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14. ABSTRACT

TGF-β signaling represents a major tumor suppressor pathway. Loss of the TGF-β response is a hallmark in human cancer. However, the mechanisms underlying TGF-β resistance in breast cancer have not been elucidated. Anaplastic Lymphoma Kinase (ALK) is a tyrosine receptor kinase of insulin superfamily. IBC is relatively rare but the most lethal subtype of breast cancer. Thus, it is important to identify biomarkers, understand better current therapies and find new potential therapies for IBC. Our long-term goal is to understand the mechanisms underlying TGF-β resistance in human cancer. The short-term strategy of our research is to focus on ALK-induced inactivation of Smad4 in breast cancer. Our unifying hypothesis is that ALK causes TGF-β resistance through Smad4 inactivation and disrupts the growth constraints exerted by TGF-β signaling to promote breast tumorigenesis. To test our hypothesis, we propose the following specific aims to achieve our goals: 1. Investigate *in vivo* and clinical relevance of Smad4 tyrosine phosphorylation in breast cancer; 2. Determine the role of ALK-mediated Smad4 phosphorylation in TGF-β resistance in IBC; 3. Elucidate the molecular mechanisms underlying Smad4 tyrosine phosphorylation. This proposal will contribute significantly to breast cancer prevention and treatment.

15. SUBJECT TERMS

Smad4, ALK, breast cancer

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INTRODUCTION:

TGF-β exerts its tumor suppressing function by inhibiting the growth of normal epithelial cells. Loss of the TGF-β antiproliferative response is a hallmark in human cancers [1-3]. In TGF-β signaling pathway, tumor suppressor Smad4 plays a central role in TGF-β actions. Smad4 is frequently mutated or deleted in gastrointestinal and pancreatic cancer, which counts for TGF-β resistance in these cancers. However, not all types of cancers harbor deletion or mutations in the Smad4 gene. Inactivating mutations in the Smad4 gene are rare in breast cancers [4], but TGF-β response is attenuated [5,6], indicating that the tumor suppressor activity of Smad4 is abrogated by other mechanisms.

ALK is a tyrosine receptor kinase. Abnormal expression of ALK has been reported in numerous tumors including a significant fraction of breast cancer especially triple-negative breast cancer and inflammatory breast cancer [7]. ALK activation triggers major signaling pathways (MEK/ERK, STAT3, PI3K/Akt), which promote cell proliferation while preventing cell death [8-19]. However, the effect of ALK on TGF-β action, a major anti-proliferation function in cell, has not been explored.

In our preliminary studies, we have for the first time discovered that ALK could inactivate Smad4 tumor suppressive function. In this proposal, we propose to investigate how ALK-driven inactivation of Smad4 tumor suppressor contributes to TGF-β resistance in breast cancer. We hypothesize that ALK causes TGF-β resistance through Smad4 tyrosine phosphorylation and inactivation; thus, aberrant ALK activation in breast cells disrupts Smad4-exerted growth constraints to promote tumorigenesis. Consequently, suppression of ALK activity both restores Smad4 function and blocks other oncogenic activities of ALK, thus suppressing breast tumor formation.

Specifically, in this proposal, we will determine whether aberrant activation of ALK causes TGF-β resistance by Smad4 tyrosine phosphorylation and inactivation in breast cancer cell lines. Next, we will elucidate the molecular mechanism by which ALK-mediated Smad4 tyrosine phosphorylation affects Smad4 signaling. Finally, we will determine the impact of ALK activation on Smad4 Y95 phosphorylation, mammary tumor initiation, and progression, using human tissues and mouse models (including patient-derived xenografts).

BODY:

In our preliminary studies, we found that ALK can phosphorylate Smad4 on a particular tyrosine residue and inactivate Smad4 activity. During the first funding year of this proposal, we have investigated whether forced activation and knockdown of ALK affect TGF-β responses in breast cancer cell lines. We have also examined whether Smad4 Y95 phosphorylation disrupts TGF-β-induced cellular responses in breast cancer cell lines. In addition, we have determined if ALK-resistant Smad4 mutant restores the TGF-β responses in ALK-activated breast cells lines. Thus, during the second funding year of this proposal, we further characterize the impact of ALK and Smad4 tyrosine phosphorylation on Smad4 signaling and transcriptional responses. For example, we determine how specific is Smad4 tyrosine phosphorylation by ALK or other tyrosine kinases? What steps in the TGF-β signaling pathway does Smad4 tyrosine phosphorylation affect? What is the global

effect of Smad4 Y95 phosphorylation on TGF-β mediated responses? We have completed our proposed work for year 2016-2017 as presented below:

Task 1: Examination of cellular responses to TGF-β

We have mostly reported the effect of aberrant activation of ALK on TGF-β signaling in 2015-2016 report. Like in ALK-activated lymphoma and lung cancer cells, activated ALK markedly attenuated SMAD4 responses in MDA-MB-468 cells. As shown in Figure 1, putting back of SMAD4 into breast cancer MDA-MB-468 cells, which are SMAD4-deficient, could restore TGF-β transcriptional responses. In contrast, expression of ALK could significantly block SMAD4-mediated TGF-β response. Furthermore, as we know the tyrosine phosphorylation on SMAD4 (by using mass spectrometry), we created tyrosine-to-glutamate (YE) and tyrosine-to-phenylalanine (YF) substitutions and tested their ability to restore TGF-β response in MDA-MB-468 cells. The YE mutant completely failed to restore the TGF-β response. The YF mutant, however, not only induced the highest TGF-β response, but not became resistant to the blockade by ALK.

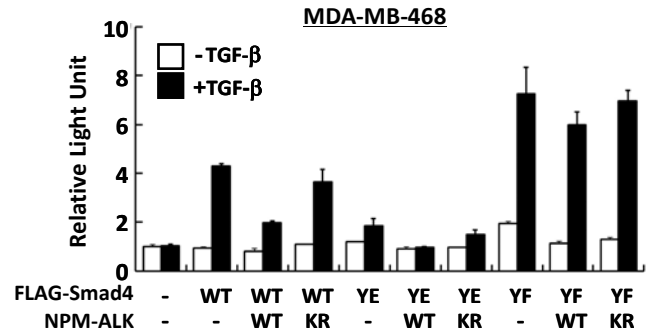

Fig. 1. ALK-mediated SMAD4 phosphorylation impairs its transcriptional activity. SMAD4-deficient breast cancer MDA-MB-468 cells were transfected with SMAD4 or Y95 mutants, NPM-ALK (WT or KR) together with TGF-β-responsive reporter SBE-luc for transcription readout and Renilla Luciferase for normalization. Dual-luciferase activities were measured 18 h after TGF-β treatment (2 ng/ml). Values are the means (±s.e.) of three separate experiments performed in triplicates.

Task 2: Examining the effect of ALK on Smad signaling activities.

Subtask 1: Examining Smad4 phosphorylation by different protein tyrosine kinases (PTK).

The human genome encodes 90 protein tyrosine kinases (PTK), including 58 receptor tyrosine kinases and 32 non-receptor tyrosine kinases. Activation of many of these kinases (e.g. EGFR, Her2, BCR-Abl, c-Src, just name a few) has been associated with tumorigenesis, and thus may also interplay with Smad proteins. Hence, we were wondering if Smad4 is phosphorylated and its activity is regulated by different protein tyrosine kinases. This investigation will provide insights into the mechanisms underlying TGF-β resistance in many cancer types.

Specifically, in our study, we have constructed an expression library of all PTKs. Out of 90 PTKs, 80 PTKs were expressed in transfection experiments. We thus screened the effect of these PTKs and examined their ability to phosphorylate Smad4 using phosphor-Tyr Western blotting analysis. We found a few of PTKs can phosphorylate Smad4, including c-Abl (Fig. 2). More importantly, using PY95 antibody, we

could detect Smad4 phosphorylation in breast cancer tissues, where EGFR family members are also activated. We are now testing whether EGFR family members can phosphorylate other sites of Smad4 or other Smads.

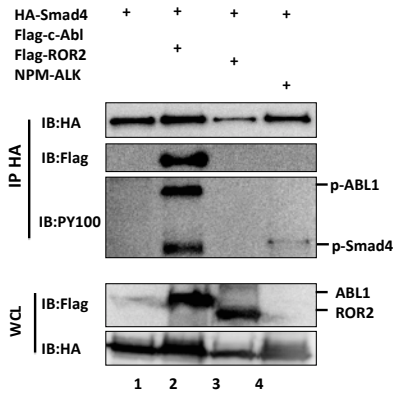

Fig. 2. NPM-ALK and c-Abl, but not ROR2, phosphorylate Smad4 on tyrosine. Co-IP experiments were done in transfected 293T cells.

Subtask 2: Examining the effect of ALK on other Smads and the effect of overexpressed ALK on potential tyrosine phosphorylation of Smad2/3.

Smad4 belongs to the structurally related Smad family of intracellular signal transducers for TGF-β superfamily. The human genome encodes eight Smad proteins. Besides co-Smad Smad4, there are five receptor-activated Smads (R-Smads), which are serine phosphorylated by specific type I receptors upon ligand stimulation (i.e. TGF-β/activin-specific Smad2/3 and BMP-specific Smad1/5/8). After identifying the phosphorylation of Smad4 by ALK, we were interested in determining if ALK phosphorylates Smad4 specifically or also targets other Smad proteins.

We used cell transfection, immunoprecipitation and Western blot with specific antibodies, endogenous Smad tyrosine phosphorylation, and *In vitro* kinase assay to examine the effect of ALK on other Smads and the effect of overexpressed ALK on potential tyrosine phosphorylation of Smad2/3. We found that ALK

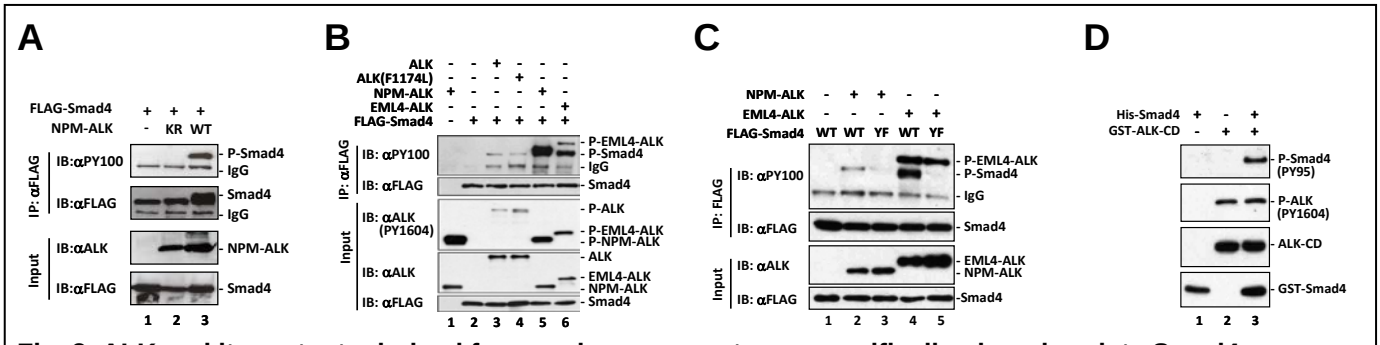

Fig. 3. ALK and its mutants derived from various cancer types specifically phosphorylate Smad4 on tyrosine. (A) NPM-ALK, but not its kinase-dead mutant (KR), could phosphorylate Smad4. **(B)** Like NPM-ALK, EML4-ALK and ALK(F1174L) could also phosphorylate Smad4. **(C)** Wildtype Smad4, but not its tyrosine-to-phenylalanine substitution (YF), could be phosphorylated by NPM-ALK and EML4-ALK. **(D)** Recombinant ALK cytoplasmic domain containing the kinase domain (ALK-CD) efficiently phosphorylated recombinant Smad4 protein on tyrosine.

specifically tyrosine-phosphorylated Smad4 but not other intracellular mediators of TGF-β signaling such as Smad2 and Smad3, suggesting the specific regulation of Smad4 activity by ALK (Fig. 3).

Subtask 3: Examine the effect of Smad4 tyrosine phosphorylation on Smad4 intracellular localization.

TGF-β treatment promotes nuclear accumulation of Smad4 and the subsequent transcriptional activation of target genes. To further characterize in detail how ALK regulates Smad4 activity, here we examined if ALK and resulted Smad4 tyrosine phosphorylation changes the subcellular distribution of Smad4 using conventional methods such as immunofluorescence staining (Fig. 4A) or cell fractionation assay (Fig. 4B). We found that overexpression of ALK had no effect on Smad4 nuclear-cytoplasmic partitioning. Furthermore, we made mutation of Y95 into acidic E (i.e. Y95E to mimic phosphorylation) or F (Y95F to cause phosphorylation-defective) on Smad4, and found that these mutations did not impact the intracellular localization of Smad4.

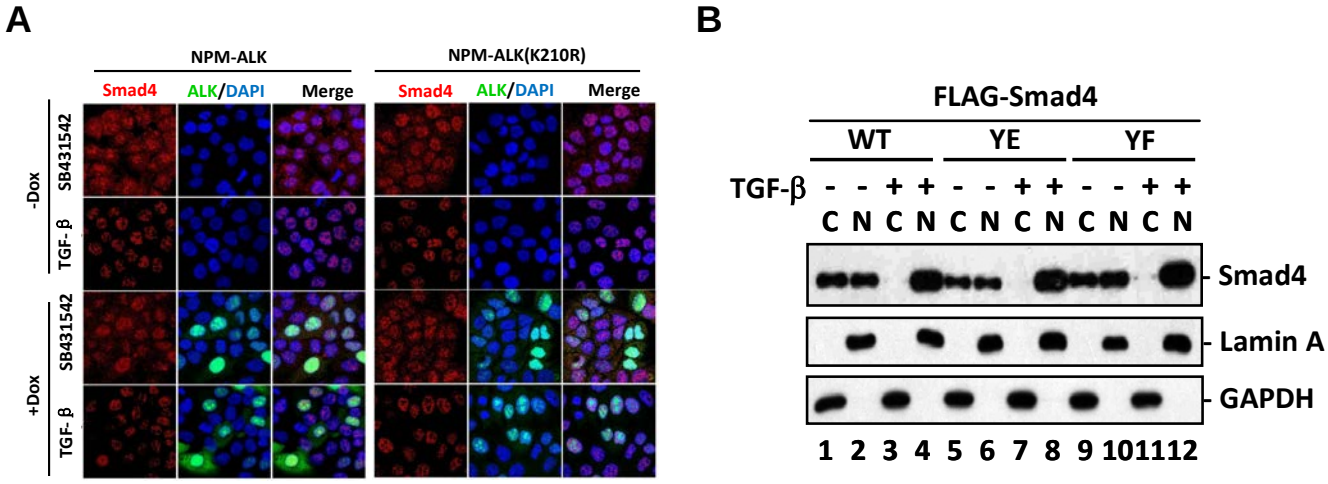

Fig. 4. Phosphorylation of SMAD4 has no effects on its nuclear vs. cytoplasmic localization. (A) NPM-ALK exhibits no effects on nuclear translocation of SMAD4. HaCaT cells stably expressing NPM-ALK or NPM-ALK(K210R) upon Dox induction were treated with SB431542 or TGF-β for 2 h, and then subjected to immunofluorescence with indicated antibodies. Red, immuno-stained SMAD4; purple, DAPI nuclear staining. **(B)** Mutation at Y95 of SMAD4 has no effect in its nuclear-cytoplasmic accumulation. HeLa cells were transfected with FLAG-tagged SMAD4 (WT, Y95E or Y95F) and then treated with TGF-β (2 ng/ml, 1 h). Nuclear (N) and cytoplasmic (C) fractions were isolated and subjected to Western blotting. Lamin C is a nuclear marker, while GAPDH is a cytoplasmic marker.

Subtask 4: Examine the effect of Smad4 tyrosine phosphorylation on Smad4-Smad2/3 complex formation.

Since the function of Smad4 in mediating TGF-β signaling relies largely on its association with Smad2 and Smad3 to form a large transcriptional factor complex, we determined the effect of ALK on TGF-β-induced complex formation between Smad4-Smad2/3 in BC cell lines with/without ALK knockdown or with ALK overexpression. For this purpose, we immunoprecipitated Smad4 with Smad4-specific antibody from a variety of cell lines (e.g. MDA231 cells), and the presence of Smad2 in the precipitated complex was determined by Western blot with Smad2-specific antibody. We found that overexpression of active ALK

(NPM-ALK) did not alter the interaction between Smad4 and Smad2 at endogenous levels (Fig. 5A) or in transfected cells (Fig. 5B). Likewise, overexpression of active ALK did not alter the interaction of Smad4 with Smad3 (Fig. 5C). Furthermore, the YE or YF substitution had no effect on the Smad2/3-Smad4 interactions (Fig. 5B and 5C).

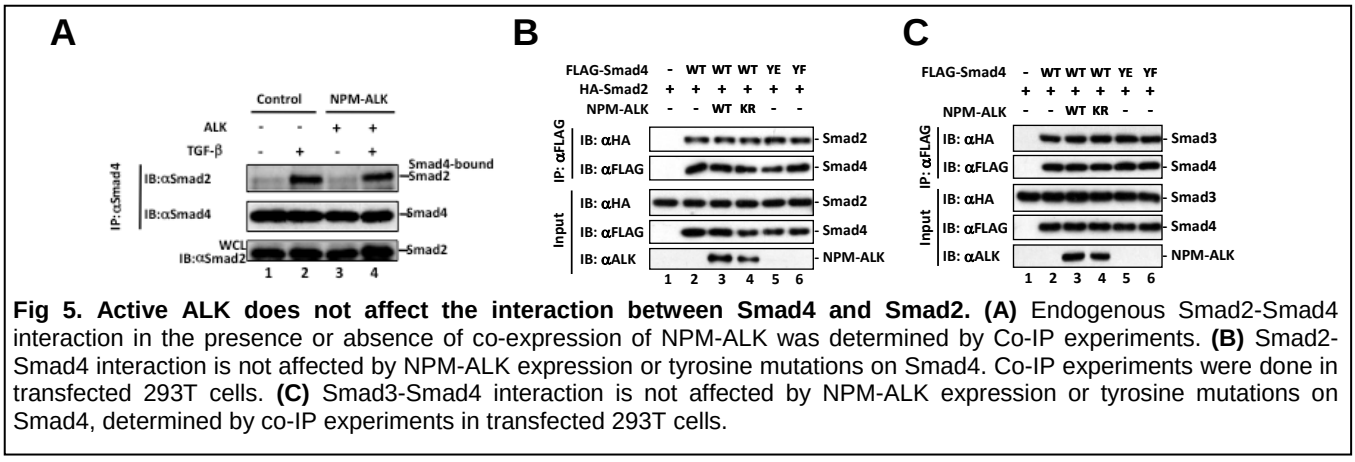

Fig 5. Active ALK does not affect the interaction between Smad4 and Smad2. (A) Endogenous Smad2-Smad4 interaction in the presence or absence of co-expression of NPM-ALK was determined by Co-IP experiments. **(B)** Smad2-Smad4 interaction is not affected by NPM-ALK expression or tyrosine mutations on Smad4. Co-IP experiments were done in transfected 293T cells. **(C)** Smad3-Smad4 interaction is not affected by NPM-ALK expression or tyrosine mutations on Smad4, determined by co-IP experiments in transfected 293T cells.

Subtask 5: Analysis of Smad4 WT, Y95E or Y95F DNA-binding activity using Electrophoretic Mobility Shift Assay (EMSA).

We have examined the effect of Smad4 tyrosine phosphorylation on its binding to DNA by using two DNA-binding assays: EMSA and DNA pull-down. Specifically, EMSA detects the retarded mobility of DNA-protein complexes in comparison to free DNA probes. As expected, SMAD4 effectively binds to Smad-binding element (SBE). Notably, this gel-shifting ability of SMAD4 was nearly abolished by NPM-ALK in the presence of ATP (Fig. 6A). In DNA-pulldown assays, wild-type SMAD4 efficiently bound to biotin-labeled SBE, which was inhibited by NPM-ALK, but not kinase-dead NPM-ALK(K210R). The Y95E mutant was unable to bind to SBE (Fig. 6B, lane 6). Interestingly, the Y95F mutant exhibited a higher level of SBE-binding activity than wild-type SMAD4, which is not affected by NPM-ALK.

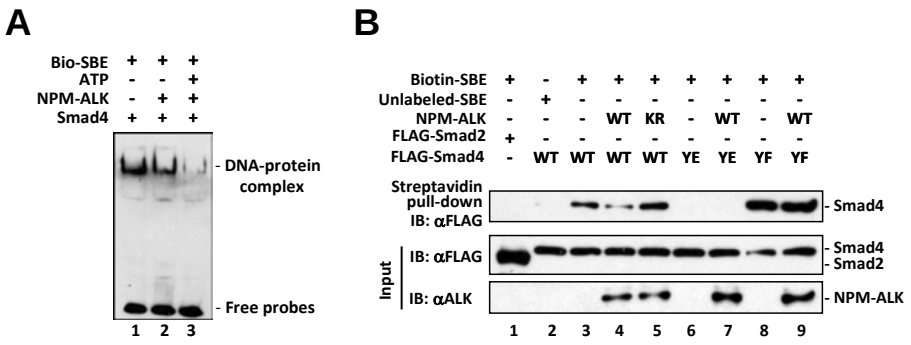

Fig 6. Phosphorylation of SMAD4 on Y95 impairs its DNA binding activity. (A) NPM-ALK impairs DNA-binding ability of SMAD4 in an EMSA assay. His-SMAD4 and NPM-ALK were separately immune-purified from HEK293T cells. The binding of SMAD4 to biotin(Bio)-labeled SBE DNA probe was first done by incubation with NPM-ALK in the presence or absence of ATP and then detected by Streptavidin-HRP. SBEm: a mutated SMAD-binding element (SBE). **(B)** NPM-ALK or Y95E mutation abolished DNA binding of SMAD4 in a pull-down assay. HEK293T cells were transfected with FLAG-SMAD2, FLAG-tagged SMAD4 (WT, Y95E or Y95F) and/or NPM-ALK (WT or KR), treated with TGF-β (2 ng/ml, 1 h) and cell lysates harvested. DNA-bound proteins in cell lysates were incubated with biotin-labeled SBE DNA probes and pulled down on Streptavidin-beads. Retrieved proteins were determined by Western blotting using indicated antibodies. Unlabeled SBE probe is a negative control.

Subtask 6: Examine the effect of Smad4 tyrosine phosphorylation on Smad4 binding to chromatin.

We have further examined the effect of Smad4 tyrosine phosphorylation on its binding to chromatin by using a more physiological relevant approach: Chromatin Immunoprecipitation (ChIP). Specifically, the effect of phosphorylation on Smad4 binding to endogenous TGF-β target gene promoters was examined. Cell lysates was collected and sonicated to generate chromosomal fragments. Anti-Smad4 or anti-Smad4(pY95) antibody was used to immunoprecipitate total Smad4 or Smad4(pY95), and the recruitment of Smad4 or Smad4(pY95) to chromatin was quantitatively analyzed by qPCR with primers covering a specific promoter region of TGF-β target genes of p15 (Fig. 7A), p57 (Fig. 7B), p21 and PAI-1 (data not shown). Immunoprecipitation with control IgG, qPCR with primers covering a distal region or the promoter region of GAPDH was included as negative controls.

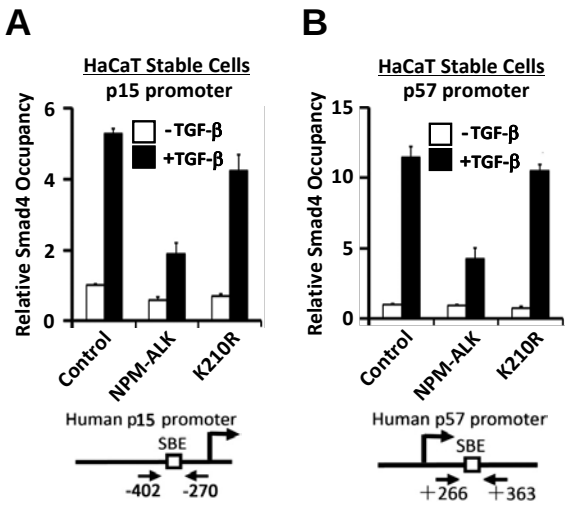

Fig 6. Phosphorylation of SMAD4 on Y95 impairs its DNA binding activity. (A) NPM-ALK impedes SMAD4 binding to the promoter region of endogenous *p15* gene. HaCaT tet-on cells (NPM-ALK or K210R) were pre-treated with Dox for 24 h, and then stimulated with TGF-β (2 ng/ml, 1.5 h). Chromatin IP (ChIP) was carried out with anti-SMAD4 antibody and PCR analysis using primers specific to *p15* as shown. Values are the means (±s.e.) of three separate experiments performed in triplicates. **(B)** NPM-ALK impedes SMAD4 binding to the promoter region of endogenous p57 gene. Cell treatment, ChIP and data analysis were done as described in Panel A.

Task 3: Analysis of the global effect of ALK on Smad4 genomic and transcriptional responses

These studies are underway.

Task 4: To test whether ALK activation leads to Smad4 Y95 phosphorylation in vivo

TGF-β signalling plays paradoxical roles in tumourigenesis, promoting both tumour suppression and metastasis. The current paradigm is that tumour suppression is the dominant activity of TGF-β in normal tissues and tumours at early stages. As a tumour suppressor, SMAD4 can potently suppress proliferation and promote apoptosis. We established cell lines stably expressing wild-type SMAD4, its Y95E mutant, or GFP (as control) in breast cancer MDA-MB-468 cells. These cells were injected subcutaneously into nude

mice to induce tumours. About 40 days later, the nude mice were executed and tumours were dissected and weighed. Results show that parental or control GFP cells led to obvious early tumour growth. Expression of wild-type SMAD4 moderately inhibited the growth of tumours, consistent with its tumour suppressor function. In contrast, the ability of SMAD4 to inhibit tumour growth was greatly compromised by the phospho-mimicking Y95E mutation (Fig. 7A). In MDA-MB-468 cells, while SMAD4 restored TGF-β response, NPM-ALK blocked SMAD4 transcriptional response (Fig. 7B).

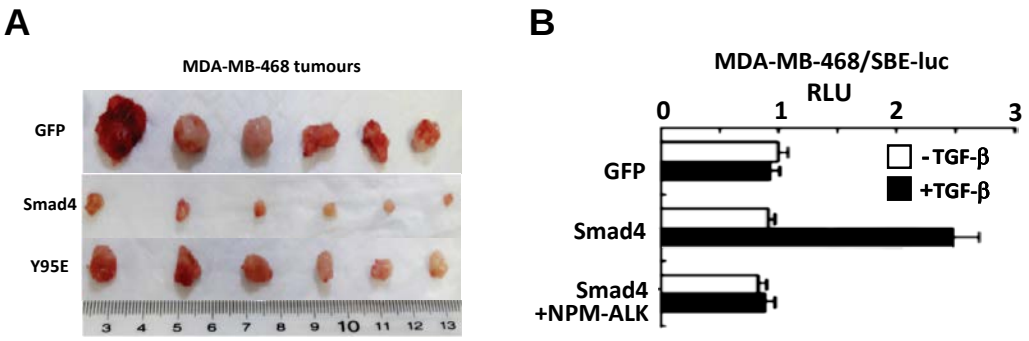

Fig 7. Phosphorylation of SMAD4 impairs its tumor suppressor activity. (A) SMAD4(Y95E) has weakened tumour suppressor activity. SMAD4-null MDA-MB-468 cells reconstituted with GFP, SMAD4 or SMAD4(Y95E) were injected into nude mice subcutaneously to determine their tumourigenic activity. MDA-MB-468 cells stably expressing SMAD4(Y95E) generate larger tumours than those expressing SMAD4 as evaluated by tumour size. **(B)** NPM-ALK blocks SMAD4 transcription activity. MDA-MB-468 cells were transfected with SBE-luc together with GFP, SMAD4 wild-type, or SMAD4 wild-type plus NPM-ALK. Relative luciferase activity was measured 18 h after TGF-β treatment (2 ng/ml). Values are the means (±s.e.) of three separate experiments performed in triplicates and normalized for transfection efficiency against Renilla Luciferase activities.

KEY RESEARCH ACCOMPLISHMENTS:

- Determined the specific regulation of ALK on Smad4.
- Determined that Smad4 is phosphorylated by other protein tyrosine kinases establishing a general regulatory mechanism of TGF-b activity by protein tyrosine kinases.
- Determined that how Smad4 Y95 phosphorylation regulates Smad4 activity. Tyrosine phosphorylation of Smad4 doesn't affect its binding to other Smad partners or its nuclear translocation upon TGF-β stimulation.
- Determined that how Smad4 Y95 phosphorylation blocks the DNA-binding activity of Smad4.
- Determined the effect of ALK and Smad4 Y95 phosphorylation on TGF-β tumor suppressing and transcriptional responses.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

- TGF-β Meeting, Uppsala, August 31-September 2, 2017
- TGF-β Meeting, Xiamen, September 26, 2017.

CONCLUSION:

The oncogenic action of ALK has been believed to be through the signaling pathways (MEK/ERK, STAT3, PI3K/Akt), which promote cell proliferation while preventing cell death. Through our study, we for the first time revealed that ALK inhibited TGF-β signaling pathways. Furthermore, we found that ALK inhibited TGF-β signaling by tyrosine phosphorylating Smad4 at Y95. Mechanistically, Smad4 tyrosine phosphorylation completely wiped out its DNA-binding activity and transcriptional responses. Consequently, tyrosine phosphorylation disables Smad4 to inhibit tumor growth. Therefore, our findings decipher a novel crosstalk between ALK and TGF-β pathway in tumorigenesis and reveal a potential TGF-β-related effects in patients with ALK treatment.

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APPENDICES: N/A