Identification of the transforming *STRN-ALK* fusion as a potential therapeutic target in the aggressive forms of thyroid cancer

Lindsey M. Kelly^{a,1}, Guillermo Barila^{b,1}, Pengyuan Liu^{c,1}, Viktoria N. Evdokimova^a, Sumita Trivedi^d, Federica Panebianco^a, Manoj Gandhi^a, Sally E. Carty^e, Steven P. Hodak^f, Jianhua Luo^a, Sanja Dacic^a, Yan P. Yu^a, Marina N. Nikiforova^a, Robert L. Ferris^d, Daniel L. Altschuler^b, and Yuri E. Nikiforov^{a,2}

^aDepartment of Pathology and Laboratory Medicine, ^bDepartment of Pharmacology and Chemical Biology, ^cDepartment of Otolaryngology, ^dDepartment of Surgery, Division of Endocrine Surgery, and ^eDepartment of Medicine, Division of Endocrinology and Metabolism, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213; and ^fDepartment of Physiology and Cancer Center, Medical College of Wisconsin, Milwaukee, WI 53226

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Thyroid cancer is a common endocrine malignancy that encompasses well-differentiated as well as dedifferentiated cancer types. The latter tumors have high mortality and lack effective therapies. Using a paired-end RNA-sequencing approach, we report the discovery of rearrangements involving the anaplastic lymphoma kinase (*ALK*) gene in thyroid cancer. The most common of these involves a fusion between *ALK* and the striatin (*STRN*) gene, which is the result of a complex rearrangement involving the short arm of chromosome 2. *STRN-ALK* leads to constitutive activation of *ALK* kinase via dimerization mediated by the coiled-coil domain of *STRN* and to a kinase-dependent, thyroid-stimulating hormone-independent proliferation of thyroid cells. Moreover, expression of *STRN-ALK* transforms cells in vitro and induces tumor formation in nude mice. The kinase activity of *STRN-ALK* and the *ALK*-induced cell growth can be blocked by the *ALK* inhibitors crizotinib and TAE684. In addition to well-differentiated papillary cancer, *STRN-ALK* was found with a higher prevalence in poorly differentiated and anaplastic thyroid cancers, and it did not overlap with other known driver mutations in these tumors. Our data demonstrate that *STRN-ALK* fusion occurs in a subset of patients with highly aggressive types of thyroid cancer and provide initial evidence suggesting that it may represent a therapeutic target for these patients.

Thyroid cancer is a common type of endocrine neoplasia and typically arises from follicular thyroid cancer (FTC) cells. It encompasses well-differentiated papillary thyroid cancer (PTC) and FTC, which can dedifferentiate and give rise to poorly differentiated thyroid cancer (PDTC) and anaplastic thyroid cancer (ATC). Some cases of PDTC and ATC are believed to develop de novo (i.e., without a preexisting stage of well-differentiated cancer). Although only a small proportion of well-differentiated thyroid cancer tumors have aggressive biological behavior, PDTC has a 10-y survival rate of ~50% and ATC is one of the most lethal types of human cancer, with a median patient survival of 5 mo after diagnosis (1–3). Such low survival of patients who have dedifferentiated tumors is due to the propensity of the tumors for extrathyroidal spread and loss of the ability to trap iodine, which confers tumor insensitivity to the standard radioiodine therapy. Therefore, better understanding of the genetic mechanisms of tumor dedifferentiation and unraveling of effective therapeutic targets for these tumors are important for improving outcomes for these patients.

Currently, well-characterized driver mutations are known to occur in ~70% of PTC and ~50% of PDTC and ATC, including point mutations, such as those of v-Raf murine sarcoma viral oncogene homolog B1 (*BRAF*) and *RAS*, and chromosomal rearrangements involving rearranged during transfection (*RET*), peroxisome proliferator-activated receptor γ (*PPAR* γ), and neurotrophic tyrosine kinase, receptor, type 1 (*NTRK1*) genes (4, 5).

However, a significant proportion of thyroid cancers have no known driver mutations. The discovery of novel genetic events has been accelerated more recently due to the availability of next-generation sequencing approaches that allow investigators to obtain information on the entire genome, exome, or transcriptome of tumor cells (6). In this study, we used whole-transcriptome [RNA-sequencing (RNA-Seq)] analysis to identify novel gene fusions in thyroid cancer. We report the discovery and characterization of the recurrent striatin (*STRN*) gene and anaplastic lymphoma kinase (*ALK*) gene fusion, which may represent a previously unknown mechanism of thyroid cancer dedifferentiation and may be exploited as a potential therapeutic target for the most aggressive forms of thyroid cancer.

Results

Identification of *ALK* Fusions in Thyroid Cancer Using RNA-Seq. To search for novel driver gene fusions in thyroid cancer, we studied a group of 446 PTC cases with snap-frozen tumor tissue available. Tumors were prescreened for common known mutations believed to be driver events in thyroid cancer (*BRAF*, *NRAS*,

Significance

Thyroid cancer is common and has an excellent outcome in many cases, although a proportion of these tumors have a progressive clinical course and high mortality. Using whole-transcriptome (RNA-sequencing) analysis, we discovered previously unknown genetic events, anaplastic lymphoma kinase (*ALK*) gene fusions, in thyroid cancer and demonstrate that they occur more often in aggressive cancers. The most common fusion identified in these tumors involved the striatin (*STRN*) gene, and we show that it is transforming and tumorigenic in vivo. Finally, we demonstrate that the kinase activity of *STRN-ALK* can be blocked by *ALK* inhibitors, raising a possibility that *ALK* fusions may be used as a therapeutic target for patients with the most aggressive and frequently lethal forms of thyroid cancer.

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¹L.M.K., G.B., and P.L. contributed equally to this work.

²To whom correspondence should be addressed. E-mail: nikiforove@upmc.edu.

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HRAS, *KRAS*, *RET/PTC*, and *PAX8-PPAR γ*). Overall, 317 (71%) cancers were found to carry one of these mutational events (Table S1). The remaining 129 (29%) mutation-negative tumors were selected for further analysis. Among those, 21 cases were used for the paired-end whole-transcriptome sequencing (RNA-Seq) on an Illumina HiSeq sequencing system (Table S2). Of those, three tumors were found to have fusions involving the *ALK* gene (Fig. 1). One of these was a fusion between the echinoderm microtubule-associated protein-like 4 (*EML4*) and *ALK* genes. The fusion point in the chimeric transcript was located between exon 13 of *EML4* and exon 20 of *ALK*, identical to variant 1 of the *EML4-ALK* fusion previously described in lung cancer (7). The other two tumors showed a fusion between exon 3 of the *STRN* gene and exon 20 of *ALK*. Both fusion partners are located on the short arm of chromosome 2 (2p22.2 and 2p23, separated by ~7.5 Mb), indicating that the fusion is a result of intrachromosomal paracentric rearrangement. RT-PCR followed by Sanger sequencing confirmed the fusion breakpoints in all three tumors identified by RNA-Seq, and all rearrangements were validated at the DNA level by FISH, using the break-apart and fusion probe designs (Fig. 1). Using tumor DNA and an array of primers located in the respective gene introns, unique genomic fusion points positive for *STRN-ALK* were identified for both tumors (Fig. S1).

However, both tumors carrying *STRN-ALK* revealed no reciprocal fusions detected by RNA-Seq, RT-PCR, or PCR. Instead, they showed additional fusions involving genes located in this region of chromosome 2p, indicating that *STRN-ALK* is part of a complex rearrangement involving this chromosomal region. On RNA-Seq analysis, one tumor carrying *STRN-ALK* revealed five additional fusions involving transcripts of nine genes located within the 15-Mb region of chromosome 2p (Fig. 1D). This was further confirmed by FISH, which showed several smaller signals from the fragmented *ALK* and *STRN* probes in addition to the fusion between the portions of *STRN* and *ALK* (Fig. 1E). The clustering of breakpoints of multiple rearrangements in this region on chromosome 2p raises the possibility that a recently

described phenomenon of chromothripsis (8) may be responsible for the generation of *STRN-ALK* fusions in thyroid cells.

The *STRN* gene encodes STRN, a member of the calmodulin-binding WD repeat protein family believed to act as Ca²⁺-dependent scaffold proteins (9, 10). It contains four putative protein-protein interaction domains, including a caveolin-binding domain (55–63 aa), a coiled-coil domain (70–166 aa), a calcium-dependent calmodulin-binding domain (149–166 aa), and the WD-repeat region (419–780 aa). The predicted fusion protein retains the N-terminal caveolin-binding and coiled-coil domains of STRN fused to the intracellular juxtamembrane region of ALK (Fig. 2A). Western blot analysis of the tumors carrying *STRN-ALK* using an antibody to the C terminus of ALK showed a band of ~75 kDa, corresponding to the predicted molecular mass of 77 kDa for the fusion protein (Fig. 2B). No ALK protein was detected in normal thyroid tissue or in thyroid tumors lacking this fusion. These results were confirmed by quantitative RT-PCR; although WT ALK is expressed in normal thyroid cells at a very low level, thyroid tumors carrying the *STRN-ALK* or *EML4-ALK* fusion showed, on average, a 55-fold (range: 34.3- to 82.2-fold) increase in the expression of the 3'-portion of *ALK* (Fig. 2C). In all tumors examined, the fusion point between exons 19 and 20 of *ALK* is expected to result in the loss of its extracellular and transmembrane domains, and thus its cell membrane anchoring. This was confirmed by immunohistochemistry with ALK antibody, which showed diffuse cytoplasmic localization of both *STRN-ALK* and *EML4-ALK* fusion proteins in tumor cells (Fig. 2D). The nucleotide sequence of *STRN-ALK* was deposited in the GenBank database (Fig. S2).

Biochemical and Biological Characterization of *STRN-ALK*. To study functional consequences of *STRN-ALK* fusions, we generated the HA epitope-tagged expression plasmids for *STRN-ALK*: *STRN-ALK* (K230M), in which Lys230 (Lys1150 in the WT ALK) in the ATP-binding site is substituted by Met, which is known to produce a kinase-dead protein (7); *STRN-ALK* (Δ CB), a mutant with internal deletion of the caveolin-binding domain

Fig. 1. *ALK* gene fusions in thyroid cancer. (A) Chromosomal location of *ALK* and its fusion partners, *EML4* and *STRN*, involved in gene rearrangements identified in PTC by RNA-Seq. (B) Confirmation of the *EML4-ALK* fusion by RT-PCR, Sanger sequencing, and FISH with the break-apart *ALK* probe, showing splitting of one pair of red and green signals (arrows). L, 100-bp ladder; N, normal tissue; NC, negative control; T, tumor. (C) Confirmation of the *STRN-ALK* fusion by RT-PCR, Sanger sequencing, and FISH with the break-apart *ALK* probe, showing the loss of green signal in one of the signal pairs (arrows). (D) Scheme of gene fusions identified by RNA-Seq in a 15-Mb region of chromosome (Chr.) 2p in a tumor carrying the *STRN-ALK* fusion. (E) FISH with probes for *STRN* (green) and *ALK* (red) showing fusion between the two probes (arrows) and several small fragments of each probe in the tumor cell nuclei, indicating further rearrangements of the part of each probe not involved in the *STRN-ALK* fusion.

To examine whether the increased kinase activity of STRN-ALK affects cell proliferation and transformation of thyroid cells, rat thyroid PCCL3 cells were transfected with STRN-ALK and kinase-dead STRN-ALK (K230M) plasmids and assessed for cell proliferation using BrdU labeling. Cells expressing STRN-ALK showed increased thyroid-stimulating hormone (TSH)-independent cell proliferation that was dependent on ALK kinase activity (Fig. 4*A* and *B*). Moreover, cells expressing STRN-ALK developed a spindle-shaped and birefringent appearance typically associated with a transformed-like phenotype. The tumorigenicity of STRN-ALK was assayed by injecting 1×10^7 transfected NIH 3T3 cells into nude mice. The cells transfected with STRN-ALK developed s.c. tumors (seven of eight inoculations) that were recognizable 13 d after inoculation, whereas untreated NIH 3T3 cells and cells transfected with kinase-dead STRN-ALK (K230M) did not develop tumors (none of eight inoculations for each) (Fig. 4*C* and *D*). Microscopic examination of tumors that arose in the inoculated cells expressing STRN-ALK revealed a fibrosarcoma-like appearance with high mitotic activity and focal tumor necrosis, which are the features of high-grade malignancy

Fig. 4. STRN-ALK increases proliferation and induces cell transformation and tumor formation. PCCL3 cells transfected with HA-STRN-ALK and kinase-dead HA-STRN-ALK (K230M) showed cytosolic expression of the introduced protein on HA immunofluorescence and the kinase-dependent transformed-like phenotype seen as spindle-shaped and birefringent cells (*A*) and kinase-dependent proliferative response as assessed by BrdU labeling (*B*). (*C–E*) Transformation and tumorigenic properties of NIH 3T3 cells transfected with empty vector, STRN-ALK, and kinase-dead STRN-ALK (K230M). (*C*) Kinetics of tumor growth in xenografts of NIH 3T3 cells in nude mice injected s.c. in the neck with 1×10^7 cells expressing STRN-ALK, kinase-dead STRN-ALK (K230M), or nontransfected NIH 3T3 cells. (*D*) Representative mice showing tumor formation (arrow) at the site of injections of cells expressing STRN-ALK and no tumor formation at the site of injection of kinase-dead STRN-ALK (K230M). (*E*, Left) Microscopic appearance of tumors formed at the site of inoculation showing sheets of spindle cells with five or more mitoses (arrows) seen per one high-power field. H&E stain. The tumor cells express the HA-STRN-ALK construct as seen by immunofluorescence with anti-HA antibody (Center) and immunohistochemistry with anti-ALK antibody (Right). (Magnification: 200 $\times$.)

(Fig. 4*E*). These results demonstrate that STRN-ALK fusion leads to the activation of ALK, increased cell proliferation, and cell transformation in vitro and in vivo.

Prevalence of ALK Fusions in Various Types of Thyroid Cancer and Association with Aggressive Disease. Screening of an additional 235 well-differentiated PTCs by RT-PCR revealed one other tumor positive for STRN-ALK, resulting in a total finding of three STRN-ALK and one EML4-ALK fusions, an overall frequency of four (1.6%) fusions in 256 samples of this tumor type. Further analysis detected STRN-ALK in three (9%) of 35 PDCs and one (4%) of 24 ATCs (Fig. S3). Other types of thyroid cancer, including 36 FTCs and 22 medullary carcinomas, were negative. All detected ALK fusions were STRN-ALK, and no additional cases of EML4-ALK were found. The prevalence of ALK fusions was significantly higher in tumors prone to dedifferentiation ($P < 0.05$, Fisher's exact test) (Fig. 5*A*).

Phenotypically, PTC positive for ALK fusions had a predominantly or entirely follicular growth pattern with small areas of papillae formation (Fig. 5*B*). Two of the four tumors had aggressive features at presentation, such as extrathyroidal extension and/or lymph node metastasis. These two tumors had an advanced stage [tumor, node, metastasis (TNM) stage III] at presentation, whereas two other PTCs were TNM stage I–II. Among the three PDCs carrying STRN-ALK, two had areas of residual well-differentiated PTC with a follicular growth pattern (Fig. 5*C*). Two of the patients had widely disseminated disease at presentation. The STRN-ALK-positive ATC had a large area of residual follicular variant PTC (Fig. 5*D*). This patient died 6 mo after the diagnosis due to widely metastatic disease. All eight tumors carrying ALK fusions were negative for BRAF, RAS, or other driver mutations known to occur in $\sim 70\%$ of thyroid cancers. None of these patients had a documented history of radiation exposure. These findings indicate that ALK rearrangements occur in well-differentiated PTC with a predominantly follicular growth pattern, as well as in dedifferentiated tumors that are likely to develop from preexisting PTC with follicular architecture. The fact that ALK fusions do not overlap with other driver mutations in thyroid cancer suggests that they are likely to be independent driver events that may govern dedifferentiation of PTC with a characteristic follicular phenotype.

Inhibition of STRN-ALK Kinase and Cell Growth by ALK Inhibitors. A number of ALK tyrosine kinase inhibitors are readily available, including the aminopyridine ALK inhibitor crizotinib, which has been approved by the US Food and Drug Administration for treatment of EML4-ALK-positive lung cancer due to a significant response rate and low toxicity (14). Therefore, STRN-ALK fusions may potentially represent a promising therapeutic target for thyroid cancer. An in vitro immunoprecipitation-coupled kinase assay using the synthetic YFF (Tyrosine–Phenylalanine–Phenylalanine) peptide substrate (12) was optimized for inhibitor testing. As shown in Fig. 6*A*, kinase-dependent YFF phosphorylation can be detected with linear product accumulation up to 20 min. Next, sensitivity to ALK inhibitors was assessed in STRN-ALK and crizotinib-resistant STRN-ALK (G349S) mutant protein, in which Gly349 (Gly1269 of the WT ALK) in the ATP-binding pocket is replaced with Ser, resulting in a loss of sensitivity to crizotinib but not to a diaminopyrimidine ALK inhibitor, TAE684 (15). Dose-responses (measured at 15 min) confirmed that STRN-ALK is sensitive to the ALK inhibitors crizotinib and TAE684, with an $IC_{50} \sim 250$ nM and ~ 8 nM, respectively (Fig. 6*B* and *C*). Although TAE684 showed similar potency to STRN-ALK (G349S) and STRN-ALK, the former was resistant to the effect of crizotinib up to 3 μ M, a concentration that fully inhibited WT STRN-ALK.

Further, we studied the effect of ALK inhibitors on the proliferation of thyroid cells driven by STRN-ALK. Thyroid PCCL3 cells expressing either STRN-ALK or crizotinib-resistant STRN-ALK

Fig. 5. Prevalence and phenotypic features of thyroid cancer associated with *ALK* fusions. (A) Prevalence of *ALK* fusions in PTC, PDTC, and ATC. (B) Well-differentiated PTC with a predominantly follicular growth pattern and focal papillary structures. (C) PDTC with areas of residual well-differentiated PTC with a follicular growth pattern (arrows). (D) ATC with a neighboring area of well-differentiated PTC with a follicular growth pattern (arrows). H&E stain. (Magnification: 100 $\times$.)

(G349S) cultured in the absence of TSH were treated with different concentrations of crizotinib. Growth inhibition was observed in cells expressing STRN-ALK with an IC_{50} of $\sim 0.2 \mu M$ (Fig. 6D), similar to the levels found to block the kinase activity of ALK. The inhibition of growth of cells expressing STRN-ALK (G349S) by crizotinib was not observed in concentrations below $2 \mu M$. These results provide *in vitro* evidence that STRN-ALK kinase activity and thyroid cell growth may be blocked specifically by chemical inhibitors of ALK, raising the possibility that STRN-ALK may serve as a therapeutic target for thyroid cancer.

Discussion

Gene fusions, which result from intrachromosomal or interchromosomal rearrangements, are an important mechanism of oncogene activation in human cancer (6, 16, 17), including thyroid tumors (18–20). Next-generation sequencing, particularly the whole-genome and whole-transcriptome analyses, is an efficient tool for the discovery of novel driver gene fusions (6, 21, 22). Importantly, many gene fusions are successfully used as therapeutic

targets, such as BCR-ABL fusion in chronic myelogenous leukemia, which served as a target for one of the first tyrosine kinase inhibitors, imatinib, introduced as a frontline therapy for patients with cancer (23).

Although the majority of thyroid cancers are effectively treated with surgery and radioactive iodine, some well-differentiated cancers and most poorly differentiated and anaplastic cancers have a high mortality rate. In this study, we used RNA-Seq to identify *STRN-ALK*, a previously unknown recurrent chromosomal rearrangement involving the *ALK* gene, and to show that it occurs with higher prevalence in dedifferentiated types of thyroid cancer.

The first *ALK* fusion, *NPM-ALK*, was discovered in anaplastic large-cell lymphoma (24), followed by identification of other *ALK* fusion partners in lymphomas and different tumor types, including *EML4-ALK* rearrangement in non-small-cell lung cancer (7). In addition, activation of *ALK* via point mutation has been demonstrated in tumors originating from tissues with a high level of expression of endogenous *ALK* (i.e., neuroblastomas) (13). Of interest, the occurrence of point mutations in the tyrosine

Fig. 6. Inhibition of STNRN-ALK kinase activity and thyroid cell growth by ALK inhibitors. (A) Immunoprecipitation-coupled kinase assay from HEK-transfected cells using the synthetic YFF peptide substrate showing kinase-dependent YFF phosphorylation (pYFF) with linear product accumulation up to 20 min. KD, kinase-dead STNRN-ALK (K230M); WT, STNRN-ALK. Inhibition of substrate phosphorylation by crizotinib (B) and TAE684 (C) in HEK 293 cells expressing STNRN-ALK (WT) and STNRN-ALK (G3495) mutant (IR) measured at 15 min. (D) Inhibition of growth in PCCL3 thyroid cells expressing HA-STNRN-ALK (WT) and HA-STNRN-ALK (G3495) mutant (IR) by crizotinib. Cells cultured in cell media containing 5% (vol/vol) FBS and no TSH were treated with different concentrations of crizotinib for 24 h, and BrdU was added for the last 4 h of crizotinib treatment. Cell proliferation was assessed as a percentage of HA/BrdU-positive cells. Lines are the curve fitting to a dose-response curve.

kinase domain of ALK in two ATCs has been reported in one observation, and these mutations appear to increase ALK kinase activity in NIH 3T3 cells (25). It remains to be further demonstrated if ALK is expressed in ATC, and whether or not such mutations contribute to anaplastic transformation. In addition, one observation exists reporting a high frequency of *EML4-ALK* rearrangement in PTC from atomic bomb survivors in Japan detected by a highly sensitive RT-PCR assay from archival paraffin blocks and not confirmed by FISH or other methods. However, we were not able to detect any *EML4-ALK* fusions in frozen tissues from >60 well-characterized post-Chernobyl radiation-induced thyroid cancers (26).

Most importantly, *ALK* rearrangements that occur in other cancer types are an effective therapeutic target, and a number of small-molecule inhibitors of ALK kinase have been developed and characterized in preclinical and clinical studies. One of them is crizotinib, a potent, orally available ATP-competitive aminopyridine inhibitor of the ALK and MET kinases that is used for treatment of lung cancer carrying *EML4-ALK* rearrangements (14, 27, 28). It inhibits tyrosine phosphorylation of activated ALK with an IC_{50} of 20–40 nM and showed a therapeutic response in 57% of patients with ALK rearrangement-positive lung cancer (14). Several other compounds showing selective and highly potent inhibition of ALK kinase have been developed, and some are in clinical trials for treatment of patients with *ALK*-positive lung cancer and lymphoma (29, 30).

Although all types of ALK fusions identified so far were found to increase cell proliferation and induce cell transformation, some variability in the potency of these effects was observed (31). Moreover, *ALK* fusions involving different partners or even different fusion points with the same partner showed differential sensitivity to the structurally diverse ALK kinase inhibitors (15).

In this study, we observed that in vitro inhibition of kinase activity of STRN-ALK was achieved by both tested ALK inhibitors, crizotinib and TAE684. Although STRN-ALK responds to TAE684 with an IC_{50} close to reported values for other ALK fusion proteins (32), its in vitro sensitivity to crizotinib is approximately eight- to 10-fold lower than reported (33). Whether this represents an inherent property of STRN-ALK fusion protein remains to be investigated.

In summary, we report the discovery and characterization of a novel type of ALK fusion in thyroid cancer, which may serve as a driver of tumor dedifferentiation and anaplastic transformation. Furthermore, our in vitro data demonstrate that this fusion protein is sensitive to already available ALK inhibitors. Preclinical studies using animal tumor models will further explore the potential use of *STRN-ALK* as a therapeutic target, which may lead to clinical trials for patients with the most lethal, dedifferentiated types of thyroid cancer.

Materials and Methods

Tissue samples were collected using a protocol approved by the University of Pittsburgh Institutional Review Board. Paid-end sequencing was performed using an Illumina HiSeq200 sequencer. Detailed information about the tissue samples, RNA-Seq analysis, detection and validation of mutations and gene fusions, FISH, immunohistochemistry, Western blotting, expression vectors, cell transfection, cell growth, transformation, and tumorigenicity assays, as well as dimerization and kinase assays, can be found in *SI Materials and Methods*.

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