET/PTC1, RET/PTC3*, and *PAX8/PPARg* rearrangements were detected by real time reverse transcriptase PCR with primers designed to flank the respective fusion point on ABI 7500 (Applied Biosystems, Waltham, MA, USA), as described previously [16]. The adequacy of samples for molecular testing was based on the quantity and quality of isolated nucleic acids and the proportion of epithelial cells in the sample. Two controls (GAPDH and KRT7) were used to assess quality and quantity of sample as described previously [16].

Next generation sequencing assay for thyroid cancer (ThyroSeq v2)

An extended assay of mutations and chromosomal rearrangements were tested using NGS (DNA/RNA ThyroSeq v2) on Ion Torrent Personal Genome Machine (Life Technologies) with Sanger sequence confirmation using FFPE or frozen tissue on cases that were either negative by the standard 7-gene mutation panel ($n = 7$) or were without prior testing ($n = 8$). ThyroSeq v2 assay was used to test for point mutations and indels (i.e., insertions and deletions) in 14 genes and for 42 types of gene fusions known to occur in thyroid cancer as described previously [18]. Briefly, point mutations and indels were studied in >1000 hotspots of the following genes: *AKT1*, *BRAF*, *NRAS*, *HRAS*, *KRAS*, *PTEN*, *TP53*, *TSHR*, *GNAS*, *CTNNB1*, *RET*, *PIK3CA*, *TERT*, and *EIF1AX*. The analytic sensitivity of the NGS method was 3% of mutant alleles, and the clinical sensitivity was 5%. The NGS assay also tested for 42 types of gene fusions involving the *RET*, *BRAF*, *NTRK1*, *NTRK3*, *ALK*, *PPARG*, and *THADA* genes fused to different partners by sequencing of the fusion transcripts. The presence of at least 50 reads of the fusion transcript constituting 5% or more of all sequencing reads for a given tumor was required to consider the test positive.

Amplification of the *GAPDH* and *KRT7* genes was used to control quality and quantity of RNA and amount of epithelial cells present in the specimen. To assess the proportion of epithelial cells within a FNA sample, the expression of the housekeeping gene *GAPDH*, which is uniformly expressed in all cell types, was compared to the expression of the cytokeratin gene *KRT7*, which is expressed only in few distinct types of epithelial cells including thyroid cells. Expression of both genes was detected by real-time RT-PCR on ABI7500. The difference in expression between *KRT7* and *GAPDH* (i.e., CtKR7 - CtGAPDH) was used to correlate with adequacy of thyroid FNA sample.

RESULTS

Clinical characteristics

A total of 18 patients (15 [83%] females and 3 [17%]males; age range, 8–17 years; median, 13 years) underwent informative molecular testing (Table 1). The most frequent presenting

feature was a neck mass or a thyroid nodule ($n = 13$, 72%). PTC was noted in 17 patients, including four with encapsulated follicular variant PTC and one with a follicular carcinoma with capsular microinvasion (Table 1). Tumor size ranged from 0.8 to 7.0 cm, with a median tumor size of 2.2 cm. Two patients had a family history of PTC in a mother (case 10) and a paternal aunt (case 17). None of the patients had a documented history of radiation exposure.

Molecular analysis

Samples from 15 patients were analyzed previously using the standard 7-gene panel. Of these 15 cases, molecular alterations were identified in 9 (60%). These nine positive cases were not retested by the ThyroSeq v2 NGS assay (Table 1). In the six previously negative cases, the ThyroSeq v2 NGS assay revealed new chromosomal rearrangements in 4 (67%). Samples from three untested cases were analyzed using only the ThyroSeq v2 NGS and revealed a point mutation in one (33%) case (Table 1).

The ThyroSeq v2 NGS assay identified the novel *ETV6/ NTRK3* gene fusion in three cases that were negative previously by the standard panel. This fusion involves the translocation between exon 4 of the ETS variant gene 6 (*ETV6*) on chromosome 12p13, which encodes an ETS-related transcription regulator and exon 14 of the neurotrophin receptor 3 (*NTRK3*) gene on chromosome 15q25, which encodes a tyrosine kinase receptor. Additionally, the ThyroSeq v2 NGS assay also detected a *TPR/NTRK1* gene fusion in one case that previously was negative by the standard panel. This fusion involves an intrachromosomal inversion between exon 21 of *TPR* gene to exon 12 of neurotrophic tyrosine kinase receptor type 1 (*NTRK1*) on chromosome 1q. The ThyroSeq v2 NGS assay also revealed a *BRAF-V600E* point mutation in one previously untested case.

No molecular alterations were found in 4 cases, regardless of methodology, although in one of these cases, RNA amplification failed (Table 1, case 15). In the cases tested previously by the standard 7-gene panel, together with the ThyroSeq v2 NGS assay, 13 of 15 cases (87%) now revealed a molecular alteration, including chromosomal rearrangements in 53% ($n =$ 8/15) and gene mutations in 33% ($n = 5/15$). The 9 positive cases by the standard 7-gene panel were not retested with the NGS assay; thus, we do not know if there would have been any false-negative results (Supplemental Figure; http://dx.doi.org/10.2350/15-07-1667-OA.S1).

Histopathologic features of DTC with chromosomal rearrangements

The three cases with the *ETV6/NTKR3* rearrangement showed a PTC with an unencapsulated follicular variant (FV) pattern with minor components of solid, insular, or trabecular patterns, along with bands of thick fibrosis (Fig. 1). The cases showed variable angiolymphatic invasion and involvement of margins, with one case also showing diffuse bilateral involvement of the thyroid (Table 1).

The *TPR/NTRK1* case showed a similar morphologic pattern with an unencapsulated FV pattern of PTC with a minor trabecular pattern and thick fibrotic bands within the tumor (Fig. 1). The single *PAX8/PPARg* case showed an encapsulated FV pattern of PTC; however, over half of the nodule also displayed a striking solid to insular pattern (Fig. 1).

There was no evidence of necrosis or high-grade nuclear features to suggest a poorly-differentiated TC, but focal capsular break with microinvasion was noted (Table 1).

These chromosomal rearrangement cases showed a variable degree of follicular differentiation with more aggressive histologic qualities (i.e., nonencapsulation, solid growth pattern, extensive gland involvement, thick tumor fibrosis, margin involvement, and/or lymph node metastasis (Table 1) than is seen in typical follicular variant PTC (FVPTC).

The *RET/PTC* cases, while having a conventional PTC pattern, also showed minor solid or squamoid patterns with thick fibrotic bands and positive lymph nodes (Fig. 1, Table 1).

Histopathologic features of DTC with point mutations

The three cases with *RAS* mutations showed a uniform follicular variant pattern without evidence of a solid, insular, or trabecular pattern or intratumoral fibrosis (Fig. 1, Table 1). Central neck dissection at the time of initial surgery was not performed in these cases. The three cases with *BRAF-V600E* mutation showed conventional PTC with angiolymphatic invasion and positive lymph nodes when sampled (Table 1).

Two of the cases negative for mutations with the ThyroSeq v2 NGS assay showed a FVPTC with total encapsulation and no aggressive histologic features (Table 1, cases 16 and 17). However, one negative case by NGS (Table 1, case 18) did share similar histologic features with the chromosomal rearrangement cases, including extensive capsular invasion and thick tumor fibrosis.

Clinicopathologic correlations and outcomes

There were too few cases to draw significant conclusions about the impact of the preoperative mutation status/ indeterminate FNA cytology on surgical management outcomes in this small series. Of note, one case with indeterminate cytology and negative preoperative molecular testing by the standard 7-gene mutation panel underwent two surgeries (e.g., lobectomy, followed by completion thyroidectomy); however, a chromosomal rearrangement (*TPR/NTRK1*) was detected subsequently by the ThyroSeq v2 NGS assay.

All patients were alive at follow-up with no distant metastasis. Residual disease and/or recurrence were noted in three patients, whose tumors had molecular abnormalities, including chromosomal rearrangements (Case 1: *ETV6/NTRK3* and Case 4: *RET/PTC1*) and a point mutation (Case 10: *BRAF-V600E*).

DISCUSSION

This study demonstrated that the use of the ThyroSeq v2 NGS assay detects additional molecular alterations in pediatric DTC compared to a limited gene panel. In the 15 cases tested by the standard panel, molecular alterations were detected in 60%. With the addition of the ThyroSeq v2 NGS assay, the percentage with molecular alterations in this cohort (n = 15) increased to 87%, as the ThyroSeq v2 NGS assay revealed gene fusions in four previously negative cases. The ThyroSeq v2 NGS assay was able to reduce the percentage of

cases with an unknown oncogenic driver, similarly to large population-based studies, which also have shown significant reductions in the negative mutation rate of PTC (from 25% to 3.5%), with the use of NGS using both DNA and RNA assays [19]. In the cohort tested by both methods, there were a higher percentage of chromosomal rearrangements (53%) compared to point mutations (33%). We also report the discovery of the gene fusions *ETV6/NTRK3* and *TPR/NTRK1* among this small pediatric cohort, which has been associated previously with radiation-associated PTC in children [8,10]. No patient in our cohort had an identifiable history of radiation exposure. In large population based NGS studies of sporadic PTC, these gene fusions are rare events (<1%) [19].

While our study was too small to make definite conclusions, histopathologic findings suggestive of more aggressive disease (i.e., extensive gland involvement, positive margins/extracapsular spread, angiolymphatic invasion, and/or positive lymph nodes, along with a mixed pattern of papillary, follicular, and solid/insular/trabecular patterns) were noted among some of the gene fusion positive cases. Interestingly, similar histopathologic features also are described in radiation induced PTC with chromosomal rearrangements, which showed solid and follicular patterns with thick intratumoral fibrosis and inflammation, in addition to a conventional papillary pattern [7–10,20]. Also, our sporadic pediatric DTCs with the *ETV6/NTRK3* gene fusion showed an unencapsulated FV pattern with foci of solid growth, similar to the histopathology described in radiation associated PTC with a *ETV6/NTRK3* gene fusion [8].

In post-Chernobyl radiation induced TC, the *ETV6/NTRK3* gene fusion was identified with a NGS transcriptome RNA-Sequencing approach with fusion detection software and was validated by two independent groups [8,10]. The breakpoints of the *ETV6/NTRK3* gene fusion in thyroid carcinomas are different than other tumors with the same gene fusion (i.e., infantile fibrosarcoma, secretory breast carcinoma, acute myeloid leukemia). Nonthyroidal tumors show fusion of exons 1 to 4 or 1 to of *ETV6* with exons 13 to 18 of *NTRK3*. The typical fusion results in a constitutively tyrosine phosphorylated insulin receptor substrate 1 (*IRS1*) that functions as an adaptor protein linking the fusion protein to Ras-MAPK and the phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathways [10,21,22]. In radiation induced TC, Ricarte-Filho et al. [10] reported fusion of *ETV6* exons 1 to 4, which encodes a sterile a motif dimerization domain, to exons 12 to 18 of *NTRK3*. Expression of this thyroid specific fusion oncogene in radiation-exposed TC was able to activate the PI3K and MAPK signaling pathways through a constitutively active tyrosine kinase product. Similar to our cases, Leeman-Neill et al. [8] reported fusion of *ETV6* exon 4 or 5 to exons 14 to 18 of *NTRK3*. Their group showed evidence of tumor induction by ^{131}I or γ-radiation in vitro, and also showed that this fusion was the second most common rearrangement after *RET/PTC* in pediatric PTC associated with radiation exposure [8].

The high prevalence of fusion transcripts *ETV6/NTRK3*, *TPR/NTRK1*, *RET/PTC*, and our unique *PAX8/PPARg* case, together with the corresponding histopathologic features may suggest that these tumors have an increased susceptibility to chromosomal fragility potentially inducing such a change. To generate a chromosomal rearrangement, partner genes must be in close spatial proximity during interphase. As demonstrated previously in *RET/PTC* and *NTRK* rearrangements in thyroid cells [23,24] the spatial organization of the

genome is likely having a critical role in the generation of specific gene fusions [25,26]. More advanced epidemiologic investigation of these cases would be necessary to understand if a potential carcinogenic source or unrecognized radiation exposure could be driving these particular gene fusions in children, especially those with the *ETV6/NTRK3* rearrangement. It is important to note that case 18, while sharing histopathologic features similar to the chromosomal rearrangement cases, was negative for molecular alterations with the ThyroSeq v2 NGS assay. This case suggests that there still may be uncharacterized molecular alterations that could be driving pediatric DTC cases with aggressive features.

Strong evidence exists in the adult literature indicating that a refined molecular classification scheme reflects differences between subsets of PTCs, which could focus a more "personalized" surgical and medical treatment plan based on the underlying signaling pathways and biologic potential of these tumors [19]. Our findings emphasized the importance of using a molecular approach capable of detecting a wide range of molecular abnormalities in pediatric DTC, especially gene fusions. The benefits of this approach include better characterization of the molecular changes that will likely provide information regarding the biologic behavior of the tumor. Detection and characterization of biomarkers of aggressive disease on preoperative FNA evaluation will enable the most appropriate operation (e.g., lobectomy versus total thyroidectomy). In addition, identification of aggressive tumors will outline optimal strategies for long-term management, compared to the majority who may likely benefit from "lower-intensity" therapy [1]. This is especially important for more indolent tumors, such as encapsulated FVPTC and those associated with *RAS* mutations, where conservative treatment without completion lobectomy or RAI therapy may be sufficient [19,27]. None of our *RAS* mutated cases had overly aggressive qualities, although one did show focal capsular and vascular invasion (Case 12). Thus, the ability to characterize and distinguish between indolent encapsulated FVPTC and more aggressive PTC, with the help of molecular markers has an essential role in clinical management [11,19].

In conclusion, the molecular diagnostic approach to pediatric thyroid cancer should include broad DNA/RNA gene panels capable of detecting point mutations and the increasing number of gene fusions identified in pediatric patients. Our group has shown previously in the pediatric and adult literature that the identification of a positive molecular marker in the preoperative FNA cytology material provides an invaluable adjunct to plan appropriate surgical management [13,15,17]. We hope that our new data together with our collective institutional experience, provides additional support for the use of molecular studies in the evaluation of pediatric patients with thyroid cancer and thyroid nodules, which currently is not recommended by recent ATA Guidelines Task Force on Pediatric Thyroid Cancer [1].

The size of our study precludes conclusions on the behavioral statistics of these sporadic pediatric DTCs. Future investigations will be helpful to assess for a possible relationship between our apparently sporadic *ETV6/NTRK3* rearrangements and radiation-induced TC. In addition, the retrospective nature of the study limited the number of cases available for testing due to archival cases that failed to yield amplifiable DNA/RNA, as there was a high percentage of cases with uninformative results (Supplemental Figure; http://dx.doi.org/10.2350/15-07-1667-OA.S1). Prospective studies with good quality nucleic acid are needed

to improve the yield in future studies. In summary, our data support the use of broad DNA/RNA molecular panels to enhance the identification of molecular changes, which may aid future classification, treatment, and clinical management recommendations as we better characterize the natural history and basic biology of sporadic pediatric DTC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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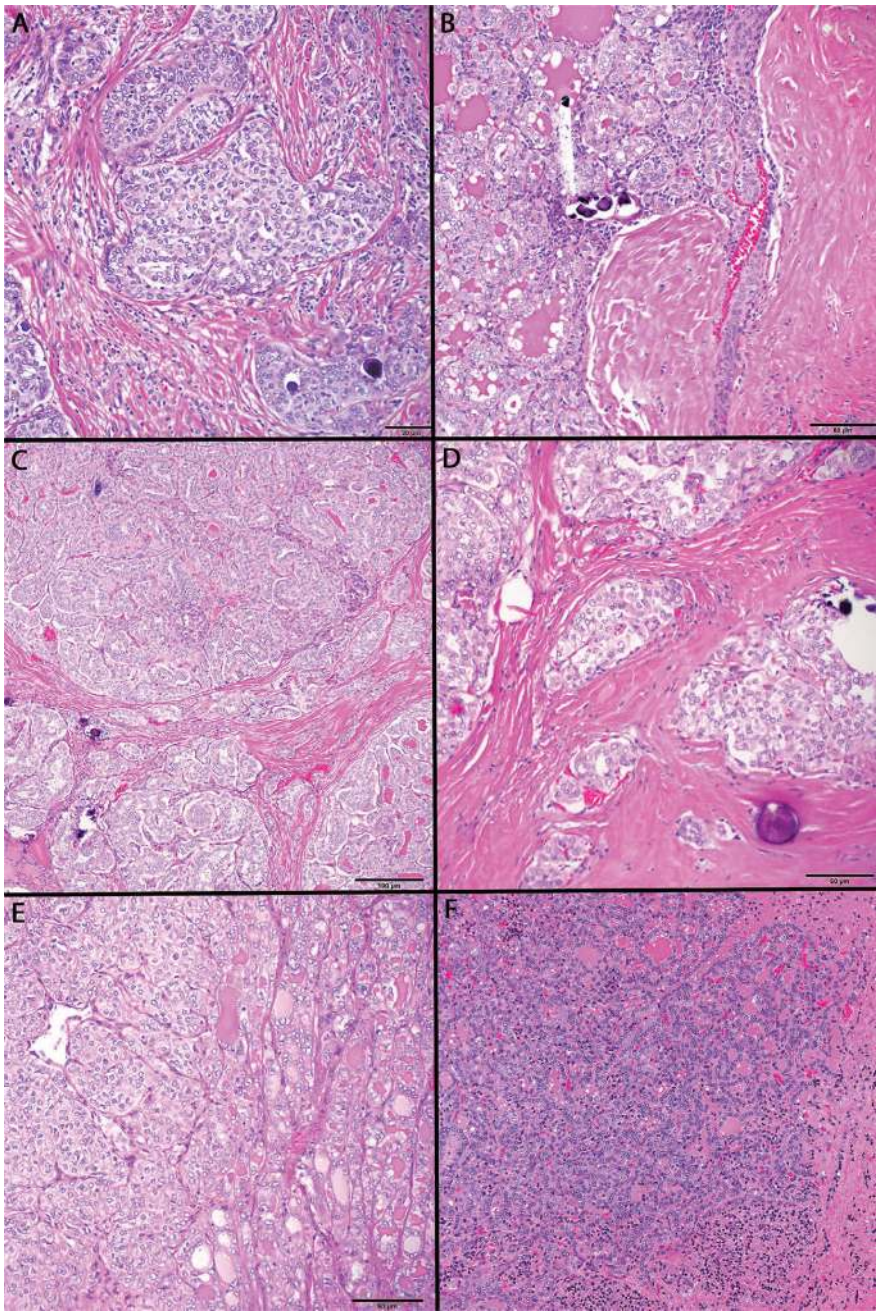

Figure 1.

Histologic patterns of sporadic pediatric papillary thyroid carcinoma (PTC) correlated with chromosomal rearrangements and point mutations. **A**. Case 1 PTC with *ETV6/NTRK3*, solid foci with thick bands of fibrosis and psammoma bodies. Hematoxylin and eosin (H&E), ×400. **B**. Case 3 PTC, with *ETV6INTRK3*, unencapsulated follicular variant pattern with thick bands of fibrosis and psammoma bodies. Hematoxylin and eosin (H&E,) ×200. **C**. Case 7 PTC with *TPRINTRK1*, admixed abortive papillae, follicular and trabecular patterns, fibrosis and psammoma bodies. H&E, ×100. **D**. Case 6 PTC with *RET/ PTC3*, solid and follicular foci, thick fibrosis, and psammoma bodies. H&E, ×200). **E**. Case 8 PTC with

PAX/PPARg, an encapsulated follicular variant with admixed solid/insular pattern. H&E, ×200). **F**. Case 13 PTC with *NRAS*, unencapsulated follicular variant without intratumoral fibrosis. H&E, ×100).

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Table 1

Molecular, cytologic, and histopathologic characteristics of sporadic pediatric differentiated thyroid carcinoma

Case	Age	Sex	7-Gene mutation panel (source)[a]	ThyroSeq v2NGS	FNA diagnosis	Histopathologic features	LN	ALI	Margin	pTNM stage
1	13	F	NEG (FNA)	ETV6/NTRK3	SM	PTC mixed FV unencap with insular/solid; bilateral-diffuse; fibrosis; PB; lymphocytic inflammation	43/98	Y	Y	T3aN1b
2	8	F	NEG (FNA)	ETV6/NTRK3	PM	PTC mixed FV unencap with trabecular/solid; fibrosis; lymphocytic inflammation surrounding tumor	0/5	N	Y	T2N0
3	17	F	NEG (FFPE)	ETV6/NTRK3	NA	PTC mixed FV unencap with foci of solid; fibrosis; few PB; mild lymphocytic inflammation surrounding tumor	NA	Y	N	T2Nx
4	12	M	RET/PTC1 (FNA)	ND	PM	PTC classic with closely packed papillae, with squamoid foci; bilateral-diffuse; fibrosis; PB	39/125	Y	Y	T3N1b
5	16	F	RET/PTC1 (FNA)	ND	SM	PTC classic with FV, solid; R lobe-diffuse; fibrosis; few PB	1/16 (+2 PB)	N	N (close)	T1N1
6	12	F	RET/PTC3 (FNA)	ND	PM	PTC classic with FV, solid; multifocal; fibrosis; PB; inflammation surrounding tumor	14/52	N	Y	T2N1b
7	8	F	NEG (FNA)	TPR/NTRK1	SM (FNA/bx)	PTC unencap FV, trabecular, abortive papillae, multifocal, fibrosis, PB	2/3	Y	N	T1bN1a
8	12	F	PAX8/PPARg (bx)	ND	SM (bx)	PTC encap FV with solid/insular; capsular microinvasion	0/9	N	N	T1bN0
9	15	F	BRAF (FNA)	ND	PM	PTC conventional; thin fibrous bands	3/7	Y	N	T2N1a
10	13	F	BRAF (FNA)	ND	PM	PTC conventional with focal trabecular pattern; multifocal fibrosis	14/14	Y	N (<1 mm)	T2N1a
11	14	F	ND	BRAF (FFPE)	PM	PTC conventional; multifocal PB	NA	Y	N	T1Nx
12	13	F	HRAS (FNA)	ND	AUS/FLUS (FNA) SFON (bx)	PTC encap FV with focal capsular invasion	NA	Y	N	T2Nx
13	16	M	NRAS (FNA)	ND	AUS/FLUS -> FN/SFN (2nd)	PTC unencap FV (only partially encap)	NA	N	N (<1 mm)	T1aNx
14	17	F	NRAS (FFPE)	ND	NEG/BEN	PTC encap FV	0/4[b]	N	N	T2N0
15	13	F	NEG (FNA)	DNA NEG; RNA-NA	AUS/FLUS -> SMAL (2nd)	FC, capsular microinvasion	0/1	N	N	T1bN0
16	10	M	NEG (FFPE)	NEG (FFPE)	NA	PTC encap FV	0/1	N	N	T2N0
17	14	F	ND	NEG (FFPE)	NEG/BEN	PTC encap FV	NA	N	N	T3Nx
18	17	F	ND	NEG	SM	PTC unencap FV (partially encap with extensive invasion); multifocal, fibrosis	0/3	Y	N	T1N0

LN indicates lymph node involvement; ALI, angiolymphatic invasion; pTNM Stage, AJCC Cancer Staging, Tumor, Nodal, Metastasis; NEG, negative; ND, testing not done previously; PM, positive for malignant cells; SM, suspicious for malignant cells; FN/SFN, follicular or oncocytic neoplasm/suspicious for follicular or oncocytic neoplasm; AUS/FLUS, atypia or follicular lesion of undetermined

significance; NEG/BEN, negative or benign; NA, data not available; PTC, papillary thyroid carcinoma; FV, follicular variant; FC, follicular carcinoma; encap, total encapsulation; unencap, unencapsulated or partial encapsulation; PB, psammoma bodies; R, right; L, left.

[a] Unless otherwise specified, source is frozen tissue.

[b] Lymph node dissection at completion thyroidectomy.