Oncogenic TPM3-ALK activation requires dimerization through the coiled-coil structure of TPM3

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ABSTRACT

Inflammatory myofibroblastic tumor (IMT) is a mesenchymal tumor that can arise from anywhere in the body. Anaplastic lymphoma kinase (ALK) gene rearrangements, most often resulting in the tropomyosin 3 (TPM3)-ALK fusion gene, are the main causes of IMT. However, the mechanism of malignant transformation in IMT has yet to be elucidated. The purpose of this study was to clarify the role of the TPM3 region in the transformation of IMT via TPM3-ALK. Lentivirus vectors containing a TPM3-ALK fusion gene lacking various lengths of TPM3 were constructed and expressed in HEK293T and NIH3T3 cell lines. Focus formation assay revealed loss of contact inhibition in NIH3T3 cells transfected with full-length TPM3-ALK, but not with ALK alone. Blue-native polyacrylamide gel electrophoresis (BN-PAGE) revealed that TPM3-ALK dimerization increased in proportion to the length of TPM3. Western blot showed phosphorylation of ALK, ERK1/2, and STAT3 in HEK293T cells transfected with TPM3-ALK. Thus, the coiled-coil structure of TPM3 contributes to the transforming ability of the TPM3-ALK fusion protein, and longer TPM3 region leads to higher dimer formation.

1. Introduction

Inflammatory myofibroblastic tumor (IMT) is a rare disease that is defined as a true neoplasm of myofibroblastic spindle cells accompanied by a variable inflammatory infiltrate of plasma cells, eosinophils, and lymphocytes, and occurs in various organs [1]. Since the first report of anaplastic lymphoma kinase (ALK) gene rearrangement caused by chromosomal translocation by Griffin et al. [2] and the detection of the tropomyosin 3 (TPM3)-ALK fusion gene by Lawrence et al. [3], about half of all IMT cases have been found to exhibit ALK gene rearrangements [4]. As fusion gene partners of ALK, TPM3, TPM4, CTLC, ATIC, CARS, RANBP2, SEC31L1,

and PPFIBP1 have so far been reported in patients with IMT, with TPM3 being the most frequent [4,5].

These fusion genes have been described to provide malignant potential through two mechanisms: 1) the promoter of the upstream fusion partner promotes the constitutive transcription of the fusion gene in cells that originally express small amounts of the carboxyl-terminal gene; and 2) the dimerization domain of the upstream fusion partner induces the dimerization of the fusion protein, causing the auto-phosphorylation of the carboxyl-terminal gene [4,6]. However, few reports have demonstrated the dimerization of fusion proteins and the relationship between dimerization and malignant potential [7,8]. The fusion partner may also define the cellular localization of the fusion protein [6].

While ALK is normally silent in adults except in the central nervous system, TPM3 is constitutively expressed in mesenchymal cells. TPM3 is composed of a coiled-coil structure and may promote dimerization of TPM3-ALK fusion protein and may promote auto-phosphorylation of the ALK catalytic domain. Although these two mechanisms are presumed to lead to the generation of IMT [3,9], it has never been shown. In the present study, we elucidated the role

of the TPM3 region by visualizing the dimerization of the TPM3-ALK fusion protein using blue-native polyacrylamide gel electrophoresis (BN-PAGE).

2. Materials and methods

2.1. TPM3-ALK fusion gene cloning and lentivirus vector constructs

We obtained the TPM3-ALK fusion gene from a surgical specimen of a lung inflammatory myofibroblastic tumor from a 42 year-old man, after obtaining written informed consent and approval from the ethical committee of our institute (Fig. 1). The fusion gene was gene confirmed using the 5′-rapid amplification of cDNA ends (5′-RACE) technique with SMARTer® PCR cDNA Synthesis Kit according to the manufacturer's instruction (Clontech Laboratories, Mountain View, CA, USA). After cloning the coding sequence of the TPM3-ALK fusion gene using pGEM-T easy Vector Systems (Promega, Madison, WI, USA) (D0), various lengths (165, 330, 495, 660 bp) were deleted from the 5′ end immediately after the first ATG (D1, D2, D3, D4 in Fig. 1) using an inverse PCR [10,11]. D*1 consisted of a fusion gene lacking 165 bp of the TPM3 region from the 3′ end. The primers used for cloning were sense 5′-CACCATGGCTGGGATCACC-3′ and antisense GGAAGAGAAGTGAGTGTGCGACC; and the primers used for inverse PCR were sense 5′-CAGCTGGTTGAAGAAGAGCTGG-3′ (D1), 5′-CTCAAAGAAGCTAAGCACATTGCAG-3′ (D2), 5′-AACCT-GAAGTGTCTGAGTGCTGCT-3′ (D3), and 5′-GTGTACCGCCGGAAG-CACC-3′ (D4, D*1) and antisense 5′-CATGGTGAATCGAATTCCCG-3′ (D1, D2, D3, D4) and 5′-CTGGTCCATCAGTCTAATCTGCTCAT-3′ (D*1). All the products were ligated to FLAG-tag and incorporated into a CSII-CMV-MCS-IRES2-Bsd lentiviral plasmid (Riken BioResource Center, Tsukuba, Ibaraki, Japan).

2.2. Cell cultures

NIH3T3 and HEK293T cell lines were obtained from American Type Culture Collection (Manassas, VA, USA) and Lenti-X HEK293T cell line was purchased from Clontech Laboratories. Cells were cultured in DMEM supplemented with 10% bovine serum (NIH3T3) or 10% fetal bovine serum (HEK293T, Lenti-X HEK293T) and antibiotic-antimycotic mixed stock solution (Nakarai Tesque, Kyoto, Japan) at 37 °C in a 5% CO_2 incubator. Lenti-X HEK293T was transfected with lentiviral plasmid vectors and lipofection reagent Hilymax (Dojindo, Kumamoto, Japan) to package lentivirus.

2.3. Protein assays

Protein was extracted from HEK293T cells 4 days after infection with a lentivirus carrying TPM3-ALK. For BN-PAGE, protein was extracted using NativePAGE™ sample buffer (Life Technologies, Grand Island, NY, USA) without sodium dodecyl sulfate (SDS). BN-PAGE was performed using the NativePAGE™ Novex® Bis-Tris Gel system (Life Technologies), according to the manufacturer's instructions. After transfer to a PVDF membrane, the membrane was incubated in 8% acetate for crosslinking and in methanol to remove Coomassie Brilliant Blue G-250. Anti-FLAG-tag antibody (1:1000; Sigma-Aldrich, St. Louis, MO, USA) was used to detect the fusion protein. For SDS-PAGE with western blotting, the following primary antibodies were used: anti-FLAG-tag antibody (1:1000; Sigma-Aldrich), anti-phospho-ALK antibody (1:1000), anti-ALK antibody (1:1000), anti-phospho-ERK1/2 antibody (1:600), anti-ERK1/2 antibody (1:1000), anti-phospho-STAT3 antibody (1:600), anti-STAT3 antibody (1:3000; Cell Signaling Technology, Danvers, MA, USA), and anti-β-actin antibody (1:20,000; Santa Cruz Biotechnology, Dallas, TX, USA).

2.4. Focus formation assay

Lentiviruses were used to infect NIH3T3 cells in the presence of blasticidin (10 μg/mL; Sigma-Aldrich, St. Louis, MO, USA). After 6 days of exposure to blasticidin, the infected NIH3T3 cells were seeded on a 6-well plate at 1000 cells per well, cultured in blasticidin-free medium for 28 days, and the foci were counted. Green fluorescence protein (GFP) and mutant KRAS (G12C) vectors were used as negative and positive controls, respectively.

3. Results

3.1. Transforming activity of TPM3-ALK requires the TPM3 region

To evaluate the transforming activity of the TPM3-ALK gene, we transfected a lentivirus vector of the TPM3-ALK gene (D0) or the
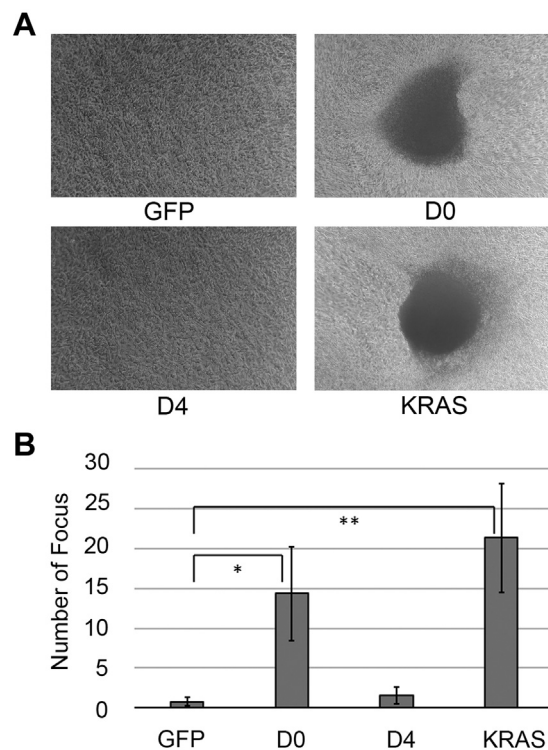

Fig. 1. Diagram of tropomyosin 3 (TPM3), anaplastic lymphoma kinase (ALK), TPM3-ALK fusion gene, and TPM3-ALK lacking various lengths of the TPM3 region. Coiled-Coil: coiled-coil structure, Ig-like: immunoglobulin-like domain, TM: transmembrane domain, TK: tyrosine kinase domain.

Fig. 2. Focus formation assay. (A) Representative images of the focus. (B) Histogram depicting the number of foci. The focus formation assay described above was performed in triplicate. *P < 0.05, **P < 0.01.
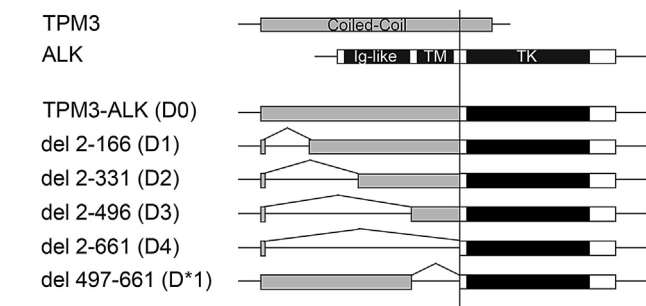
TPM3-region-deleted fusion gene (D4) into NIH3T3 cells. Focus formation assay revealed that TPM3-ALK-transfected NIH3T3 cells (D0) formed similar number of foci compared with mutant KRAS-transfected cells and more foci compared with green fluorescent protein (GFP) control. Cells transfected with a TPM3-region-deleted-fusion-gene (D4) formed only a few foci (Fig. 2). Thus, the transforming activity of the TPM3-ALK fusion gene required the existence of the TPM3 region.

3.2. TPM3-ALK dimerization requires the coiled-coil structure of TPM3

TPM3 is an actin filament-binding protein with a dimeric α-helical coiled-coil structure along its entire length. The coiled-coil structure is based on a repeated pattern of seven amino acids with hydrophobic residues at the first and fourth positions; this repeated pattern is known to contribute to dimerization [12—14]. Therefore, previous reports have speculated that the TPM3-ALK fusion gene may form dimers that contribute to the constitutive activation of ALK and its signal cascade [3,9]. To confirm this speculation, we transfected lentivirus vectors containing TPM3-ALK fusion genes lacking various lengths of the TPM3 coiled-coil structure into HEK293T cells and visualized the resulting dimerization using blue-native polyacrylamide gel electrophoresis (BN-PAGE) (Fig. 3A). BN-PAGE detected TPM3-ALK dimers but no monomers after transfecting full-length TPM3-ALK. Dimer formation decreased and the quantity of monomers increased with increasingly larger deletions of the TPM3 region from the amino terminal, and no dimer formation was detected when the entire length of TPM3 was deleted. When a quarter of the carboxyl region of TPM3 was deleted, a dimer band could still be detected using BN-PAGE. Thus, no specific domain within TPM3 was responsible for dimerization, but the length of TPM3 proportionately influenced the extent of the dimerization of the TPM3-ALK fusion protein.

3.3. TPM3-ALK dimer activates downstream signal pathways

Finally, we analyzed the effect of dimerization on downstream signal pathways using SDS-PAGE with western blotting. Phosphorylated ALK was detected in full-length TPM3-ALK transfected HEK293T cells, but not in HEK293T cells transfected with TPM3-region-deleted fusion gene. Three signal pathways involving ALK have been reported: the PI3K/Akt, JAK/STAT, and Raf/MEK/ERK pathways. Among them, phosphorylation of Akt was similar between GFP control-transfected and fusion gene-transfected HEK293T cells (data not shown). Phosphorylation of STAT3 and ERK1/2 was detected in full-length TPM3-ALK-transfected HEK293T cells, but not in TPM3-region-deleted or control cells (Fig. 3). STAT3 and ERK1/2 phosphorylation was also seen in HEK293T cells transfected with TPM3-ALK fusion genes with incomplete lengths of the TPM3 region (D1, D2, D3). These results suggest that the dimerization of TPM3-ALK induces ALK phosphorylation and activates the JAK/STAT and Raf/MEK/ERK pathways.

4. Discussion

In the present study, we visualized the dimerization of the oncogenic fusion protein TPM3-ALK using BN-PAGE. BN-PAGE is a native-PAGE technique that was established by Schagger et al. in 1991 [15,16]. Unlike other native PAGE techniques, in which no charged dye is used, Coomassie Brilliant Blue is used as a charge shift molecule in BN-PAGE. Therefore, the size resolution of the technique is as high as that of SDS-PAGE while the protein is kept in its native state [17]. Thus, BN-PAGE is a useful and simple technique for analyzing the protein complexes. In previous reports, oncogenic dimerization was demonstrated using a more complicated procedure: Bischof et al. performed coimmunoprecipitation experiments using anti-ALK and anti-NPM antibodies to detect a heterodimer between NPM-ALK and wild-type NPM and also performed a
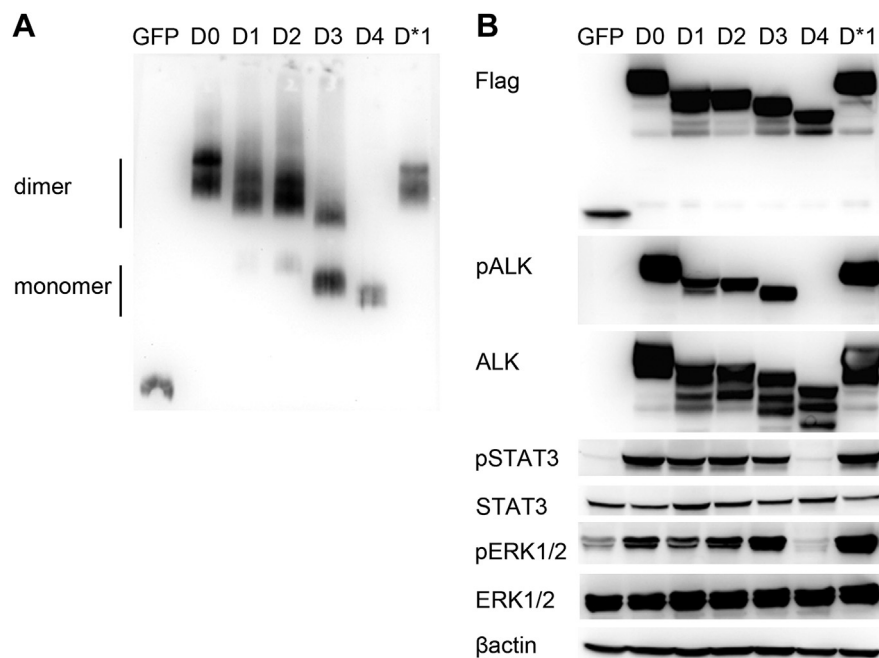

Fig. 3. Protein assays. (A) Blue-native polyacrylamide gel electrophoresis (BN-PAGE) detected dimer and monomer of the TPM3-ALK fusion protein. The proportion of dimer to monomer decreased with increasingly larger deletions of the TPM3 region. (B) SDS-PAGE with western blotting revealed that the phosphorylation of ALK, STAT3 and ERK1/2 decreased dramatically when the full length of the TPM3 region was deleted.

sucrose gradient sedimentation analysis using an *in vitro* kinase assay to reveal a kinase activity in the gradient fraction that was larger than that for the NPM-ALK monomer [7]; McWhirter et al. demonstrated oligomerization by applying a glutaraldehyde cross-linking technique to purified Bcr peptide, thereby revealing the presence of Bcr-Abl fusion protein oligomerization [8]. Successful use of this simple BN-PAGE technique to visualize the dimerization of an oncogenic fusion gene was to our knowledge for the first time.

Overexpression of TPM3-ALK led to phosphorylation of ALK, STAT3, and ERK1/2 and loss of contact inhibition, whereas phosphorylation and focus-forming potential were lost when the full length of the TPM3 region was deleted. This suggests that overexpression of ALK alone is not sufficient to activate the ALK signaling. Although Caren et al. [18] reported that 5% of sporadic neuroblastomas had exhibited amplification of the ALK gene locus without ALK gene mutation, Soda et al. [19] denied the transforming activity of NIH3T3 cells arising from ALK gene overexpression alone. Our study revealed again the importance of the fusion gene partner in the constitutive activation of the oncogenic signal pathway through the induction of dimerization.

Conflict of interest

The Authors declare no conflict of interest.

Transparency document

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