Identification of Anaplastic Lymphoma Kinase as a Potential Therapeutic Target in Ovarian Cancer

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ABSTRACT

Ovarian cancer is the leading cause of death from gynecological cancer. Improvement in the clinical outcome of patients is likely to be achieved by the identification of molecular events that underlie the oncogenesis of ovarian cancer. Here we show that the anaplastic lymphoma kinase (ALK) is aberrantly activated in ovarian cancer. Using an unbiased and global phospho-proteomic approach, we profiled 69 Chinese primary ovarian tumor tissues and found ALK to be aberrantly expressed and phosphorylated in 4 tumors. Genetic characterization of these ALK positive tumors indicated that full length ALK expression in two serous carcinoma patients is consistent with ALK gene copy number gain while a stromal sarcoma patient carries a novel trans-membrane ALK fusion gene: FN1-ALK. Biochemical and functional analysis demonstrated that both full length ALK and FN1-ALK are oncogenic and tumors expressing ALK or FN1-ALK are sensitive to ALK kinase inhibitors. Furthermore, immunohistochemical analysis of ovarian tumor tissue microarray detected aberrant ALK expression in 2-4% serous carcinoma patients.

Our findings provide new insights into the pathogenesis of ovarian cancer and identify ALK as a potential therapeutic target in a subset of serous ovarian carcinoma and stromal sarcoma patients.

INTRODUCTION

Ovarian cancer is the fifth leading cause of death from cancer in women, following lung, breast, colon and pancreatic cancers. In 2011, 21,990 new cases and 15,460 deaths of ovarian cancer are projected in the United States (The SEER Program of NCI). Over 90% ovarian cancer cases are epithelial cancer, which represents a series of molecularly and etiologically distinct diseases (1). Epithelial ovarian cancer can be grouped into two types. Type I tumors are genetically stable and confined to the ovary at presentation. It includes low-grade serous, low-grade endometrioid, clear cell, mucinous and Brenner carcinomas, 90% of which are curable. In contrast, type II tumors, including high-grade serous carcinoma, undifferentiated carcinoma and malignant mixed mesodermal tumors, are highly aggressive and usually present in advanced stages (2). Most type II tumors bear TP53 mutations and share no genetic alterations found in type I tumors (2, 3). High-grade serous carcinoma stands out from other subtypes since it accounts for 60-80% of...
ovarian cancer cases and most deaths from ovarian cancer. Recent studies have shown that high-grade serous carcinoma may arise from fallopian tube epithelium other than ovary itself (1, 4, 5). Due to the subtle nature of symptoms and inadequate screening tools, most serous ovarian carcinoma patients present at advanced stages with poor prognosis (2, 6). Although initial response rates to standard surgery and chemotherapy are high, the disease recurs in the majority of patients and eventually becomes chemo-resistant (7). The fact that the overall survival rate for women with ovarian cancer has not changed over the last 30 years demands new therapeutic approaches. With advances in understanding the genetics and molecular biology of ovarian cancer, targeted therapy will likely have a significant impact on overcoming chemo-resistant disease and improvement of patient outcome (1, 6, 8).

Oncogenic receptor tyrosine kinases (RTKs) have been implicated in many types of solid tumors including ovarian cancer. A recent report by Sheng et al. demonstrated that an NRG1-Her3 autocrine loop could promote ovarian cancer cell proliferation (9). Over-expression of other RTKs, such as EGFR, Her2, Her3, PDGFR and EphA2 were reported in ovarian cancer (10-13). However, it remains to be determined whether these RTKs play any roles in tumor initiation and progression in ovarian cancer.

In this report, we used an immuno-affinity profiling approach (14-16) to identify activated tyrosine kinases in 69 primary ovarian tumors. Our results indicate that many RTKs are aberrantly phosphorylated in tumors. Most interestingly, phosphorylation of anaplastic lymphoma kinase (ALK) is identified in three high-grade serous carcinoma patients and one stromal tumor patient. When activated by mutations or translocations, ALK can drive oncogenesis in non-Hodgkin’s lymphoma (17), neuroblastoma (18-21), non-small cell lung cancer (15, 22), inflammatory myofibroblastic tumor (23) and renal cell carcinoma (24, 25). Our biochemical and functional analysis suggests that, as it does in other malignancies, ALK is likely driving a subset of ovarian cancers and could be a new drug target in this devastating disease.

MATERIALS AND METHODS
Patients, tissue specimens and pathologic data.

Ovarian tissues were collected randomly from 69 ovarian tumor patients and 19 patients with benign gynecological conditions who needed oophorectomy in the Xiangya Hospital (Changsha, Hunan, China) from February 2009 to February 2010 with written consent from the patients (IRB approval). Tissue specimens were taken intra-operatively and were either formalin-fixed and paraffin-embedded (FFPE) or snap frozen in liquid nitrogen. Histological typing and grading were performed according to International Federation of Gynecology and Obstetrics (FIGO) guidelines. General pathologic information of the 69 ovarian tumor patients is listed in Supplemental Table 1. FFPE ovarian tumor tissue microarray (TMA) slides were purchased from Folio Biosciences (Powell, Ohio) and Biochain Institute, Inc. (Hayward, CA).

Cell lines and antibodies.
Ovarian cancer cell lines Ovsaho, Ovman, and Ovmiu are purchased from Japanese Collection of Research Bioreresources/Health Science Research Resources Bank. Antibodies against ALK (D5F3 XP™), phospho-ALK (Y1278/1282/1283), SHC, phospho-SHC (Y234/240), Erk, phospho-Erk (T202/Y204), Stat 3, phospho-Stat3(Y705), phospho-AKT (S473), phospho-S6 (S235/236), CDC2 (CDK1), phospho-CDC2 (Y15) and β-Actin are from Cell Signaling Technology, Inc. Danvers, MA.

Phospho-tyrosine peptide profiling by PhosphoScan®.
An average of 15 milligrams of peptides were prepared from 0.2-0.5 grams of resected frozen ovarian tissues by homogenization, trypsin digestion and Sep-pak C18 column purification as described previously (14-16). Peptides containing phospho-tyrosine (pY) were isolated by immuno-precipitation with a monoclonal antibody against phospho-tyrosine (pY100), concentrated on reverse-phase micro tips, and analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). Briefly, samples were collected with an LTQ-Orbitrap mass spectrometer, using a top-ten method, a dynamic exclusion repeat count of 1, and a repeat duration of 30 seconds. MS and MS/MS spectra were collected in the Orbitrap and LTQ component of the mass spectrometer,
respectively. SORCERER-SEQUENT (TM, v4.0.3 (c) 2008, Sage-N Research, Inc., Milpitas, CA) searches were done against the NCBI human RefPept database downloaded on Jan. 6, 2009 (containing 37,742 proteins) or Mar. 1, 2010 (containing 36,500 proteins), allowing for serine, threonine and tyrosine phosphorylation (STY+80) and methionine oxidation (M+16) as differential modifications. The PeptideProphet probability threshold was chosen to give a false positive rate of 5% for the peptide identification (26).

**Clustering and ranking analysis.**

To assess potentially aberrant tyrosine phosphorylation of receptor tyrosine kinases (RTK) in tumor tissues, spectral counts per RTK were summed and normalized to the amount of peptide subjected to pY immuno-precipitation (15 mg), elevated spectral count in each tumor sample was calculated by subtracting average spectral count in 19 normal ovarian tissues. Elevated pY spectral counts of RTKs, representing elevated tyrosine phosphorylation, observed in 60 tumor samples were used as basis for hierarchical Clustering using the Pearson correlation distance metric and average linkage (MultiExperiment Viewer version 4.4). RTKs with elevated phosphorylation in tumors are ranked based on the average value of elevated spectral count in corresponding tumors.

**Western blot.**

Frozen ovarian tumor tissue were minced in liquid nitrogen and re-suspended in 1x cell lysis buffer. Tissue or cell suspension was then sonicated and cleared by centrifugation. Immunoblotting analysis was carried out according to the manufacturer’s standard protocol (www.cellsignal.com).

**5’ Rapid Amplification of Complementary DNA Ends (RACE).**

RNeasy Mini Kit (Qiagen, Valencia, CA) was used to extract RNA from human tumor samples. Rapid amplification of cDNA ends was performed with the use of 5’ RACE system (Invitrogen, Carlsbad, CA) with primer 5’GCAGTAGTTGGGGTTGTAGTC for cDNA synthesis and 5’GCAGTGCTTGGTGAGGCCTC for nested PCR reaction, followed by cloning and
sequencing the PCR products.

**RT-PCR.**

RNA was extracted from ovarian tumor tissue or cell lines using RNeasy Mini Kit (Qiagen). For RT-PCR, first-strand cDNA was synthesized from 2.5 mg of total RNA using SuperScript™ III first-strand synthesis system (Invitrogen) with oligo (dT)20. Primer pairs used to amplify wild type ALK and FN1-ALK transcripts were 5’GAGGATATATAGGCGGCAAT, 5’TGCAGCTCCTGGTGCTTCC and 5’TAAAGCTGGGTGTACGA CCAA, 5’TGCAGCTCCTGGTGCTTCC, respectively. Transcripts from the control gene GAPDH was amplified using primer pairs 5’GATTCCACCCATGGCAAATTCC and 5’GATTCCACCCATGGCAAATTCC.

**Quantitative PCR assay.**

DNeasy Blood & Tissue Kit (Qiagen) was used to extract genomic DNA from frozen ovarian tumor samples. Quantitative PCR (qPCR) was performed using iQ™ SYBR Green Supermix (Bio-Rad, Hercules, CA). Each qPCR reaction contains 10μl of SYBR Green Master Mix, 1μl of 10 nM forward primer, 1μl of 10 nM reverse primer, 6μl of nuclease free water and 2μl of genomic DNA at 10 ng/ml. qPCR reaction was carried out with the following parameters: 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 65°C for 15 seconds. A dissociation run from 55°C to 95°C was added to the end of qPCR reaction for melting curve analysis. Non-specific amplification was not detected with the primers used in the study. PCR reactions were set up in duplicates for each sample tested. Data was exported from CFX96 Real-Time PCR Detection System and analyzed using Microsoft Excel. We used an Ultra Conservative Element (UCE) located on human Chromosome 7 (Chr7: 1,234,865-1,235,026) that has been identified to have a copy number of two as reference DNA segment, and calculated the ΔCt by subtracting the Ct value of the UCE reactions from that of the ALK reactions in each sample. This normalizes the variation in the amount of templates between samples. We then used ΔCt values from each individual to calculate relative copy number against calibrator DNA isolated from NA10851 (Coriell Cell Repository, Camden, NJ). Calculations were performed to normalize a copy number of two to a value of 1 in the bar
graph. Standard deviations were based on normalized Ct values of duplicates. The sequences of the primers that are used to amplify ALK gene are:

5’ACAAGGTCCACGGATCCAGAAACA,  
5’AGTCTCCCAGTTGCAACGTTAGGT.

Constructs and the establishment of 3T3 cell lines stably expressing FN1-ALK and wild type ALK.

The coding sequences of wild type ALK and FN1-ALK were cloned from primary tumor samples OC26 and OC19, respectively, into retroviral vector MSCV-Neo. The expression plasmids were transfected into 293T cells by FuGene 6 (Roche Diagnostics, Indianapolis, IN) and retrovirus was harvested 48 hours later. NIH3T3 cells were infected with MSCV-neo/ALK or MSCV-neo/FN1ALK and selected in G418-containing media for 7 days. The cells were then cultured in soft agar or injected subcutaneously into nude mice.

Tumor grafts and in vivo drug sensitivity studies.

1.5-2x10^6 transduced 3T3 cells expressing Src, FN1-ALK or ALK were resuspended in Matrigel (BD Biosciences) and injected subcutaneously into the upper or lower right flank of 6-8 weeks old female NCR NU-F mice purchased from Taconic (Hudson, NY). Mice were randomized to 3 treatment groups (n=6 per group) once the tumors became palpable (~50 mm^3) and gavaged orally with vehicle (10% 1-methy-2-pyrrolidinone/90% PEG-300) alone, 100mg/kg/day Crizotinib or 10mg/kg/day TAE684 (NVP-TAE684) for 7-11 days. Crizotinib and TAE684 were purchased from ChemieTek (Indianapolis, IN). Tumors were measured every other day using calipers, and tumor volume was calculated as 0.52xwidth^2xlength. Mice were monitored daily for general conditions. The experiment was terminated when the mean size of the treated or control mice reached 1500 mm^3. Animal protocol is in accordance with Cell Signaling Technology Animal Care and Use Committee with approval ID 650.
RESULTS

Identification of ALK phosphorylation in ovarian tumor tissues by LC-MS/MS mass spectrometry.

To identify oncogenic kinases in ovarian tumors, we performed immuno-affinity profiling of tyrosine phosphorylation in 69 tumor and 19 normal tissues with PhosphoScan® (14). The characteristics of the 69 ovarian cancer patients are summarized in Table 1. Most of the 43 serous type tissues are high-grade serous carcinoma characterized by cancerous spreading in the pelvic area. LC-MS/MS identified 2484 tyrosine phosphorylation sites in 1349 proteins, with global false discovery rate to be less than 5% using peptide prophet (26). These data are available at PhosphoSitePlus™ (www.phosphosite.org). Spectral counts for all phospho-tyrosine (pY) sites identified in tyrosine kinases across the 88 ovarian tissues are listed in Supplemental Table 2.

Previous studies have demonstrated that elevated tyrosine phosphorylation of RTKs may represent aberrant kinase activity in tumor tissues, which could drive oncogenesis (15). Using RTK tyrosine phosphorylation in 19 normal ovarian tissues as basal phosphorylation level, we assessed potentially aberrant tyrosine phosphorylation of RTKs in tumor tissues. As shown in Fig. 1A, we observed elevated tyrosine phosphorylation in many RTKs across the 60 tumor tissues including discoidin domain receptors (DDRs), ephrin receptor kinases (EphA, EphB), as well as EGFR and FGFR family kinases. Interestingly, ALK was ranked as the top kinase with the highest elevated tyrosine phosphorylation among other RTKs (Fig. 1B).

ALK phosphorylation was found in 4 patients, including 3 serous carcinoma patients OC07, OC16 and OC26 and a stromal sarcoma patient OC19 (Fig. 1C). Hyper-phosphorylation of ALK in OC19 is indicated by multiple pY sites in the juxtamembrane, kinase and C-terminal regulatory domains, many of which were seen previously in other type of cancers (data not shown). Phosphorylation of Y1507, which is equivalent to Y567 in NPM-ALK found in anaplastic large cell lymphoma, was detected in all four patients (Fig. 1C). This phospho-site was also observed in primary tumor tissues and cell lines of non-small cell lung cancer bearing EML-ALK fusions, as well as neuroblastoma cell lines bearing ALK activating mutations (data not shown). Extracted ion chromatograms,
MS2 spectra of two tryptic peptides containing phosho-Y1507 indicated the detection of ALK peptides in these patients (Suppl. Fig. 1A and 1B).

Previous studies have shown that Y1507 (Y527 in NPM-ALK) located at the carboxyl-terminal region of ALK is a direct docking site for SH2 domain-containing transforming protein (SHC1). Recruitment and phosphorylation of SHC1 activate Ras/extracellular signal regulated kinase (ERK) pathway and promotes tumorigenesis (27, 28). Consistent with these observations, using label free quantification analysis, we found hyperphosphorylation of SHC1, MAPK14 and CDK1 at specific sites (Suppl. Fig. 2, Suppl. Table 3) in the three serous carcinoma patients bearing ALK phosphorylation. In addition, phosphorylation of other ALK downstream signaling molecules, including those that are involved in Jak/Stat3, PI3K/AKT pathways, are up-regulated in these tumors (Suppl. Fig. 2, Suppl. Table 3). These results suggest that in serous carcinoma patients OC07, OC16 and OC26, ALK might contribute to activation of multiple signaling pathways that promote tumor cell survival, growth and proliferation.

Taken together, we found 4 out of 69 (5.8%) ovarian cancer patients carrying phosphorylated ALK at sites that correlate with ALK oncogenesis, including 3 out of 43 (7.0%) serous carcinoma and one stromal sarcoma patients. To our knowledge, this is the first time that ALK tyrosine phosphorylation is described in ovarian cancer.

Using an antibody directed against the ALK intracellular domain, we examined ALK protein localization and expression by immunohistochemical and western blot analysis with patient tissue (Fig. 2). Similar to neuroblastoma cells and tumor tissues that over-express ALK (21), we observed a diffused ALK staining at the cytoplasm of serous carcinoma OC26, but not in other serous carcinoma tissues such as OC29a (Fig. 2A). In the stromal tumor OC19, strong ALK signal is present at both the plasma membrane and the cytoplasm, with membrane accentuation in some cells (Fig. 2A). Western blot analysis detected mature full-length ALK at 220 kd in OC16 and OC26, and the potential proteolytically cleaved 140 kd form (29) in OC26, which might be too weak to be detected in OC16. We identified multiple ALK signals of variable sizes in OC19, with the most prominent signal at ~78 kd, suggesting the presence of a novel form of ALK different from the EML4-ALK Variant 3 fusion protein in the lung cancer cell line H2228.
(Fig. 2B). These results suggest that OC19 might carry a different gene translocation involving ALK.

As a neuronal receptor kinase, ALK is not normally expressed in the ovary. Expression and phosphorylation of ALK in four ovarian cancer patients prompted us to test if the ALK gene in these patients may have undergone genetic alterations. As the activating mutations previously reported in neuroblastoma (18-20) were not detected in the cytoplasmic region of ALK in OC07, OC16, OC26 and OC19 (data not shown), we set out to examine other possible genetic alterations of ALK.

**ALK gene copy number gain in serous carcinoma tissues.**
In neuroblastoma, ALK was identified as a recurrent target of copy number gain and gene amplification (18, 19, 21). Recent study conducted by The Cancer Genome Atlas (TCGA) research network indicated a remarkable degree of genomic disarray in serous carcinoma (30). To examine whether there is copy number variation (CNV) of ALK in ovarian tumors where ALK phosphorylation was detected, we employed real time quantitative PCR with genomic DNA isolated from serous carcinoma OC07, OC16, OC26, and stromal sarcoma OC19, as well as serous carcinoma OC08, in which ALK phosphorylation is not detected. While no obvious CNV of ALK was detected in OC08 and OC19, ALK copy number gains of 1.5 and 1.7 fold were detected in OC16 and OC26, respectively (Fig. 3A). The result is consistent with our high-resolution single nucleotide polymorphism (SNP) array analysis, which indicated a copy number of 3 in the ALK region in Patient OC26 (data not shown). The ALK copy number gain in OC16 and OC26 may account for the aberrant expression of ALK in these serous carcinoma patients. Surprisingly, we observed a loss of ALK gene copies in serous carcinoma OC07 (Fig. 3A), in which ALK phosphorylation (Fig. 1A and 1C, Suppl. Fig. 1) and mRNA expression (see below) were detected, suggesting that aberrant ALK expression and phosphorylation is independent of ALK copy number in this patient.

**Identification of a novel fusion protein FN1-ALK in a malignant stromal sarcoma patient.**
Fusion of ALK kinase domain to a partner protein, resulting in constitutive activation of ALK and oncogenesis in non-Hodgkin’s lymphoma (17), non-small cell lung cancer (15, 22, 31), inflammatory myofibroblastic tumor (23) and renal cell carcinoma (24, 25), has been reported previously. Given the presence of highly phosphorylated ALK with differing sizes in Patient OC19 and the fact that no ALK mutation or copy number gain was detected in this patient, we suspect the presence of an ALK gene translocation. To test this possibility, we performed 5’ rapid amplification of cDNA ends (5’RACE) with RNA isolated from OC19 tumor. Since the majority of previously reported ALK fusions have a common break point between exons 19 and 20 (15, 17, 22-25, 31), we searched the sequences upstream of this common break point using a primer annealing to the 5’ end of exon 20. Cloning and sequence determination of the RACE product revealed that ALK is fused in frame to fibronectin 1 (FN1), a ubiquitous component of extracellular matrix (ECM) and plasma (32), at a novel break point between ALK exons 18 and 19. The resultant fusion protein FN1-ALK contains the amino-terminal 1201 amino acids of FN1 and the carboxyl-terminal 598 amino acids of ALK, which include the transmembrane and cytoplasmic regions (Fig. 3B). Encoded by the first 23 exons, the FN1 portion of FN1-ALK retains a diverse set of binding domains involved in fibronectin self-association and interaction with other ECM components, which could potentially provide strong activating signal to ALK (Fig. 3B). Unlike previous ALK fusions, the novel break point in FN1-ALK allows the ALK transmembrane region to be retained in the fusion protein (Fig. 3B). The predicted protein sequence of full length FN1-ALK is shown in Suppl. Fig. 3B.

To test mRNA expression of FN1-ALK or full length ALK in tumors with ALK phosphorylation, we performed RT-PCR analysis with primers that specifically amplify fragments from FN1-ALK or full length ALK cDNA (Fig. 3C). ALK mRNA expression was observed in the three serous carcinoma patients, OC07, OC16 and OC26, as well as in two ovarian cell lines OVMANA and OVSAHO (Fig. 3C). In contrast, only the FN1-ALK fusion gene, but not the full length ALK gene, was detected in OC19 (Fig. 3C). As expected, neither full length ALK nor FN1-ALK was detected in OC08 and the ovarian cancer cell line Ovmiu. These results indicate that FN1-ALK fusion is present in the
stromal sarcoma patient OC19 whereas full length ALK expression resides in serous carcinoma patients OC07, OC16 and OC26.

To examine the FN1-ALK gene fusion at genomic level, we used PCR primers annealing to the 3’ end of FN1 Exon 23 and the 5’ end of ALK Exon 19 to amplify DNA sequence spanning the joint of these two genes. Sequences of the PCR product revealed that the first 946 base pairs of FN1 Intron 23 were fused to the last 14 base pairs of ALK Intron 18 (Suppl. Fig. 3A). Given that ALK and FN1 map to the short and long arm of Chromosome 2 (2p23 and 2q34), respectively, in the same orientation, it remains to be determined whether the formation of the FN1-ALK fusion was caused by a large interstitial deletion spanning the centromere or other chromosomal changes.

**Transforming potential of ALK and FN1-ALK.**

To determine whether full length ALK and FN1-ALK have transforming potential, we generated NIH3T3 cells stably expressing full length ALK or FN1-ALK (Fig. 4A, B). As expected, we observed both full length ALK (220 kd) and the 140 kd cleaved ALK (29, 33) in 3T3/ALK cells. Interestingly, in addition to full length FN1-ALK proteins (predicted molecular weight: 198.82 kd), which migrates at ~225 kd, we observed a prominent ALK signal of ~78 kd in 3T3/FN1-ALK cells, which is a reminiscence of the strong ALK signal in Patient OC19 tumor (Fig. 4B, Fig.2B). We suspect this ~78 kd protein is a fragment derived from full length FN1-ALK. Both ALK and FN1-ALK showed intracellular reticulum/Golgi localization (Fig. 4C) as indicated by immunofluorescence analysis, probably due to a defect of glycosylation of these over-expressed proteins in 3T3 cells (33). When 3T3/Neo, 3T3/ALK or 3T3/FN1-ALK cells were injected subcutaneously into nude mice, we observed tumor growth induced by both 3T3/ALK and 3T3/FN1-ALK cells, with the FN1-ALK tumors growing more aggressively than the ALK tumors (Fig. 4C). The average tumor sizes 12 days after injection are around 2000 mm$^3$ and 100 mm$^3$ for FN1-ALK and ALK tumors, respectively. If allowed to grow, ALK tumors could reach ~2000 mm$^3$ 20 days after injection (data not shown). These results indicate that both the full length ALK and the FN1-ALK fusion gene isolated from the ovarian cancer patients have transforming potential and that FN1-ALK is more oncogenic than the full length ALK.
In order to investigate downstream signaling pathways activated by full length ALK and FN1-ALK, we analyzed endogenous signaling molecules of 293T cells when they over-express full length ALK and FN1-ALK. Both full length ALK and FN1-ALK showed constitutive activation as indicated by the phosphorylation of tyrosine sites in their kinase domain (Suppl. Fig. 4). Interestingly, the 140 kd cleaved ALK and the ~78 kd FN1-ALK fragment had stronger phosphorylation than their full length forms. Consistent with ALK activation, phosphorylation of Stat3, SHC and Erk is up-regulated, with FN1-ALK cells showing stronger up-regulation of these molecules than ALK cells (Suppl. Fig. 4). ALK inhibitor Crizotinib abolished phosphorylation of both ALK and FN1-ALK. As expected, phosphorylation of Stat3, SHC and Erk was also diminished upon Crizotinib treatment (Suppl. Fig. 4). Another ALK inhibitor TAE684 showed similar results (data not shown). These results indicate that, similar to what we observed in patient tumors using phospho-proteomic approach, expression of both ALK and FN1-ALK in 293T cells leads to activation of multiple downstream signaling molecules, which can be inhibited by ALK inhibitors. In addition, the stronger oncogenic activity of FN1-ALK than full length ALK might be attributed to its stronger potential in activating downstream molecules, such as Stat3, SHC and Erk, whose activation contributes to cell growth and proliferation.

**ALK and FN1-ALK as potential therapeutic target.**

Both PF-02341066 (Crizotinib) and TAE684 are potent and selective ALK inhibitors that have been shown to suppress tumor cell growth in several types of cancers expressing different ALK fusion proteins (34-38). We tested the effects of these inhibitors on the growth of ALK and FN1-ALK tumors in nude mice. As shown in Fig. 5A, administration of Crizotinib (100mg/kg/day) drastically inhibited the growth of both ALK and FN1-ALK tumors, but not the 3T3/SRC tumors. Consistent with these observations, western blot analysis using antibodies specific for ALK and phospho-ALK revealed that Crizotinib abolished phosphorylation of both full length FN1-ALK and the ~78 kd ALK variant 24 hours after treatment (Fig. 5B). We also observed an ALK signal with lower molecular weight next to the full length FN1-ALK in crizotinib treated tumor, which is likely to be a signal of non-phosphorylated FN1-ALK (Fig. 5B). Similar tumor growth-
inhibiting effects were observed with TAE684 (Suppl. Fig. 5). These results suggest that ALK and FN1-ALK tumors are highly sensitive to ALK inhibitors.

Detection of ALK expression in a larger patient cohort
To test possible aberrant ALK expression in a larger patient cohort, we performed immunohistochemical analysis on ovarian tissue microarrays (TMA). Similar to what we observed in the Chinese cohort (69 tumors) using PhosphoScan®, while no ALK staining was detected in normal tissues, granulosa-theca cell tumors or clear cell carcinomas, ALK staining was detected in 14 out of 353 (4%) serous carcinoma and 3 out of 37 endometrioid carcinoma specimens, with half of the specimens having strong ALK signal (Fig. 6A, 6B). We observed mostly weak ALK staining in some mucinous carcinoma specimens (Fig. 6A, images not shown). Consistent with what we found in Patient OC26, most of the ALK staining observed in serous carcinomas in TMA is diffusely localized in the cytoplasm (Fig. 6B) This result suggests that ALK might be activated and signaling in these tumors, which has been also been observed in neuroblastomas where wild type ALK is overexpressed (21). Considering that a significant number of high-grade serous carcinoma has been misclassified as endometrioid carcinoma (1, 39), our data suggest that strong ALK expression resides mainly in a portion (~2%) of serous carcinoma.
DISCUSSION

Oncogenic role of ALK has been well characterized in many types of hematopoietic and solid tumors, in which ALK is deregulated by gene translocations, activating mutations and amplification (15, 17-25, 31, 40, 41). Although ALK transcript was reported to be present in epithelial ovarian cancer (42), ALK protein expression and phosphorylation in ovarian cancer has not been reported. Using a phospho-proteomic approach, we identified ALK tyrosine phosphorylation in 4 of 69 primary ovarian tumor tissues. Further investigation revealed ALK gene copy number gain in two serous carcinoma patients and a novel ALK gene translocation (FN1-ALK) in a stromal sarcoma patient. Mouse 3T3 fibroblasts over-expressing full-length ALK or FN1-ALK generated subcutaneous tumor in nude mice. In addition, we showed that ALK inhibitors dramatically suppressed the growth of both 3T3 ALK and 3T3 FN1-ALK tumors. This study provides new insight into the molecular pathogenesis of ovarian cancer and compelling rationale for extending targeted therapy against ALK to this new type of solid tumor.

Oncogenic role of full length ALK has been implicated in neuroblastoma (21), glioblastoma (43) and breast cancer (44). Consistent with a recent report from The Cancer Genome Atlas Research Network, where 489 high-grade serous ovarian adenocarcinomas were analyzed (30), we did not find any activating mutation or significant gene amplification of ALK in 41 serous carcinoma patients. However, our data demonstrated phosphorylation of full length ALK at Y1507 in 3 serous carcinoma patients, two of which have ALK gene copy number gain. The phosphorylation of multiple down stream signaling molecules such as SHC1, STAT3, tyrosine kinases (SYK, FRK and FYN), SHIP 2 (INPPL1), MAPK14, CDK1, and PI3K, are upregulated compared to other serous carcinoma patients. These results indicate that previously reported Ras/ERK, Jak3/Stat3 and PI3K/Akt pathways, which mediate the effect of ALK activity and promote tumor cell proliferation and survival (27, 28), are activated in ALK-bearing serous carcinoma patients. Our proteomic approach also identified many other molecules such as NEDD9 and PKCδ (PRKCD) that are involved in transformation (45, 46) in these patients, although their relevance to ALK activity has yet to be determined.
Aberrant expression, phosphorylation, cytoplasmic localization, as well as activation of ALK downstream signaling pathways suggest an oncogenic role of full length ALK in serous ovarian carcinoma. Interestingly, unlike 3T3/FN1-ALK cells, 3T3/ALK cells did not generate transformed colonies in soft agar assay (data not shown). However, they generated tumors in nude mice. We suspect that full length ALK could be activated through paracrine signaling under *in vivo* conditions. Functional validation using more pathologically relevant models such as primary tumor xenografts or fallopian tube secretory epithelial cell (FTSEC) (4) models will help us assess the transformative effects of ALK and determine how full length ALK is activated in serous carcinomas.

The novel fusion protein FN1-ALK identified in a stromal sarcoma patient represents the first ALK fusion protein bearing the trans-membrane domain of ALK itself. Fusion to the fibronectin 1 gene that encodes a ubiquitously expressed ECM protein likely causes high-level expression of the ALK kinase. Unlike most ALK fusion partner proteins that render ALK constitutive activity through homo-dimerization, FN1 may do so through its strong interactions with ECM structures and components such as fibrin, heparin, collagen and gelatin. Since fibronectin is subjected to proteolytic breakdown by a number of neutral proteases (47), the ~78 kd fragment of FN1-ALK that we observed in both the patient tumor and transfected 3T3 or 293T cells is likely a proteolytically cleaved product of full length FN1-ALK (~220 kd). Strong expression, hyper-phosphorylation and membrane/cytoplasmic localizations of ALK suggest constitutive internalization and activation of FN1-ALK, which may lead to its strong oncogenic potential, as evidenced by the strong activation of down stream molecules in 293T cells and the *in vivo* tumorigenicity assay. Considering the sensitivity of 3T3/FN1-ALK tumors to ALK inhibitors and the recent promising results of treating inflammatory myofibroblastic tumor (IMT) with ALK inhibitor Crizotinib (38), we expect optimistic response of stromal sarcoma patients carrying FN1-ALK to targeted therapy against ALK. Our discovery broadens the scope of ALK fusion oncoproteins in human cancer and identifies a compelling therapeutic target in a new type of mesenchymal neoplasm.

To overcome chemo-resistance and to improve the overall survival of ovarian cancer patients, targeted therapy has come to the forefront of investigation. Among reported
oncogenes (8) associated with ovarian cancer identified to date, EGFR received substantial attention as a therapeutic target due to its over-expression in 70% ovarian cancer patients (48). However, inhibitors and monoclonal antibodies against EGFR only resulted in marginal efficacy due to toxic side effects and lack of inhibitor-sensitizing EGFR mutations in ovarian cancer patients (49). Unlike EGFR, which is ubiquitously expressed in normal tissue, ALK expression is restricted to certain parts of the brain (50). This might explain why inhibition of ALK has little toxic effects in patients (28). As demonstrated recently in non-small cell lung cancer and IMT patients, ALK inhibitor Crizotinib only showed grade 2 side effects at a dose of 250 mg twice daily (37, 38). Compared to 10% response rate to second-line chemotherapy, the response rate of non-small cell lung cancer patients carrying the EML4-ALK fusion to Crizotinib is 57% (37). Given the presence of an ALK-driven subgroup of ovarian cancer especially serous carcinoma patients and the fact that Crizotinib inhibited ALK or FN1-ALK tumor growth and activation of down stream signaling molecules, we speculate that Crizotinib or other ALK-targeting agent may become effective treatment in these patients.

Besides ALK inhibitors, other therapeutic options for ALK bearing ovarian cancer patients might also be considered. Firstly, co-expression/activation of other RTKs in ALK bearing serous carcinoma patients, such as Ret (in OC26) (21), suggests that combinational therapy against multiple RTK could provide better outcome. Secondly, since ALK expression is tumor specific in ovarian cancer, antibody-mediated immuno-therapy against ALK could be highly efficient with little damage to normal tissue. Thirdly, as previously shown in anaplastic large cell lymphoma (ALCL) (28), ALK is likely to be highly immunogenic in ovarian cancer patients and thus could be an ideal target for anti-tumor vaccination in these patients.

Combining our results from the Chinese patient cohort and the immunohistochemical analysis of TMA containing over 400 ovarian tumors of a distinct patient cohort, we estimate that 2-4% high-grade serous ovarian carcinoma patients have aberrant ALK expression. Given the annual ~140,000 world wide new cases and ~7000 deaths from high-grade serous ovarian carcinoma, the number of patients who might benefit from targeted therapy against ALK could be substantial.
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<table>
<thead>
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<tbody>
<tr>
<td></td>
<td>No.</td>
</tr>
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<td></td>
</tr>
<tr>
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<td>40</td>
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<tr>
<td>&gt;55</td>
<td>13</td>
</tr>
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<td><strong>II. Histologic type</strong></td>
<td></td>
</tr>
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</tr>
<tr>
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</tr>
<tr>
<td>Endometrioid</td>
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</tr>
<tr>
<td>Mucinous</td>
<td>5</td>
</tr>
<tr>
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<tr>
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<tr>
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</tr>
<tr>
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<tr>
<td>Others</td>
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<td>5</td>
</tr>
<tr>
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</tr>
<tr>
<td>Teratoma</td>
<td>3</td>
</tr>
<tr>
<td>Squamous Carcinoma</td>
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TABLES
Table 1. Characteristics of Ovarian Cancer Patients

FIGURE LEGENDS
Figure 1. Identification of aberrantly phosphorylated receptor tyrosine kinases (RTKs) in 60 ovarian cancer patients.

A, Elevated tyrosine phosphorylation (pY) of RTKs in 60 ovarian tumors. Hierarchical clustering of elevated tyrosine phosphorylation in 26 RTKs across 60 ovarian tumors using MultiExperiment Viewer Version 4.4 (MeV). Elevated pY level of 0.2 or higher are shown with indicated colors. Grey spots represent data points with no pY level increase or no assigned pY spectrum. B, Ranking of RTK tyrosine phosphorylation in ovarian cancer. RTKs are ranked based on the average value of elevated pY count in tumors with increased pY of each RTK. Data was visualized using MeV with the same color scale as in A. The number of tumors with elevated pY level of each RTK is shown in parentheses. C, ALK phospho-tyrosine sites identified by LC-MS/MS mass spectrometry in 4 ovarian tumor patients. Peptide sequences and spectral counts of juxtamembrane sites (Y1078 1092, 1096), kinase domain sites (Y1278, 1282, 1283) and carboxyl terminal sites (Y1507, 1586) are shown.

Figure 2. ALK protein expression in ovarian cancer patients.
A, Immunohistochemical staining of paraffin sections of ovarian tissue from serous carcinoma patients OC29a, OC26 and stromal sarcoma patient OC19 with an antibody against ALK kinase domain. Images represent 40X magnification B, Western blot analysis of protein lysates from serous carcinoma tissue OC16, OC26 and OC29a or stromal sarcoma patient OC19, as well as a lung cancer cell line H2228 carrying EML4-ALK fusion using antibodies against ALK and β-Actin as a loading control. Positions of wild type (WT) ALK (both full length and the 140 kd cleaved forms), EML4-ALK and ~78 kd ALK variant are indicated.

Figure 3. Genetic characterization of ovarian tumors with ALK phosphorylation.
ALK gene copy number variation in serous carcinoma patients. qPCR analysis was performed with genomic DNA isolated from tumor tissue OC07, OC08, OC16, OC19, and OC26. The value of relative gene copy number of ALK to that of UCE in each gDNA sample is shown. Calculations were performed to normalize a copy number of 2 to a value of 1 in the bar graph. Standard deviations were based on duplicate normalized Ct values. This result represents 3 independent experiments. B, Identification of a novel fusion protein, FN1-ALK, in a stromal sarcoma patient, OC19. Fusion of the amino-terminal 1201 amino acids of fibronectin 1 (FN1) to the carboxyl-terminal 598 amino acids containing the transmembrane (TM) and the intracellular kinase domain of ALK. The type I, II and III modules in fibronectin 1 (FN1) are shown in rectangles, diamonds and ovals, respectively, with domains required for fibrillogenesis shaded in blue. Domains subjected to alternative splicing in various fibronectin isoforms are shaded in light yellow (B, A and V). The positions of FN1 domains involved in binding to various extracellular matrix proteins are indicated. The ALK amino acid positions of the novel breakpoint (Novel BP, black arrow) in FN1-ALK and the common breakpoint (Common BP, gray arrow) are indicated. C, RT-PCR analysis of full length ALK or FN1-ALK expression in ovarian tumor tissue and cell lines. The positions of PCR primers used to amplify the ALK cDNA region from Exon 16 to Exon 20 and the FN1-ALK cDNA region from FN1 Exon 21 to ALK Exon 20 are indicated. Exons in ALK and FN1 are indicated in red and blue, respectively. Amplification of GAPDH is used as a qualitative control of the cDNA samples.

Figure 4. Oncogenic potential of ALK and FN1-ALK.

A, Schematic diagram of MSCV constructs used to generate stably transfected NIH 3T3 cells. ALK and FN1 cDNA sequences are indicated with red or blue boxes, respectively. ALK kinase domain and transmembrane domain are shaded in red and black, respectively. Note the joint of FN1 and ALK, which is indicated with an arrow. Neo, neomycin; LTR, long terminal repeat. B, Western blot analysis of protein lysates prepared from 3T3 cells stably transfected with Neo, ALK or FN1-ALK. Positions of the full length ALK or FN1-ALK, 140 kd cleaved ALK in 3T3/ALK cells and ~78 kd FN1-ALK fragment in 3T3/FN1-ALK cells, respectively. Blot with anti-β-Actin antibody was
C, Immuno-fluorescence analysis of stably transfected 3T3 cells and in vivo tumorigenecity assay. Immunofluorescent analysis was performed with stably transfected 3T3 cells expressing neomycin (Neo), ALK or FN1-ALK using D5F3 XP™ antibody against ALK (green), DY-554 phalloidin for actin filaments (red), and DRAQ5® for DNA (blue). 3T3 cells expressing Neo, ALK or FN1-ALK are injected subcutaneously into nude mice. The tumor images, the rate of tumor growth and the average size of tumors are reported 12 days after injection. The results are representative of two independent experiments.

Figure 5. ALK and FN1-ALK tumors are sensitive to an ALK inhibitor, Crizotinib. A, Four to six nude mice carrying 3T3 tumors expressing Src, ALK or FN1-ALK are treated with vehicle or 100mg/kg/day Crizotinib by oral gavage when tumors are palpable (~50 mm³). The tumors are measured every other day until the mean tumor size of the vehicle treated mice reaches 1500 mm³. B, Mice carrying FN1-ALK tumors were treated with vehicle or Crizotinib for 24 hours before the tumors were harvested and analyzed by western blot assay using antibodies against ALK, phospho-ALK (Y1278/1282/1283) and β-Actin. Arrows indicate the positions of the full length FN1-ALK and the ~78 kd truncated ALK variant. Note an ALK signal with lower molecular weight (*) next to the FN1-ALK, which is likely non-phosphorylated FN1-ALK.

Fig. 6. Aberrant ALK expression in a larger patient cohort detected by immunohistochemistry analysis of ovarian TMA. A, Immunohistochemical analysis of FFPE ovarian tissue microarrays (TMA) were performed ALK (D5F3) XP™ rabbit mAb as described in Materials and Methods. Total specimen numbers of each subtype and numbers of ALK weak (+) or strong (++ or ++++) specimens are listed. B, Images from 4 serous carcinoma specimen that are ALK+++ (a and b) and ALK++ (c and d), representing 40X magnification.
pY, phospho-tyrosine. "y" in the peptide sequence represents phospho-tyrosine.
<table>
<thead>
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<th>Ovarian Tissue Type</th>
<th>Total</th>
<th>ALK + (%)</th>
<th>ALK ++/ +++ (%)</th>
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<tr>
<td>Serous Carcinoma</td>
<td>353</td>
<td>7 (2.0%)</td>
<td>7 (2.0%)</td>
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<td>37</td>
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</tr>
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<td>Clear Cell Carcinoma</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Granulosa-theca Cell Tumor</td>
<td>19</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Normal</td>
<td>37</td>
<td>0</td>
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Hong Ren, Zhi-Ping Tan, Xin Zhu, et al.

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