Anaplastic Thyroid Cancers Harbor Novel Oncogenic Mutations of the ALK Gene

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Running title: ALK mutation in thyroid cancer

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Precis:

This study identifies novel oncogenic mutations in the ALK kinase in anaplastic thyroid cancer, a deadly endocrine cancer, suggesting new opportunities for therapeutic management using ALK kinase inhibitors that are presently in clinical development.

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Abstract

Thyroid cancer is the most common endocrine cancer and targeted approaches to treat it pose considerable interest. In this study, we report the discovery of \textit{ALK} gene mutations in thyroid cancer that may rationalize clinical evaluation of ALK inhibitors in this setting. In undifferentiated anaplastic thyroid cancer (ATC), we identified two novel point mutations in exon 23 of the \textit{ALK} gene, C3592T and G3602A, with a prevalence of 11.11%, but found no mutations in the matched normal tissues or in well-differentiated thyroid cancers. These two mutations, resulting in L1198F and G1201E amino acid changes, respectively, both reside within the ALK tyrosine kinase domain where they dramatically increased tyrosine kinase activities. Similarly, these mutations heightened the ability of ALK to activate the PI3K/Akt and MAP kinase pathways in established mouse cells. Further investigations demonstrated that these two ALK mutants strongly promoted cell focus formation, anchorage-independent growth, and cell invasion. Similar oncogenic properties were observed in the neuroblastoma-associated ALK mutants K1062M and F1174L, but not in wild-type ALK. Overall, our results reveal two novel gain-of-function mutations of ALK in certain ATCs and they suggest efforts to clinically evaluate the use of ALK kinase inhibitors to treat patients who harbor ATCs with these mutations.
Introduction

Anaplastic lymphoma kinase (ALK) is a member of the insulin receptor sub-family of receptor tyrosine kinases (RTK) with its encoding gene located on the short arm of chromosome 2 (1, 2). ALK was initially identified as part of an oncogenic fusion gene, NPM1-ALK (also known as NPM-ALK), in anaplastic large-cell non-Hodgkin's lymphomas (ALCL) (3). It is also part of the fusion gene EML4-ALK in non-small-cell lung cancer (NSCLC) (4). There are a few other ALK fusion genes, such as TMP3/4-ALK and RANBP2-ALK in inflammatory myofibroblastic tumors (IMT) (5). The tyrosine kinase activities of these fusion ALK proteins are aberrantly activated and promote cell proliferation and survival (6, 7). ALK fusion proteins have also been shown to activate various signaling pathways, among which are the phosphatidylinositol 3-kinase (PI3K)/Akt pathway and the Ras → Raf → MEK → ERK (MAP kinase) pathway with multiple interaction points to mediate the ALK signaling (8, 9).

Recently, ALK mutations were found in 6-14% of sporadic neuroblastomas (10-14). ALK mutations were also reported in familial neuroblastomas (13, 14). Moreover, genetic amplification of the ALK gene could also occur in neuroblastomas or cell lines derived from this tumor (10, 11, 13, 15). Except for occasional mutations in the juxtamembrane domain, most ALK mutations identified so far are within the tyrosine kinase domain of ALK. ALK mutations and/or copy gain were found particularly in advanced and metastatic neuroblastomas, and patients with ALK mutations had a worse prognosis (11, 12, 14). Several common ALK mutations were demonstrated to be functional. For example, short interfering RNA-mediated knockdown of the ALK expression in cell lines harboring ALK mutants F1174L or R1275Q caused cell apoptosis
and suppression of cell proliferation (12-14). The F1174L and another mutant ALK, K1062M, were shown to display increased tyrosine kinase activity and promote cell focus formation, cell transformation, and xenograft tumorigenesity in nude mice (10). The oncogenicity of ALK F1174L and R1275Q were also demonstrated in another study (12). Genetic copy gain of the ALK is also functionally important as suggested by the demonstration that inhibition of ALK in neuroblastoma cell lines harboring ALK copy gain induced cell apoptosis through reduced signaling of the PI3K/Akt and MAP kinase pathways (15).

Mutations of the ALK gene have not been reported in human cancers other than neuroblastomas. In the present study, we investigated the mutation status of the ALK gene in various thyroid cancers, including well-differentiated papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC) and undifferentiated anaplastic thyroid cancer (ATC). Prompted by our finding of ALK mutations in ATC, a rapidly aggressive and deadly human cancer (16), we also examined melanoma and colon carcinoma for ALK mutation.
Materials and Methods

Tumor samples, cell lines, and DNA isolation

A large number of human tumor samples, as indicated in the Results, and human thyroid cancer cell lines K1, BCPAP, K5, FTC133, OCUT-1, OCUT-2, FB-1, SW1736, HTh7, HTh74, KAT18 and C643 (with the later 8 cell lines being derived from ATC), melanoma cell lines M14, A375 and UACC62, and colon carcinoma cell lines T84, RKO and HT-29 were used for mutational analysis of the ALK gene. The original sources of the cell lines used in this study were as follows: K1 and K5 from Dr. David Wynford-Thomas (University of Wales College of Medicine, Cardiff, UK); BCPAP from Dr. Massimo Santoro (University of Federico II, Naples, Italy); FTC133 from Dr. Georg Brabant (University of Manchester, Manchester, UK); OCUT-1 and OCUT-2 from Dr. Naoyoshi Onoda (Osaka City University Graduate School of Medicine, Osaka, Japan); KAT18 from Dr. Kenneth B. Ain (University of Kentucky Medical Center, Lexington, KY); FB-1 from Dr. Riccardo Giannini (Department of Surgery, University of Pisa, Pisa, Italy); SW1736, HTh7, HTh74, and C643 from Dr. N. E. Heldin (University of Uppsala, Uppsala, Sweden). These cell lines have recently been tested and authenticated to be distinct thyroid cancer cell lines (17). The melanoma cell lines UACC62 and M14 were obtained from the cell bank of National Cancer Institute (Bethesda, MD) and the melanoma cell line A375 and the colon cancer cell lines T84, RKO and HT-29 were obtained from American Type Culture Collection (ATCC, Manassas, VA). Use of tumor samples was Institutional Review Board-approved as reported previously (18). Except for FTC133 cells cultured in DMEM/HAM’S F-12 medium, all tumor cell lines were cultured in RPMI-1640 medium, supplemented with 10% fetal bovine serum (FBS), 2
mM L-glutamine, streptomycin (100µg/mL) and penicillin (100 U/mL). Genomic DNA from cell lines and tumors was isolated by phenol-chloroform extraction using MaXtract high density gel tubes (Qiagen, Valencia CA) as described previously (19).

**PCR amplification and sequencing**

PCR amplification of exons 23, 24 and 25 of the *ALK* gene was performed using the primers and conditions as described previously (10). The amplified PCR products were directly sequenced using a BigDye terminator v3.1 cycle sequencing ready reaction kit (Applied Biosystems) and an ABI PRISM 3730 automated next generation genetic analyzer (Applied Biosystems). Gene Bank accession number is NM_004304.3 for ALK.

**Multiple amino-acid sequence alignment**

Original amino-acid sequences of ALK of various species were obtained from NCBI database as follows: *H_sapiens* (NP_004295.2), *C_lupus* (XP_540136.2), *B_taurus* (XP_616782.3), *M_musculus* (NP_031465.2), *G_gallus* (XP_419364.2) and *D_rerio* (XP_691964.2). These amino-acid sequences were compared using a computer based multiple sequence alignment program. 

**Expression vectors and site-directed mutagenesis**

The expression vector pcDNA3 carrying wt-ALK and mutant ALKs (K1062M and F1174L) are a kind gift from Profs. Yasuhide Hayashi and Seishi Ogawa at The University of Tokyo, Japan (10). The expression vector pcDNA3 carrying wt-ALK was used to generate the novel ALK mutants L1198F and G1201E discovered in the present
study with a Quick Change XL II Site-Directed mutagenesis kit (Stratagene, Lajolla USA) according to the instructions of the manufacturer. The primers were designed using a template specific mutagenic primer design program. The primer sequences are as follows: for L1198F: sense, ALK-C3592T_F 5’-CATCCTGCTGGAGTTCATGGCGGGGG-3’; antisense, ALK-C3592T_R 5’-CCCCCGCCATGAACTCCAGCAGGATG-3’. For G1201E: sense, ALK-G3602A_F 5’-GAGCTCATGGCGGAGGGAGACCTCAAG-3’; antisense, ALK-G3602A_R 5’-CTTGAGGTCTCCCTCCGACCAGCT-3’. The mutations were confirmed in the vectors by sequencing with the primer ALK-VEC_F 5’-TCTGCTGTGGTGACCTCTG-3’. Plasmid DNAs for the transfection experiments were purified using a mini prep kit (Catalog No. K2100-11, Invitrogen, Carlsbad, CA).

**Cell culture, transfection and pooled stable expression**

NIH3T3 cells (ATCC, Manassas, VA) were grown in DMEM medium and supplemented with fetal calf serum and plated (8.0 X 10^5 cells/well) on 6-well plates. Twenty four hours later, cells were transfected using the Lipofectamine™ 2000 Transfection Reagent (Invitrogen, Carlsbad, CA) with equal amount of empty vector or vector containing wild-type ALK or mutant ALK DNA per manufacturer’s instructions. Cells were selected with 800 µg/mL G418 (Stratagene Lajolla, USA) 48 h after transfection. Medium was changed every 3 days. After 14 days of selection, stably formed clones were pooled and integration of the plasmid was checked by PCR and expression of the ALK protein was confirmed by Western blotting. Stably transfected pooled clones were used for functional studies.
ALK kinase assay

The ALK tyrosine kinase assay was a non-radioactive solid-phase enzyme-linked immunosorbent assay, which was performed as described previously (10) using the Universal Tyrosine Kinase Assay kit (Catalog No. MK410, Takara Bio inc., USA). Briefly, cells stably transfected with empty vector, wild-type ALK, the two novel ALK mutants (L1198F and G1201E), and the two positive ALK mutant controls (K1062M and F1174L) were lysed and lysates were centrifuged at 12,000g for 10 min at 4 ºC. The supernatents were collected and protein concentration was measured using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). For each enzymatic reaction, an equal volume of cell lysate containing the same amount of protein was added to the kinase reaction buffer and the mixture was transferred to the microplate supplemented with the kit containing the substrate (immobilized peptide poly [Glu-Tyr]). The kinase reaction was initiated by adding 40 mM ATP-2Na solution, followed by incubation for 20 min at room temperature.

Western blotting

Western blotting was performed as described previously (20). Briefly, 15 µg of cell lysate proteins prepared for ALK kinase assay was separated on SDS/PAGE and transferred to PVDF membrane (Millipore Co., Bedford, MA). After transfer, the membrane was blocked with 5% skim-milk/PBS containing 0.1% Tween 20 (PBST) for 1 hour at room temperature and the membrane was sliced based on the molecular weight
and incubated with primary antibodies. Membranes were incubated overnight at 4°C with anti-Flag (Catalog No. 2368, Cell Signaling Technology), anti-phospho-Akt (Catalog No.sc-7985, Santa Cruz Biotechnology), or anti-phospho-ERK (Catalog No.sc-7383, Santa Cruz Biotechnology) primary antibodies. Membranes were incubated with anti-Akt (Catalog No.sc-8312), anti-ERK (Catalog No.sc-94), or anti-β-actin (Catalog No. sc-1616, Santa Cruz Biotechnology) primary antibodies for 1 h at room temperature. After washing four times with PBST, blots were incubated with respective HRP conjugated secondary antibodies (Catalog No. sc-2004 and sc-2005, Santa Cruz Biotechnology) for 1 h at room temperature. After washing with PBST, protein bands on the membrane were detected with enhancement chemiluminescence (ECL) reaction and exposure to X-ray films.

**Cell focus formation assay**

Cell focus formation assay was performed as described previously (21). Briefly, NIH3T3 cells were transfected with equal amount of empty vector, wild-type or each of mutant ALK expression vectors using the Lipofectamine 2000 transfection reagent following the manufacturer’s instructions (Invitrogen Life Technologies, CA, USA). Twenty four h after the transfection, cells were selected using G418 (800µg/mL) for 7 days. Selected cells were trypsinized, pooled and plated (5 X 10^5 cells) on 6-well plates. Medium was changed every 3-4 days. After 14 days, number of morphologically transformed foci was counted and photographed (Zeiss Axiovert 200M, CarlZeiss, Germany).
Soft agar colony formation assay

Soft agar colony formation assay was performed as previously described (10). Briefly, NIH3T3 cells stably expressing vector, wild-type or mutant ALKs were seeded (1.0 X 10^4 cells) on 6-well plates (Costar® Corning, NY) in 0.3% agar (Catalog No. 214010, BD Biosciences) over a bottom layer of 0.6% agar. After 4 weeks, the colonies of > 0.1 mm were counted and photographed (Zeiss Axiovert 200M, CarlZeiss, Germany).

Invasion assay

Cell invasion assay was performed as described previously (20). Briefly, the assay was performed using matrigel invasion chambers consisting of BD Falcon™ cell culture inserts containing a PET membrane with 8µm pores coated with matrigel matrix (BD BioCoat™ Matrigel™ Invasion Chamber, BD Biosciences, Bedford, MA). Cells expressing the vector, wild-type or mutant ALK were completely serum-starved for 7-8 h and then collected and re-suspended (5 X 10^4 cells) in 500 µL of serum-free DMEM with 0.1% BSA. Culture inserts were placed in the wells of a BD Falcon 24-well multi-well companion plate and 750 µL of DMEM containing 1% serum was added to the lower compartment of each well. Cell suspensions were added to each culture inserts. After a 22-h incubation at 37ºC with 5% CO₂, the non-invading cells on top of the matrigel were removed using cotton swab and invaded cells on the lower side of the membrane were fixed with 70% ethanol and stained with Coomassie Brilliant Blue (CBB). Invading cells were counted and photographed under a microscope with 10X magnification (Nikon Eclipse ME-600-DS-5M-L1, Tokyo, Japan).
All the data of assays presented represent at least two similar experiments.

**Results**

**Identification of novel somatic ALK mutations in anaplastic thyroid cancer**

We analyzed the tyrosine kinase domain exons 23 and 25 of the ALK gene for mutation in 12 thyroid cancer cell lines (including 8 ATC cell lines), 36 PTC (including 12 cases of each conventional, follicular variant and tall cell PTC), 20 FTC, and 18 ATC tumor samples. These exons were chosen for analysis because they contained the hot spots for ALK mutations found in neuroblastomas. No mutation was found in the thyroid cancer cell lines and the PTC and FTC tumor samples. However, we found two novel missense heterozygous ALK point mutations in ATC tumor samples. As shown Fig 1A, one mutation found in one case of ATC represented a C>T transition in nucleotide position 3,592. This mutation changed codon 1198 from CTC to TTC, resulting in the amino acid change from lysine to phenylalanine (L1198F) of ALK. As shown also in Fig 1A, the other mutation found in another case of ATC represented a G>A transition in nucleotide position 3,602. This mutation changed codon 1201 from GGG to GAG, resulting in the amino acid change of glycine to glutamic acid (G1201E) of ALK.

Reverse sequencing using antisense primers confirmed these two mutations (Fig 1A). The matched normal tissues in these two cases showed the wild-type ALK gene (Fig 1A), suggesting that the novel ALK mutations identified in the ATC samples were somatic mutations. We did not find these mutations in the COSMIC (Catalog Of Somatic Mutations In Cancer), a data base of Sanger Institute, UK and in the literature. We also checked the data bases of SNP, including the major website of Ensembl and NCBI, and did not find germline variations that represented these ALK mutations. Therefore,
this is the first report of these somatic mutations of the \textit{ALK} gene in human cancers. We additionally examined exon 24 of the ALK gene in the ATC samples but did not find mutations. In these ATC tumors, we did not find \textit{ALK} mutations that had been previously reported in neuroblastomas. The prevalence of \textit{ALK} mutations in ATC in the present study was 11.11\% (2/18).

We also analyzed exons 23 and 25 of the \textit{ALK} gene in 3 melanoma cell lines, 3 colon carcinoma cell lines, 44 melanomas, and 47 colon carcinoma samples. We did not find \textit{ALK} mutation in any of these samples, suggesting that \textit{ALK} mutation is not a common event in these cancers. We found a few silent mutations in these cancers (data not shown).

\textbf{Increased tyrosine kinase activities of novel somatic ALK mutants L1198F and G1201E and their activation of the PI3K/Akt and MAP kinase pathways}

As shown in Fig 1B, the somatically mutated amino acid residues L1198F and G1201E are located within the tyrosine kinase domain (amino acids1057-1383) of the ALK protein. Moreover, as shown in Fig 1C, alignment comparison of amino acid sequences of ALK proteins from 6 different species revealed that the L1198 and G1201 were evolutionarily conserved residues of the ALK proteins among various species. We therefore speculated that these novel somatic \textit{ALK} mutations likely altered the tyrosine kinase activity of ALK. To test this idea, we performed \textit{in vitro} mutagenesis to generate these two mutations and tested their tyrosine kinase activities. To this end, NIH3T3 cells stably expressing vector, wild-type and each mutant ALK were lysed and assayed for \textit{in vitro} tyrosine kinase activities. As shown Fig 2A, the novel ALK mutants L1198F and
G1201E displayed dramatically increased tyrosine kinase activities compared with the wild-type ALK. The neuroblastoma-associated ALK mutants K1062M and F1174L, as positive controls in the assay, also displayed high activities as expected (Fig 2A). Western blotting analysis confirmed the corresponding protein expression of the expression vector constructs (top panel of Fig 2B). These results demonstrated that the two novel ALK mutations found in ATC are gain-of-function mutations.

As oncogenic fusion ALK proteins were previously shown to activate the PI3K/Akt and MAP kinase pathways (8, 9), we next tested whether the novel ALK mutants discovered in this study had any impact on the signaling of these two pathways. As shown in Fig 2B, compared with wild-type ALK, phosphorylation of both Akt and ERK was elevated in NIH3T3 cells expressing the mutant L1198F or G1201E, similar to ALK mutants K1062M and F1174L tested here as positive controls. This was consistent with high immunostaining scores for the phosphorylation levels of Akt and ERK in the two cases of ATC harboring the ALK mutations, which were revealed in a previous study of ours (19). Thus, the role of the two novel ALK mutations in the tumorigenesis of ATC likely involves aberrant activation of the PI3K/Akt and MAP kinase pathways.

**Novel somatic ALK mutants L1198F and G1201E promoted cell transformation and invasion**

Given the known cell-transforming abilities of fusion ALK proteins and neuroblastoma-associated ALK mutants (10), we examined the transforming abilities of the two novel ALK mutants, L1198F and G1201E, to functionally test their oncogenic potential. To this end, we transfected NIH3TC cells with empty vector, wild-type ALK and various
ALK mutants and examined their ability to form cell focus and anchorage-independent colonies. As shown in Fig 3A, cells expressing ALK mutants L1198F and G1201E lost cell contact-mediated growth inhibition and grew foci of multi layers of cells, whereas control cells (vector and wild-type ALK) exhibited contact inhibition and grew in monolayer of cells. Moreover, ALK mutants L1198F and G1201E were also associated with a much larger number of foci of multi layers of cells than the wild-type ALK (Fig 3B). As a positive control, the previously well-characterized neuroblastoma-associated ALK mutants K1062M and F1174L showed similar cell focus-forming abilities (Fig 3A and B).

Anchorage-independent growth on soft agar also reflects a transformation property of cells. As shown in Fig 3C, like the positive control ALK mutants K1062M and F1174L, the novel ALK mutants L1198F and G1201E induced formation of much larger cell colonies on soft agar compared with vector and wild-type ALK. The number of large cell colonies induced by these mutants was also much bigger than that of the vector and the wild-type ALK (Fig 3D).

An earlier study showed cell invasion-promoting properties of various fusion ALK proteins (22). We tested such properties of the ALK mutants L1198F and G1201E in the present studies. As shown in Fig 4A, like ALK K1062M and F1174L, NIH3T3 cells transfected with the two novel ALK mutants were much more invasive on matrigel-matrix-coated membranes in comparison with vector or wild-type ALK-transfected cells. The number of invading cells was much bigger with the ALK mutants than the vector and wild-type ALK (Fig 4B).
Discussion

We for the first time analyzed the ALK gene for mutations in tumors other than neuroblastomas and identified two somatic novel missense ALK point mutations, C3592T and G3602A, in ATC. The two mutations caused amino acid change from lysine to phenylalanine (L1198F) and from glycine to glutamic acid (G1201E) of ALK, respectively. They are both in exon 23 and only three codons apart, with a relatively high ALK mutation prevalence of 11% in ATC, similar to that in sporadic neuroblastomas (10-12, 14). The novel mutations L1198F and G1201E are located in the tyrosine kinase domain of the ALK. More specifically, they are in the hinge region of this domain. It has been demonstrated that ALK binds ADP and staurosporine at the hinge region of the interlobe cleft, suggesting important specific functions of this region (23). Therefore, it may be expected that amino acid changes in this region may result in conformational change of the ALK protein with significant functional consequences to the tyrosine kinase activity of ALK. Indeed, both mutations were demonstrated to be gain-of-function mutations, conferring ALK dramatically increased tyrosine kinase activities. We also showed that, like several previously characterized ALK mutants in neuroblastomas, the two novel ALK mutants found in ATC strongly promoted cell focus formation, anchorage-independent growth, and cell invasion, effectively demonstrating their oncogenic functions. We thus report ALK mutations in ATC with a comparable prevalence and oncogenic power with those in neuroblastomas. Expression of ALK seems to be restricted to tissues originating from neuro-ectodermal developmental origin (24). Thyroid cancer cells were specifically shown to express ALK (24). Thus, the
mutant ALK gene can be expected to be expressed in thyroid cells, making such mutations relevant for thyroid tumorigenesis.

ATC is the most aggressive type of thyroid cancer; in fact, it is one of the most rapidly lethal human cancers (16). With the current available treatments, patients with ATC usually die within 5-6 months after initial diagnosis. The finding of the ALK mutations in ATC, but not in generally indolent differentiated PTC and FTC, suggests that activating genetic alterations of the ALK gene play a role in the aggressiveness of a subgroup of ATC. This is interestingly consistent with previous observations that ALK mutations were also associated with advanced disease stages and poor outcomes of neuroblastoma (11, 12, 14).

The molecular signaling pathways mediating the oncogenic role of ALK mutations in ATC involved the PI3K/Akt and MAP kinase pathways as suggested by our demonstration of the coupling of the two novel ALK mutants to increased phosphorylation of Akt and ERK in both ALK- transfected cell lines and in ATC tumor tissues. This is not surprising as tyrosine kinases, particularly receptor tyrosine kinases, typically activate these two pathways. Interestingly, in neuroblastoma, ALK mutations were also shown to be coupled to PI3K/Akt and MAP kinase signalings (8, 9). Thus, it appears that coupling to these pathways is a common mechanism involved in the oncogenic role of genetic alterations of the ALK gene in human cancer. The PI3K/Akt and MAP kinase pathways play a fundamental role in thyroid tumorigenesis (25, 26). Dual activation of the two pathways driven by genetic alterations is a fundamental mechanism for the pathogenesis of ATC (19, 27). ALK mutations discovered in the present study add a novel set of genetic alterations that contribute to this mechanism.
Development of inhibitors targeting ALK is an exciting current research area as a novel therapeutic strategy for neuroblastomas harboring ALK mutations, ALCL NPM1-ALK, NSCLC harboring EML4-ALK, and IMT harboring TMP3/4-ALK and RANBP2-ALK fusion genes (1, 2). It has been recently reported that most of the patients with NSCLC harboring EML4-ALK responded effectively to the treatment with an anti-ALK agent, crizotinib (28). Crizotinib has also been recently reported to be effective in treating a patient with IMT harboring RANBP2-ALK (29). In future studies, it will be interesting to examine the effects of this ALK inhibitor on the growth of ALK mutants in *in vitro* cell line assay and in *in vivo* animal tumor studies. This will not only test the dependence of cells or tumors on these ALK mutants but also test the therapeutic potentials of targeting these novel ALK mutants in human cancers, particularly ATC. It is therefore highly attractive to speculate that the subset of patients with ATC harboring ALK mutations may be similarly effectively treated with this and other ALK inhibitors.

In summary, in the present study we report novel ALK mutations in ATC with a comparable prevalence and oncogenic power to those in neuroblastomas. This is the first report on ALK mutations in non-neuroblastoma human cancers. These are gain-of-function mutations that cause dual activation of the PI3K/Akt and MAP kinase pathways in ATC. They may represent a novel therapeutic target in ATC for the recently developed ALK inhibitors.
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Footnotes


22. http://www.sanger.ac.uk/genetics/CGP/cosmic/


Reference


27. Xing M. Genetic alterations in the phosphatidylinositol-3 kinase/Akt pathway in thyroid cancer. Thyroid 2010;20:697-706.


Figure Legends

Figure 1. Identification of novel somatic ALK mutations in anaplastic thyroid cancer. A. Sequencing electropherogram of the ALK gene. Presented in the left portion of Fig 1A are the sequencing results of the matched normal tissues of the two ATC cases, showing the wild-type ALK gene. Presented in the middle and right portions of Fig 1A are the sequencing results of two ATC tumors: shown in the upper panel are the sequencing results of sense and antisense strands of a region of exon 23 of the ALK gene in an ATC showing the heterozygous C>T mutation at nucleotide position 3592 in codon 1198, resulting in the L1198F amino acid change of ALK; shown in the lower panel are the sequencing results of sense and antisense strands of a region of exon 23 of the ALK gene in another ATC showing the heterozygous G>A mutation at nucleotide position 3602 in codon 1201, resulting in the G1201E amino acid change. Arrows indicate the mutated nucleotides. Nucleotide numbers refer to the position within the coding sequence of the ALK gene, where position 1 corresponds to the first position of the translation initiation codon. All samples were sequenced in two repeated experiments with independent PCR by sense and antisense primers. B. Schematic diagram of the ALK. Shown are the relative positions of the novel somatic ALK mutations L1198F and G1201E and the previously characterized mutations K1062M and F1174L from neuroblastoma. L1198F and G1201E are located in the tyrosine kinase domain of the ALK. C. Amino-acid sequence alignment of the ALK proteins from 6 species. Shown are the L1198 and G1201 residues that are evolutionarily completely conserved among these different species. Numbers indicate amino acid or codon positions.
sequences are numbered with the initiation codon (methionine) of each protein defined as number 1.

**Figure 2. Increased tyrosine kinase activities of ALK mutants L1198F and G1201E and their activation of the PI3K/Akt and MAP kinase pathways.**

A. *In vitro assay of tyrosine kinase activities of ALK mutants.* NIH3T3 cells stably expressing Flag-tagged vector, wild-type ALK (wt), and each of ALK mutants as indicated were lysed. The cell lysates were assayed for tyrosine kinase activity as described in the Materials and Methods. The enzymatic activities were expressed as measured O.D. value x 20. Results represent mean ± S.D. of three independent experiments.

B. *Activation of the PI3K/Akt and MAP kinase pathways.* This is reflected by increased phosphorylation of Akt (p-Akt) and phosphorylation of ERK (p-ERK), respectively. NIH3T3 cells stably transfected with the indicated vector constructs as described in Fig 2A cell lysate proteins were subjected to Western blotting analyses for the indicated proteins using appropriate antibodies as described in the Materials and Methods. Successful protein expression of Flag-tagged wild-type ALK and each of the ALK mutants is shown in the top row of Fig 2B. The key molecules of the two pathways are shown in the subsequent rows. Total Akt, ERK and β-actin were used for quality control of loading proteins.

**Figure 3. Focus-formation and anchorage-independent growth of cells promoted by ALK mutants.**

A. *Cell focus-forming activities of ALK mutants.* Shown are images of adherent growth of NIH3T3 cells transfected with Flag-tagged vector, wild-type ALK,
and each of the ALK mutants indicated. Cells were cultured in regular medium with 10% FCS under standard conditions. Images of cell foci were photographed with 10X magnification after appropriate culture of cells as described in the Materials and Methods. **B. Number of cell foci formed with the indicated transfections.** The number of transformed foci was counted 14 days after cell transfection. Results represent mean ± S.D. of three independent experiments. **C. Anchorage-independent cell growth of ALK mutants on soft agar.** NIH3T3 cells stably transfected with Flag-tagged vector, wild-type ALK, and each of the ALK mutants indicated were seeded in soft agar and colonies formed 4 weeks later were photographed with 40X magnification. **D. Analyses of number of colonies.** The number of cell colonies corresponding to Fig 3C that were > 0.1 mm in diameter was counted. Results represent mean ± S.D. of three independent experiments.

**Figure 4. Cell invasion promoted by ALK mutants.** **A. In vitro invasion assay of NIH3T3 cells with various transfections.** Cells transfected with Flag-tagged vector, wild-type ALK, and each construct of the indicated ALK mutants. Cell invasion assay was performed as described in the Materials and Methods. Shown are the cells that invaded on the matrigel matrix-coated polycarbonate filter membrane after removal of the non-invasive cells. **B. Number of invasive cells with the indicated transfections.** Results of each column represent the mean ± S.D. of the numbers of invasive cells from three independent experiments.
Figure 1

A | Normal Tumor C3592T (L1198F) ATC #14 Sense Antisense
---|---|---|---|---|---
ATC  #14 G3602A (G1201E) 2M4L98F01E

B | ALK
---|---
H_sapiens | LLELMAGGDLKSF LRETRPRPSQPSSLAMLDDLHVARIDACGCQYLEENFIHRDIAARNCLLTCPGPRV ..1265
C_lupus | LLELMAGGDLKSF LRETRPRPSQPSSLAMLDDLHVARIDACGCQYLEENFIHRDIAARNCLLTCPGPRV ..1554
B_taurus | LLELMAGGDLKSF LRETRPRPSQPSSLAMLDDLHVARIDACGCQYLEENFIHRDIAARNCLLTCPGPRV ..1272
M_muscul. | LLELMAGGDLKSF LRETRPRPSQPSSLAMLDDLHVARIDACGCQYLEENFIHRDIAARNCLLTCRPGPRV ..1269
G_gallus | LLELMAGGDLKSF LRETRPRPSQPSSLAMLDDLHVARIDACGCQYLEENFIHRDIAARNCLLTCRPGPRV ..1227
D_rerio | LLELMAGGDLKSF LRETRPRPSQPSSLAMLDDLHVARIDACGCQYLEENFIHRDIAARNCLLTCKPGPRV ..0944
Figure 2

A

ALK kinase activity

Vector
Wt
L1198F
G1201E
K1062M
F1174L

B

Vector
Wt
L1198F
G1201E
K1062M
F1174L

β-actin
ERK
p-ERK
Akt
p-Akt
ALK-Flag
Figure 3

A

Vector  Wt-ALK

L1198F  G1201E

K1062M  F1174L

B

No. of foci/well (6-well plate)

Vector  Wt  L1198F  G1201E  K1062M  F1174L

C

Vector  Wt-ALK

L1198F  G1201E

K1062M  F1174L

D

No. of colonies/well (6-well plate)

Vector  Wt  L1198F  G1201E  K1062M  F1174L
Figure 4

A

Vector

Wt-ALK

L1198F

G1201E

K1062M

F1174L

B

No. of invasive cells

Vector  Wt  L1198F  G1201E  K1062M  F1174L

No. of invasive cells

5000

4500

4000

3500

3000

2500

2000

1500

1000

500

0
Anaplastic Thyroid Cancers Harbor Novel Oncogenic Mutations of the ALK Gene
Avaniyapuram Kannan Murugan and Mingzhao Xing

Cancer Res Published OnlineFirst May 19, 2011.