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Characterization of thyroid cancer driven by known and novel *ALK* fusions

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Abstract

ALK fusions are found in various tumors, including thyroid cancer, and serve as a diagnostic marker and therapeutic target. Spectrum and outcomes of *ALK* fusions found in thyroid nodules and cancer are not fully characterized. We report a series of 44 *ALK*-translocated thyroid neoplasms, including 31 identified preoperatively in thyroid fine-needle aspirates (FNA). The average patients' age was 43 years (range, 8–76 years); only one with radiation history. All 19 resected thyroid nodules with *ALK* fusion identified preoperatively were malignant. Among nodules with known surgical pathology ($n = 32$), 84% were papillary thyroid carcinomas (PTCs) and 16% poorly differentiated thyroid carcinomas (PDTCs). PTCs showed infiltrative growth with follicular architecture seen exclusively (30%) or in combination with papillary and/or solid growth (37%). Tumor multifocality was seen in 10 (31%) PTC cases. Most PDTC had a well-differentiated PTC component. Lymph node metastases were identified in 10/18 (56%) patients with neck dissection. The most common *ALK* fusion partners were *STRN* ($n = 22$) and *EML4* ($n = 17$). In five cases, novel *ALK* fusion partners were discovered. All five PDTCs carried *STRN-ALK* fusion. On follow-up, ten patients were free of disease at 2–108 months, whereas two patients with PDTC died of disease. In summary, *ALK* fusion-positive thyroid carcinomas are typically infiltrative PTC with common follicular growth, which may show tumor dedifferentiation associated with increased mortality. Compared to *EML4-ALK*, *STRN-ALK* may be more common in PDTC, and ~10% of *ALK* fusions occur to rare gene partners. When *ALK* fusion is detected preoperatively in FNA samples, malignancy should be expected.

Key Words

- ▶ ALK
- ▶ FNA
- ▶ papillary thyroid carcinoma
- ▶ NIFTP

Endocrine-Related Cancer
(2019) **26**, 803–814

Introduction

Thyroid cancer is one of the most quickly increasing in incidence cancer types (Siegel *et al.* 2019), with papillary thyroid carcinoma (PTC) being the predominant morphologic type. The diagnosis and management of thyroid cancer depends on clinical stage, morphologic type, and molecular profile (Fagin & Wells 2016,

Durante *et al.* 2018). Since the initial discovery and characterization of *ALK* fusions in thyroid cancer (Cancer Genome Atlas Research Network 2014, Kelly *et al.* 2014, Perot *et al.* 2014), additional details have emerged. In some prior studies, *ALK* fusions, most frequently with striatin (*STRN*) or echinoderm microtubule-associated

protein-like 4 (*EML4*), have been reported in 1–3% of PTCs and with a higher frequency in poorly differentiated thyroid carcinomas (PDTCs) (Cancer Genome Atlas Research Network 2014, Kelly *et al.* 2014, Chou *et al.* 2015, Landa *et al.* 2016, Bastos *et al.* 2018). Several anaplastic thyroid carcinomas carrying *STRN-ALK* have also been reported (Kelly *et al.* 2014, Perot *et al.* 2014, Godbert *et al.* 2015). The role of *STRN-ALK* in the progression from PTC to PDTC thyroid carcinoma has been documented (Nikitski *et al.* 2018). More recently, new fusion partners of *ALK* have been identified in thyroid cancer including *TFG*, *GTF2IRD1*, and *CCDC149* (McFadden *et al.* 2014, Landa *et al.* 2016, Vanden Borre *et al.* 2017). However, the prevalence and spectrum of specific *ALK* fusion types and impact of *ALK* fusion partners on tumor phenotype and outcomes remain unknown.

The standard approach to thyroid cancer includes surgery followed by radioactive iodine (RAI) for carcinomas with a higher risk of recurrence. In the management of thyroid nodules and cancers, *ALK* fusions represent both a diagnostic marker and a potential therapeutic target. The role of *ALK* as a therapeutic target is being highlighted by a number of case reports of patients with *ALK*-positive thyroid cancer, including cases of metastatic and RAI-resistant cancer treated with Crizotinib (Demeure *et al.* 2014, Godbert *et al.* 2015, Ji *et al.* 2015, Van Der Tuin *et al.* 2019).

We have previously shown that thyroid fine-needle aspirates (FNA) are a reliable source for the detection of *ALK* fusions, assisting in the diagnosis and management of cytologically indeterminate thyroid nodules (Nikiforova *et al.* 2018). In prior studies, *ALK*-translocated thyroid carcinomas were identified by testing resected and histologically diagnosed thyroid carcinomas. The frequency of *ALK*-translocated thyroid neoplasms prospectively identified by molecular testing of thyroid FNAs is not known and the cytological and histopathological characteristics of these nodules have not been reported.

A significant proportion of *ALK*-translocated thyroid carcinomas were reported as follicular variant of PTC prior to the reclassification of the encapsulated forms of such tumors as non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) (Nikiforov *et al.* 2016). Therefore, whether or not *ALK* fusions may occur in NIFTP remains unknown.

In this study, we report the detailed analysis of a large series of *ALK*-translocated thyroid tumors, many of which were identified preoperatively by testing thyroid FNA samples, with particular focus on (i) histopathologic characteristics of tumors, including features of NIFTP, (ii) outcomes when detected preoperatively, and

(iii) spectrum of *ALK* fusion partners and discovery of novel types of *ALK* fusions.

Methods

Patient selection and study samples

Surgically resected formalin-fixed paraffin-embedded (FFPE) tissue or thyroid FNA samples were used to identify *ALK* fusions (Tables 1 and 2, and Supplementary Table 1, see section on supplementary data given at the end of this article). Thirteen patients with *ALK*-translocated thyroid carcinomas were identified by testing FFPE tissue from surgical resections (Table 1). Surgically resected cases were tested on clinical request, as part of patient's management. Thirty-one additional patients were identified by testing thyroid FNA samples, 19 of which underwent thyroid surgery (Table 2); 12 of these patients did not have follow-up data available, and it is unknown whether thyroid surgery was performed (Supplementary Table 1). Snap-frozen tissue and FFPE tissue were collected at the Department of Pathology, University of Pittsburgh Medical Center (UPMC) following approval by the University of Pittsburgh Institutional Review Board (IRB 991206). This project used the University of Pittsburgh Medical Center, Hillman Cancer Center and Tissue and Research Pathology/Pittsburgh Biospecimen Core shared resource which is supported in part by award P30CA047904. Study was done in accordance with US Federal Policy for the Protection of Human Subjects.

Pathologic examination

Two authors (S I C and Y E N) reviewed the pathology slides. In addition to standard TNM staging parameters (American Joint Committee on Cancer, 8th edition), growth pattern, histologic variant, presence of infiltrative growth, and criteria for NIFTP were considered (Nikiforov *et al.* 2016, 2018). Follicular variant was defined as having no appreciable (<1%) papillary component. PDTCs were diagnosed following Turin criteria (Volante *et al.* 2007). For *ALK* immunohistochemical analysis, 4µm FFPE sections were hybridized with primary monoclonal antibodies (Anaplastic Lymphoma Kinase, D5F3, Cell Signaling Technology, Inc) following the manufacturer's protocol.

Detection of *ALK* fusions

Testing of thyroid FNAs and FFPE-resected thyroid carcinomas as part of clinical care was performed using

Table 1 Clinicopathologic summary of patients with ALK fusion identified by testing thyroid resections.

Case #	Age, years	Sex	FNA, BC	Type of surgery	Size, cm	Pathology	pT	pN	Type of ALK fusion	Post-operative RAI	Follow-up, months
1	58	F	n.d.	TT	1.3	iFV	1b	x	e3_STRN-e20_ALK	No	NED, 192
2	61	F	V	TT, CCND	3	Mixed	2	0	e3_STRN-e20_ALK	129.1 mCi	NED, 108
3	36	F	IV	TT	1.5	Mixed	1b	x	e13_EML4-e20_ALK	75 mCi	NED, 78
4	60	F	V	TT	2	Classic	1b	x	e3_STRN-e20_ALK	No	NED, 69
5	72	F	IV	TT	4.2	PDTC	3	x	e3_STRN-e20_ALK	107 mCi	DOD, 37
6	52	M	n.d.	HT	7.5	PDTC	3	x	e3_STRN-e20_ALK	Unknown	DOD, 32
7	29	M	IV	TT, LND	3.3	Mixed	2	1b	e3_STRN-e20_ALK	150 mCi	NED, 20
8	33	F	n.d.	TT	6.5	iFV	3	x	e6ab_EML4-e20_ALK	No	NED, 17
9	54	M	VI	TT, LND	3.8	PDTC	3	1b	e3_STRN-e20_ALK	n.a.	n.a.
10	74	F	n.d.	TT	5	PDTC	3	1a	e3_STRN-e20_ALK	n.a.	n.a.
11	16	F	III	TT, CCND	2	Mixed	3	1a	e3_STRN-e20_ALK	n.a.	n.a.
12	19	F	n.d.	TT, CCND	1.4	iFV	1b	1a	e3_STRN-e20_ALK	n.a.	n.a.
13	8	M	n.d.	TT, LND	0.9	Solid, classic	1a	1b	e3_STRN-e20_ALK	n.a.	n.a.

BC, Bethesda category; CCND, central compartment neck dissection; DOD, died of disease; F, female; HT, hemithyroidectomy; iFV, infiltrative follicular variant, 'mixed' refers to a combination of conventional and follicular growth and was ultimately classified as 'classic PTC'; LND, lateral neck dissection; M, male; n.a., not available; n.d., not done; NED, no evidence of disease; PDTC, poorly differentiated thyroid carcinoma; RAI, radioactive iodine therapy; TT, total thyroidectomy.

targeted next-generation sequencing-based ThyroSeq® v3 assay as previously described (Nikiforova *et al.* 2018, Steward *et al.* 2019). The assay uses RNA to test for all previously reported types of *ALK* fusions and also detects the expression levels of *ALK* tyrosine kinase (TK) domain and of extracellular (EC) domain. Samples with disproportional overexpression of the TK over EC domain of *ALK* and negative for known types of *ALK* fusion underwent whole transcriptome analysis (RNA-Seq).

RNA-Seq and data analysis

Total RNA was used to prepare the library for RNA exome sequencing with Illumina TruSeq™ RNA Exome Sample Preparation kit v1 (Illumina, Inc). The prepared libraries were assessed using an Agilent 2200 TapeStation, and then denatured with sodium hydroxide and loaded on Illumina HiSeq2500. Cluster generation and paired-end sequencing were performed using HiSeq Paired-End Rapid Cluster kit v2 and HiSeq Rapid SBS kit v2 (Illumina, Inc). The filtered high-quality reads were aligned to the human genome (hg19-GRCh37) using TopHat aligner (Trapnell *et al.* 2009) and number of reads mapped to each gene was calculated using RSeM (Li & Dewey 2011) and featureCounts (Liao *et al.* 2014). Gene fusions were detected using Q-score (Li *et al.* 2008), Chimerascan with custom filtering algorithm (Iyer *et al.* 2011), Atlas (Huret *et al.* 2013), CIS (RTCGD) (Akagi *et al.* 2004), and CHCG (Futreal *et al.* 2004) databases. The junctions and the individual exon expression levels were used to visualize the fusion point.

RT-PCR and Sanger sequencing

RNA was subjected to DNase treatment using the Invitrogen DNA-free™ kit (Thermo Fisher Scientific). The RNA from frozen tissues was reverse transcribed by Applied Biosystems High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). The RNA from FFPE tissues and FNA was reverse transcribed by Invitrogen SuperScript IV VILO Master Mix (Thermo Fisher Scientific). RT-PCR was conducted using HotStarTaq DNA Polymerase (Qiagen), and primers listed in Supplementary Table 2. The RT-PCR products were sequenced in both directions using the Applied Biosystems BigDye Terminator Kit and an ABI 3130xl DNA Sequencer (Thermo Fisher Scientific).

Western blot analysis

Total protein was isolated from frozen tissue homogenized in RIPA buffer (Boston BioProducts, Ashland, MA, USA)

Table 2 Clinicopathologic summary of patients with ALK fusion identified by testing fine needle aspirates.

Case #	Age, years	Sex	FNA, BC	Type of surgery	Size, cm	Pathology	pT	pN	Type of ALK fusion	Post-operative RAI	Follow-up, months
1	32	F	III	TT, LND	5.4	Mixed	3	1b	e3_STRN-e20_ALK	150 mCi	NED, 44
2	43	M	V	TT	1.3	Classic	1b	0	e3_STRN-e20_ALK	No	NED, 23
3	32	F	V	TT, CCND	3.7	Mixed	2	0	e1_CTSB (UTR)-e17_ALK	No	NED, 2
4	61	F	V	TT	2.2	iFV	2	0	e3_STRN-e20_ALK	53 mCi	NED, 6
5	22	F	III	HT	1.2	Mixed	1b	x	e6_EML4-e18_ALK	n.a.	n.a.
6 ^a	76	F	VI	HT	4.2	PDTC	3	x	e3_STRN-e20_ALK	n.a.	n.a.
7	63	F	III	HT	0.5	Classic	1a	x	e3_STRN-e20_ALK	n.a.	n.a.
8	34	F	III	HT	3	Classic	2	x	e3_STRN-e20_ALK	n.a.	n.a.
9	41	F	III	TT	3	iFV	2	x	e1_CTSB (UTR)-e16_ALK	n.a.	n.a.
10	58	F	III	TT, CCND	3.5	Mixed	2	1a	e3_STRN-e20_ALK	n.a.	n.a.
11	12	F	VI	TT, LND	2.2	Classic	2	1b	e9_PPP1R21-e20_ALK	n.a.	n.a.
12	69	F	III	TT	0.8	Mixed	1a	0	e6_EML4-e18_ALK	n.a.	n.a.
13	28	M	III	HT	3.3	iFV	2	x	e6_EML4-e20_ALK	No	n.a.
14	32	F	IV	TT, CCND	2.8	iFV	2	0	e6_EML4-e20_ALK	n.a.	n.a.
15	47	F	III	HT	2.7	Mixed	2	0	e6_EML4-e20_ALK	n.a.	n.a.
16	27	F	III	TT	1	Classic	1a	0	e13_EML4-e20_ALK	n.a.	n.a.
17	30	F	III	TT	0.7	Classic	1a	x	e20ab_EML4-e20_ALK	n.a.	n.a.
18	24	F	III	HT	1.4	iFV	1b	x	e6_EML4-e20_ALK	n.a.	n.a.
19	41	M	III	TT	2	Classic	1b	1a	e13_EML4-e20_ALK	n.a.	n.a.

^aThis poorly differentiated thyroid carcinoma also harbored *TERT* C250T, c.-146 C > T mutation BC, Bethesda category; CCND, central compartment neck dissection; F, female; HT, hemithyroidectomy; iFV, infiltrative follicular variant; 'mixed' refers to a combination of conventional and follicular growth and was ultimately classified as 'classic PTC'; LND, lateral neck dissection; M, male; n.a., not available; NED, no evidence of disease; PDTC, poorly differentiated thyroid carcinoma; RAI, radioactive iodine therapy; TT, total thyroidectomy.

using OMNI-GLH (OMNI International, Kennesaw, GA, USA); 40 µg of total protein was resolved by SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad Laboratories). The membranes were incubated overnight at 4°C with ALK (D5F3, 1:2000, Cell Signaling) or β-actin (1:1000, Cell Signaling) antibodies, followed by anti-rabbit (1:10000, Promega) HRP-conjugated secondary antibody and signals were detected by ECL (GE Healthcare).

Results

Clinical and morphologic characteristics

Forty-four patients with *ALK*-translocated thyroid neoplasms were identified in either surgically removed tumors ($n=13$) or thyroid FNA samples ($n=31$). The overall average age of patients was 43.4 years (range, 8–76 years), including two pediatric patients, an 8-year-old boy and a 12-year-old girl. Overall, female patients were predominant, 36/44 (82%). The average size of *ALK*-translocated thyroid nodules was 2.8 cm (range, 0.7–7.5 cm). Thyroid FNA was performed on 38 patients and diagnosed in cytology as Bethesda Category (BC) III (25/38, 66%), BC IV (4/38, 10%), BC V (6/38, 16%), and BC VI (3/38, 8%). Additional clinicopathologic and molecular parameters of 44 patients with *ALK*-translocated thyroid neoplasms are summarized in Tables 1 and 2, and Supplementary Table 1. Only one patient had a remote history of therapeutic exposure to RAI, reportedly for multinodular goiter (case #12, Table 2).

Surgical pathology data were available for 32 patients (Tables 1 and 2). The most common procedure was a total thyroidectomy (24/32, 75%). The most common histologic diagnosis was that of well-differentiated PTC (27/32, 84%), followed by five cases of PDTC (5/32, 16%). The PTC had a predominantly follicular growth pattern with areas of papillary and/or solid growth in 10 cases (10/27, 37%) or without papillary growth meeting the criteria for infiltrative FV PTC (8/27, 30%) (Fig. 1). The remaining 33% (9/27) of PTC had predominantly classic papillary growth pattern with no significant follicular component. Overall, although the proportion of conventional papillary component varied, 19/27 (70%) of PTCs were categorized as classic PTCs due to the presence of well-formed papillary structures. Papillae were typically tightly packed and covered by cells with well-developed nuclear features of PTC. The neoplastic follicles were typically irregularly shaped and contained a significant amount of colloid. Nuclear features of PTC were overall

less prominent in the areas of follicular growth, with nuclear pseudoinclusions only rarely found. Multifocal PTC was noted in ten cases (10/32, 31%).

PDTC carrying *ALK* fusions had predominantly solid growth with a minor component of trabecular and insular architecture in some cases (Fig. 2A and B). Necrosis was identifiable in all cases. The nuclei of PDTC cells were either of smaller size with dark uniformly dispersed chromatin (three tumors, Fig. 2C) or larger size with vesicular chromatin (two tumors, Fig. 2D). Nuclear features of PTC were lost in these areas. Four out of five PDTC tumors also contained areas of well-differentiated PTC, either follicular variant or classic type. One PDTC showed an additional genetic alteration, *TERT* C250T mutation (Case #6, Table 2), whereas all other tumors had an *ALK* fusion as the only driver mutation identified.

Figure 1

Histopathologic features of representative cases of papillary thyroid carcinoma with *ALK* fusions. (A) Papillary thyroid carcinoma with mixed conventional and follicular growth; component of follicular growth was well circumscribed and thinly encapsulated. H&E, 100x. (B) Interface between follicular (left) and tightly packed papillary (right) components. H&E, 200x. (C) Well-developed nuclear features of papillary thyroid carcinoma (including nuclear pseudo-inclusions, arrow) in the conventional component. H&E, 400x. (D) A minor (<10%) component of solid growth with mitosis (arrow; 1 in 10 high power (200x) fields). H&E, 600x. (E) Lateral neck lymph node with metastatic papillary thyroid carcinoma, mixed follicular and conventional. H&E, 40x. (F) ALK immunohistochemistry, strong diffuse cytoplasmic staining of tumor cells and weak colloid-only background staining in the adjacent normal thyroid, 400x.

Figure 2

Histopathologic features of poorly differentiated thyroid carcinomas with *ALK* fusions. (A) Solid and trabecular growth (H&E, 40×). (B) Areas of tumor necrosis (H&E, 400×). (C) This tumor shows smaller, dark nuclei and significant amount of cytoplasm (H&E, 200×). (D) This tumor has punctate necrosis, larger nuclei with vesicular chromatin, smooth nuclear contours without grooves or pseudoinclusions, and small amount of cytoplasm (H&E, 200×).

Extrathyroidal extension was identified in six cancers (6/32, 19%), four of which were PDTC. There were 11 cases with pT2, 8 cases with pT3, and 8 cases with pT1b. Lymph node dissection was performed in 18 patients and resulted in pN0 ($n=8$), pN1a ($n=5$), and pN1b ($n=5$). Of ten patients with lymph node metastases, two patients had PDTC.

ALK IHC was performed on 12 tumors and was positive in 11 cases (11/12, 92%). One case showed indeterminate ALK IHC result and the lack of staining was likely caused by suboptimal tissue preservation in a 10-year-old FFPE tissue block.

Clinical follow-up was available for 12 patients (Tables 1 and 2). Five patients received post-operative RAI therapy (dose ranging from 53 to 150 mCi). Ten patients were free of disease 2–108 months after thyroid surgery. Two patients with PDTC developed multiple distant metastases and died of disease 32 and 37 months after surgery.

Preoperative detection in thyroid FNA samples

Thirty-one nodules positive for *ALK* fusions were identified by testing thyroid FNA samples. This included 15 cases with *ALK* fusions detected prospectively by ThyroSeq testing of 11,211 consecutive thyroid FNAs with indeterminate cytologic diagnosis over an 11-month period from December 2017 to September 2018, yielding the frequency of 0.13%. Overall, among all nodules with

ALK fusions first detected in FNA samples, the cytological diagnosis of Bethesda Category (BC) III was established in 23 (74%) cases, BC IV in 1 (3%), BC V in 5 (16%), and BC VI in 2 (7%).

Nineteen nodules positive for *ALK* fusions in thyroid FNA samples had a known surgical pathology outcome. All 19 were ultimately diagnosed as malignant, including 18 PTC and 1 PDTC. In all but one case, the carcinoma was diagnosed by primary case pathologists. In Case #5 (Table 2), the 1.2 cm thyroid nodule initially diagnosed as NIFTP was mostly well circumscribed, but it was lobulated, had complex central fibrous septae outlining the initial primary capsule of a smaller nodule, and a minor conventional component of PTC (Fig. 3A, B and C). ALK immunohistochemistry highlighted foci of infiltration at the lesion-normal tissue interface (Fig. 3D).

Among patients with *ALK* fusions identified by testing preoperative FNA samples (Table 2), the frequency of known lymph node metastases was 4/19, 21%.

Spectrum of *ALK* fusions and detection of novel fusion partners

The most common fusion partners of *ALK* were *STRN* (22/44, 50%) and *EML4* (17/44, 39%). In 5/44 (11%) cases, *ALK* fusions involving novel partners were identified.

Figure 3

Histopathologic features of papillary thyroid carcinoma initially diagnosed as NIFTP (case #5, Table 2). (A) This 1.2 cm thyroid mass was mostly well circumscribed and lobulated. The center of the mass showed complex fibrous septae, perhaps outlining the initial primary capsule of the smaller nodule. H&E, 40×. (B) About 5% of the mass showed branching papillae covered by follicular cells with nuclear features of papillary thyroid carcinoma. H&E, 600×. (C) Periphery of the mass with split thin capsule and permeating tumor cells. H&E, 100×. (D) ALK immunohistochemistry highlighting the focus of infiltration shown in (C), 100×.

Table 3 New types of ALK rearrangements detected in thyroid cancer.

Fusion name	Cytogenetic characteristics	Dimerization domain of ALK fusion partner	Encoding ALK exons	Predicted protein size
<i>ITSN2</i> e29- <i>ALK</i> e18	t(2;2);(p23.3;p23.2)	Coiled-coil	e18-e29	1841 aa
<i>PPP1R21</i> e9/i9- <i>ALK</i> e20	t(2;2);(p16.3;p23.2)	Coiled-coil	e20-e29	844 aa
<i>PPP1R21</i> e7- <i>ALK</i> e20	t(2;2);(p16.3;p23.2)	Coiled-coil	e20-e29	794 aa
<i>CTSB</i> e1- <i>ALK</i> e16	t(8;2);(p23.1;p23.2)	None ^a	e16-e29	711 aa ^a
<i>CTSB</i> e1- <i>ALK</i> e17	t(8;2);(p23.1;p23.2)	None ^a	e17-e29	671 aa ^a

^aTranslation start codon in ALK exon16 or ALK exon 17; exon 1 of the partner gene untranslated

These cases initially showed a preferential overexpression of the tyrosine kinase domain of *ALK* over the extracellular domain, suggesting the presence of *ALK* fusion, with no known fusion types identified. On subsequent RNA-Seq analysis, these cases revealed in-frame *ALK* fusion to novel partner genes including cathepsin B (*CTSB*) ($n=2$), protein phosphatase 1 regulatory subunit 21 (*PPP1R21*) ($n=2$), and intersectin 2 (*ITSN2*) ($n=1$) (Table 3). Unlike *ALK*, all of these three genes were found to be expressed in normal thyroid follicular cells, driving the expression of the fused part of *ALK* coding for the tyrosine kinase domain of the protein.

Two of these tumors revealed the fusion between exon 1 of *CTSB* and either exon 17 (Fig. 4) or exon 16

(Supplementary Fig. 1) of *ALK*. Interestingly, exon 1 of *CTSB* is part of the 5'-UTR and is untranslated, suggesting the presence of an alternative translation site in the chimeric genes. Indeed, alternative translation initiation sites in *ALK* exons 16 and 17 have been reported (Gasteiger *et al.* 2003). In the *CTSB* exon 1-*ALK* exon 17 fusion, a putative translation start codon is located in *ALK* exon 17 at chr2:29,450,506-29,450,504 (GRCh37/hg19). The expression of ALK protein in this tumor was confirmed by immunohistochemistry (Fig. 1F) and Western blot analysis (Fig. 4D). In *CTSB* exon 1-*ALK* exon 16 fusion, a putative alternative translation start codon in *ALK* exon 16 is located at chr2:29,451,837-29,451,835 (GRCh37/hg19).

Figure 4

Identification and confirmation of *CTSB* (exon 1)-*ALK* (exon 17) fusion. (A) Analysis of mRNA expression levels of individual *CTSB* and *ALK* exons using RNA-Seq data showing overexpression of *ALK* exons 17–29 downstream of the fusion point. Each box represents an exon and the boxes are colored according to the logarithm of their expression levels as measured in reads per kilobase per million reads. (B) RT-PCR confirmation of *CTSB*-*ALK* fusion. L, 100-bp ladder; N, normal tissue; NC, negative control; T, tumor. (C) Sanger sequencing confirmation of *CTSB* (exon 1)-*ALK* (exon 17) fusion. Ex, exon; UTR, untranslated region. (D) Western blot analysis of papillary thyroid carcinomas positive (PTC+) and negative (PTC-) for *CTSB*-*ALK* fusion and corresponding normal tissue (N). Western blot analysis using ALK antibody showed three bands around 70 to 80 kDa, suggesting the ALK protein translation from more than one start codon in *ALK* exon 17 or post-translational protein modifications. No ALK protein was detected in normal thyroid tissue or in thyroid tumors lacking this fusion. (E) Schematic representation of the predicted fusion protein retaining the transmembrane domain (TM) and tyrosine kinase (TK) domain of ALK. Propeptide, Peptidase C1A-propeptide; Peptidase C1A, c-terminal; MAM, merpin, A5 protein and receptor protein tyrosine mu; LB, ligand-binding domain.

Another two cases showed fusions between *PPP1R21* either exon 7 (Supplementary Fig. 2) or exon 9 (Supplementary Fig. 3) and exon 20 of *ALK*. In the latter case, RT-PCR analysis showed the retention of a 17-nucleotide fragment of *PPP1R21* intron 9 at the fusion site, leading to an in-frame mRNA transcript. Finally, a fusion between exon 29 of *ITSN2* and exon 18 of *ALK* was found in one case (Supplementary Fig. 4).

Among 32 cases with surgical pathology data available, there were 18 tumors with *STRN-ALK*, 11 with *EML4-ALK*, and 3 with novel types of *ALK* fusions. All five PDTC cases carried *STRN-ALK* ($P=0.07$, Fisher exact probability test). Among PTCs, no significant variability in the predominance of follicular, solid, or classic papillary architecture was noted between tumors with different types of *ALK* fusions.

Discussion

In this study, we provide an in-depth characterization of the largest cohort of patients with *ALK*-translocated thyroid cancer reported to date. The average age of patients in our series was comparable to that of patients with PTCs unselected for genetic alterations: 43.4 (8–76) versus 46.8 (15–89) years, respectively (Cancer Genome Atlas Research Network 2014). The current study describes two pediatric patients, confirming previous observations of this fusion occurring in pediatric patients (Chou *et al.* 2015, Park *et al.* 2015, Vanden Borre *et al.* 2017, Arndt *et al.* 2018). The overall predilection for female patients appears to be more prominent in *ALK*-translocated group: 4.5:1 versus 2.7:1 among PTC patients unselected for *ALK* fusions (Cancer Genome Atlas Research Network 2014). The age and sex distribution of *ALK*-translocated thyroid carcinomas in this study are comparable to those previously reported (Table 4).

ALK-translocated thyroid carcinomas can occur both in patients with and without a history of radiation exposure. However, the prevalence of *ALK* fusions appears to be higher, up to 7.6–9% (Hamatani *et al.* 2012, Arndt *et al.* 2018, Efanov *et al.* 2018), in PTCs from radiation-exposed patients. An additional approach to increasing the chance of identifying *ALK* fusions is to exclude thyroid carcinomas with other, more common driver mutations such as *BRAF*, *RAS*, and *RET/PTC*. Of 44 *ALK*-translocated thyroid carcinomas presented here, only one PDTC showed an additional genetic alteration – *TERT* promoter mutation. Other studies identified an RAI-refractory conventional PTC with *EML4-ALK* fusion and *TERT*

mutation (Van Der Tuin *et al.* 2019), *STRN/ALK*-positive PDTC with *TERT* mutation, and an *EML4/ALK*-positive PDTC with *ATM* mutation (Landa *et al.* 2016). Whole-genome sequencing of an RAI-resistant and recurrent PTC with *EML4-ALK* fusion revealed co-existing alterations in 55 genes, many of which are likely passenger mutations (Demeure *et al.* 2014).

Since 2012, about 60 patients with *ALK*-translocated thyroid cancer have been reported in different studies, ranging from papillary thyroid microcarcinomas to anaplastic thyroid carcinomas, with a significant number of those diagnosed as a follicular variant of PTC. Cases of PTC presented in this study and in the literature (Table 4) were evaluated with attention to identifying the most characteristic histology that could help to trigger testing for *ALK* fusions and exploring whether the presence of *ALK* fusion is compatible with the diagnosis of NIFTP.

Morphologically, while not pathognomonic, *ALK*-translocated PTCs appear to be characterized either by a purely follicular architecture with infiltrative growth or predominance of follicular growth with islands of tightly packed papillae or classic morphology. Our analysis suggests that tumors with pure classic papillary growth pattern and absence of an appreciable follicular component are less likely to carry *ALK* fusions. However, we acknowledge that *ALK* fusions have been reported in tumors with other morphologies, such as diffuse-sclerosing variant of PTC (Table 4). Overall, younger to middle-aged females with infiltrative thyroid cancer showing follicular architecture and negative for *BRAF*, *RAS*, and other common genetic alterations are the likeliest to harbor an *ALK* fusion.

With the growing role of *ALK* as a diagnostic FNA marker, it was important to establish whether *ALK* fusion can be seen in benign thyroid nodules or NIFTP (Nikiforov *et al.* 2016, 2018). To date, 156 follicular adenomas were shown to be negative for *ALK* fusions (Perot *et al.* 2014, Park *et al.* 2015, Bastos *et al.* 2018, Nikiforova *et al.* 2018). Here we show that only one of 19 *ALK*-translocated neoplasms (with *ALK* fusion first identified in FNA) mimicked NIFTP. This is consistent with the data in the literature. Indeed, out of about 28 PTCs described with sufficient morphologic details (i.e., with comments on variant morphology and invasion), there were no cases of non-invasive follicular variant of PTC (Table 4), rendering the possibility of an *ALK*-positive NIFTP unlikely.

When follicular growth is predominant and infiltrative growth is subtle, *ALK* IHC represents a practical tool that helps to assess for invasion more objectively, especially if *ALK* fusion was already identified by molecular testing of preoperative FNA. In this study, *ALK* IHC was

Table 4 Summary of ALK-positive thyroid cancers from prior studies with basic clinicopathologic information.

ALK +/total, n (%), histologic type	Female/male	Age, average (range), years	Morphologic variants and features	Recurrence?	Fusion partner	Reference
11/498, 2.2%, PTC	11:0	38 (13–68)	Diffuse sclerosing, <i>n</i> = 3; iFV, <i>n</i> = 6 [§]	No	<i>EML4</i> , <i>n</i> = 8	Chou <i>et al.</i> 2015
2/129, 1.5%, FVPTC	n.a.	n.a.	PTC, <i>n</i> = 2	No (<i>n</i> = 1)	<i>EML4</i> , <i>TFG</i>	McFadden <i>et al.</i> 2014
4/392, 1%, thyroid carcinomas	3:1	37 (13–50)	Classic, <i>n</i> = 2; solid, <i>n</i> = 1; FV (ETE), <i>n</i> = 1;	No	<i>STRN</i> , <i>n</i> = 2	Park <i>et al.</i> 2015
10/116, 8.6%, PTC	8:2	42 (34–47)	iFV, <i>n</i> = 4; classic, <i>n</i> = 2; FV PTC, NOS, <i>n</i> = 4	Yes, 1/8	<i>EML4</i> , <i>n</i> = 6; <i>STRN</i> , <i>n</i> = 4	Bastos <i>et al.</i> 2018
1/15, PTC	0:1	31	Classic PTC, <i>n</i> = 1	No	<i>EML4</i>	Cipriani <i>et al.</i> 2017
1 PTC ^a	0:1	62	TCV PTC	Yes, DM	<i>EML4</i>	Demeure <i>et al.</i> 2014
1/12, PTC ^b	0:1	27	Classic PTC	No	<i>EML4</i>	Pfeifer <i>et al.</i> 2019
1/59, PTC ^c	0:1	60	Classic PTC	AWD, 5 years	<i>EML4</i>	van der Tuin <i>et al.</i> 2019
3/303, 1%, PTC	3:0	10 (7–15)	PTC, pN+, <i>n</i> = 1; PTC, NOS, <i>n</i> = 2	n.a.	<i>EML4</i> , <i>STRN</i> , <i>GTF2IRD1</i>	Vanden Borre <i>et al.</i> 2017
3/75, thyroid carcinomas	1:0 ^e	71	Classic PTC, <i>n</i> = 2; ATC, <i>n</i> = 1	AWD ^e	<i>STRN</i>	Perot <i>et al.</i> 2014, Godbert <i>et al.</i> 2015
3/84, 3.5% PDTC; 0/33 ATC	1:2	(30–89) ^f	PDTC, <i>n</i> = 3	Died, <i>n</i> = 2	<i>STRN</i> , <i>EML4</i> , <i>CCDC149</i>	Landa <i>et al.</i> 2016
7/77 (9%), PTC ^d	5:2	23 (16–34)	FV PTC, <i>n</i> = 1; PTC NOS, <i>n</i> = 6	n.a.	<i>EML4</i> , <i>n</i> = 2	Arndt <i>et al.</i> 2018

^aStable disease after 6 months of Crizotinib treatment.^bThyroid carcinomas negative for common mutations (e.g., BRAF, RAS).^cRAI-refractory thyroid carcinomas.^dPTC in radiation exposed patients.^eThe sex of two patients was not reported. Response after 6 months of Crizotinib therapy.^fAge of individual patients was not reported.[§]There were also mixed PTC (*n* = 2), tall cell variant of PTC (*n* = 1), oncocytic PTC (*n* = 1), Warthin-like PTC (*n* = 1). Only *EML4*, as *ALK* fusion partner, was specifically tested for.

ATC, anaplastic thyroid carcinoma; AWD, alive with disease; DM, distant metastasis; ETE, extrathyroidal extension; FVPTC, follicular variant of PTC; iFV, infiltrative follicular variant; n.a., not available; NOS, not otherwise specified; PDTC, poorly differentiated thyroid carcinoma; PTC, papillary thyroid carcinoma; RAI, radioactive iodine; TCV, tall cell variant.

indeterminate in 1 of 12 cases. In the literature, there are at least two cases with known *ALK* fusion (identified by FISH or other methods) and negative ALK IHC (Perot *et al.* 2014, Jeon *et al.* 2019).

Sixteen percent of *ALK*-translocated tumors in this study were poorly differentiated, fully meeting the Turin diagnostic criteria for PDTC (Volante *et al.* 2007). Interestingly, the nuclei of cells in these tumors showed two different types of morphology, correlating with the observation in the animal model of *STRN*-*ALK*-driven PDTC (Nikitski *et al.* 2019). Most PDTC had a well-differentiated PTC component, suggesting that the tumors initially developed as PTC before undergoing dedifferentiation.

Previously reported cases of *ALK*-translocated thyroid carcinomas were typically identified using surgically removed tumors. In this study, we explored the frequency of *ALK* fusions in a large consecutive series of thyroid FNA samples with predominantly indeterminate FNA cytology.

We observed that a small but distinct proportion of these aspirates were found to be positive for *ALK* fusions, and all of those nodules were cancers. Many of these FNAs were diagnosed as Bethesda III by cytology, which is likely due to the presence of abundant colloid and only a moderate degree of expression of nuclear features of PTC in many of these tumors. In another study of *ALK*-positive tumors, the pre-operative cytology of such nodules was reported in four cases and included Bethesda V in three and Bethesda III in one case (Park *et al.* 2015).

Two most common fusion partners of *ALK* were *STRN* and *EML4* in this study and in the literature (Table 4). While we did not identify previously reported *ALK* fusion partners such as *TFG*, *GTF2IRD1*, or *CCDC149*, we identified fusions with *ITSN2*, *CTSB*, and *PPP1R21* genes. Of those, *ITSN2*-*ALK* and *CTSB*-*ALK* fusions have not been previously described. The *ITSN2* gene encodes Intersectin 2, a member of multidomain adaptor/scaffold proteins that assemble multimeric complexes implicated in

clathrin- and caveolin-mediated endocytosis and rearrangements of the actin cytoskeleton (Predescu *et al.* 2003, Tsyba *et al.* 2011). *PPP1R21*-*ALK* fusion was previously reported in colorectal carcinoma (Yakirevich *et al.* 2016) and was not known to occur in thyroid cancer. *PPP1R21* is essential for endosome functions (Rehman *et al.* 2019). In *ALK* fusions discovered prior to this report, such as *STRN*-*ALK* or *EML4*-*ALK*, *ALK* kinase was known to be activated via a ligand-independent dimerization of the chimeric protein mediated by the coiled-coil (CC) domain of the fusion partner with constitutive phosphorylation on Tyr1278 of *ALK* (Kelly *et al.* 2014, Holla *et al.* 2017). Activation through CC domain dimerization is the likely mechanism of *ALK* activation in *PPP1R21*-*ALK* and *ITSN2*-*ALK* fusions. In *CTSB*-*ALK*, *ALK* is fused with an untranslated exon 1 of *CTSB*, with predicted translation starting in *ALK* exon 16 or 17, suggesting a novel mechanism of *ALK* expression in these fusions.

The availability of a large number of tumors with *STRN*-*ALK* or *EML4*-*ALK* fusion allowed us to explore for the first time whether different fusion partners are associated with specific phenotypical or clinical tumor characteristics. A potentially interesting finding was that all five PDTC in this study carried *STRN*-*ALK* fusions. The number of tumors studied here was still limited and cases of PDTC carrying *EML4*-*ALK* have been reported (Landa *et al.* 2016). Nevertheless, the results of this study raise the possibility that tumors driven by *STRN*-*ALK* fusions may have a higher propensity for dedifferentiation. Among PTCs, we observed no differences with respect to phenotypical characteristics of tumors driven by these two main types of *ALK* fusion.

Clinical behavior of thyroid cancer driven by *ALK* fusions is not well studied. In our series, two patients who died of disease had PDTC with distant metastases, whereas no mortality was observed among patients with PTC on limited follow-up. *ALK*-driven PTCs were multifocal in about one-third of the cases and frequently showed infiltrative growth and lymph node metastases. However, when follow-up was available, ten patients with PTC were found to be free of disease. In the literature, 23 patients with *ALK*-translocated thyroid cancer showed no disease recurrence, while distant metastases, persistent disease requiring Crizotinib treatment and deaths of two patients with *ALK*-translocated PDTC have been reported (Table 4).

In summary, we confirm the occurrence of *ALK* fusions in a subset of thyroid cancers, specifically in PTC and PDTC, and demonstrate that *STRN* and *EML4* are the two most common *ALK* fusion partners, with ~10%

of *ALK* fusions are to other rare partner genes. The most typical histologic appearance of *ALK*-translocated PTC is that of infiltrative follicular variant or predominant follicular growth with a component of papillary growth. We further demonstrated the utility of *ALK* fusions in identifying thyroid cancer in cytologically indeterminate FNA samples. The presence of an *ALK* fusion practically rules out the diagnosis of NIFTP. Aggressive cases of *ALK*-positive thyroid carcinomas are typically PDTC, and the results of this study raise the possibility that tumors driven by *STRN*-*ALK* fusions may have a higher predisposition to dedifferentiation (compared to thyroid carcinomas with *EML4*-*ALK* fusion). Further studies with higher numbers of distinct *ALK* fusion partners including the novel ones presented here, and more comprehensive clinical follow-up, are needed to determine the clinical behavior of *ALK*-driven thyroid carcinomas.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/ERC-19-0325>.

Declaration of interest

Drs Nikiforov and Nikiforova own IP and receive royalties related to ThyroSeq. Dr Nikiforova has a consultancy agreement with Loxo Oncology.

Funding

This work was supported in part by the NIH grant R01 CA181150 to Y E N. This project used the University of Pittsburgh Medical Center, Hillman Cancer Center and Tissue and Research Pathology/Pittsburgh Biospecimen Core shared resource which is supported in part by award P30CA047904.

Acknowledgements

The authors thank Jessica Tebbets for her excellent administrative assistance.

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Received in final form 17 September 2019

Accepted 19 September 2019

Accepted Preprint published online 19 September 2019