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Short Communication

TPM3-ALK expression induces changes in cytoskeleton organisation and confers higher metastatic capacities than other ALK fusion proteins

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ABSTRACT

Translocations of the anaplastic lymphoma kinase (ALK) gene result in the production of a number of oncogenic ALK fusion proteins implicated in tumour development.

We have previously shown that X-ALK fusion proteins have differential effects on the proliferation, transformation, and invasion properties of NIH3T3 cells *in vitro*. In the present study, we have investigated the metastatic potential of various X-ALK expressing cell lines using an experimental lung metastasis assay.

We have shown that TPM3-ALK expression bestows higher metastatic capacities than other X-ALK fusion proteins and in addition, that TPM3-ALK fusion protein expression specifically induces changes in cell morphology and cytoskeleton organisation. Co-immunoprecipitation studies demonstrate a specific interaction between TPM3-ALK and endogenous tropomyosin. Together the specific actions of TPM3-ALK on the cytoskeleton organisation offer an interesting hypothesis with respect to the higher cell motility and metastatic potential of this fusion protein.

1. Introduction

Aberrant expression of the ALK tyrosine kinase as a chimeric protein with various partners plays a key role in malignant cell transformation of T-lymphocytes and other cells. Most translocations affecting the human *ALK* gene at 2p23 have been found in inflammatory myofibroblastic tumours (IMT) and in anaplastic large cell lymphomas (ALCL).[4,5] These chromosomal abnormalities result in the production of oncogenic ALK fusion proteins. In ALCL, the most frequent chromosomal abnormality is the translocation t(2;5)(p23;q35) in which the nucleophosmin *(NPM)* multimerisation domain is fused to the *ALK* tyrosine kinase domain.[6,7] In inflammatory myofibroblastic tumours (IMT) the most fre-

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quent chromosomal abnormality is the (1;2)(q25;p23) translocation involving *ALK* and *TMP3* (non muscular tropomyosin 3). The fusion protein retains the coiled coil dimerisation domain of TPM3, playing a similar generic role as NPM. Indeed, Lawrence et al. have confirmed that the TPM3-ALK fusion proteins in IMT are phosphorylated and activated.[8] Additional *ALK* translocation products have been identified in ALCL,[4,9] as well as in B-cell non-Hodgkin lymphomas,[10] and in IMT.[11]

Most X-ALK fusion proteins posses an amino-terminal oligomerisation domain(s) that mimics ligand-mediated aggregation of wild-type ALK and results in the constitutive activation of the kinase.[4,12] Expression of X-ALK proteins in ALCL and IMT provide a rare example of a tyrosine kinase receptor implicated in the oncogenesis of haematopoietic and non-haematopoietic neoplasms.[13] Although the dissemination of the haematopoietic and non-haematopoietic tumours require different mechanisms, reorganisation of the actin cytoskeleton is a primary mechanism whereby cells obtain motility, a characteristic that is essential for cell migration.[14]

Clinically, ALK-positive ALCLs are usually presented as an aggressive disease, stage III–IV, frequently associated with extranodal involvement, especially skin, bone marrow, soft tissues, lung and liver.[15,16]

Inflammatory myofibroblastic tumours (IMTs) are neoplastic mesenchymal proliferations featuring an inflammatory infiltrate composed primarily of lymphocytes and plasma cells.[17] These tumours, frequently seen in children and young adults, were first confined to cases involving the lung or pleura, until examples were described in the abdomen, skin as well as soft tissues, spleen, heart, bladder, upper respiratory tract, mediastinum, kidney, larynx, paranasal sinuses, cervical esophagus, and the soft tissues of the neck. They include a heterogeneous group of lesions characterised by inflammatory cell infiltration and variable fibrotic reaction.[17] Inflammatory myofibroblastic tumours are now a widely recognised disease with a generally favourable prognosis, although several examples of distant metastases have occurred.[18–21]

2. Materials and methods

2.1. Cell culture

NIH3T3 cells stably transfected with recombinant vectors carrying cDNAs encoding full-length X-ALK fusion products, where X represents *NPM* (nucleophosmin), *TPM3* (non muscular tropomyosin 3), *TFG* (TRK-fused gene), *CLTC* (clathrin heavy-chain polypeptide), or *ATIC* (5-aminoimidazole-4-carboxamide ribonucleotideformyltransferase/inosine monophosphate cyclohydrolase) were previously described.[1] These cells were cultured in DMEM (GIBCO BRL) containing 10% foetal calf serum v/v (Myoclone-Invitrogen), 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 100 u/ml penicillin and 100 μg/ml streptomycin and selection was performed with 1 mg/ml geneticin (G418) at 37 °C in 5% CO_2.

2.2. Western blotting

Cell extracts were sonicated in an extraction buffer (Tris-HCl pH 7.5 20 mmol/L, EDTA 0.5 mmol/L, EGTA 0.5 mmol/L, 10% glycerol v/v, DTT 0.01 mmol/L, 1 mmol/L pefabloc, 1 mmol/L sodium orthovanadate, 4 mmol/L sodium fluoride, 2 μg/ml pepstatin, 10 μg/ml leupeptin, 2 μg/ml aprotinin and 0.2% SDS w/v). Protein quantification was performed using the Bio-Rad DC Protein Assay (Bio Rad). Samples (20 μg per lane) were separated on 7.5% polyacrylamide gels. After electrophoretic transfer, nitrocellulose membranes were blocked with 3% skimmed milk w/v and incubated with ALK1 monoclonal antibody (Dakocytomation),[1] tropomyosin polyclonal antibody (diluted 1/500) allowing the detection of tropomyosin isoforms (sc-18174 from Santa Cruz) or actin monoclonal antibody diluted 1/5000 (AC-15 Sigma). Antibody binding was detected with immunoglobulins/HRP (Dakocytomation) (diluted 1/5000), using a standard chemiluminescence Western blot protocol (ECL system; Amersham).

2.3. Experimental lung metastasis assay

5×10^6 NIH3T3 cells were labelled with PKH26-GL red fluorescent cell linker kit (Sigma) allowing general cell membrane labelling as previously described.[2] Staining, plating efficiency and cell viability were analysed. Single cell suspensions of freshly prepared PKH26-GL rhodamine-fluorescent NIH3T3 transfected cells (1×10^6 cells) were intravenously injected in the retro-orbital plexus.[2] Ten days after cell inoculation, lungs were excised, frozen and embedded using O.C.T. compound. 10 μm cryosections were dried and slides were mounted using cyanoacrylate glue (Scotch Instant Glue). Lung metastases were visualised and scored under a Leica fluorescence microscope using a standard filter setup for TRITC. Fluorescence quantification was performed using the LEICAQWin software.

2.4. Immunoprecipitation

10^7 NIH3T3 fibroblasts were washed three times with ice-cold PBS, scraped from a 100 mm dish into 1 ml of lysis buffer (80 mmol/L Tris–HCl pH 7.5, 200 mmol/L NaCl, 20 mmol/LEDTA 2%Triton X-100 v/v with 1 mmol/L pefabloc, 1 mmol/L sodium orthovanadate, 4 mmol/L sodium fluoride, 2 μg/ml pepstatin, 10 μg/ml leupeptin and 2 μg/ml aprotinin) and kept at 4 °C in rotation for 30 min. Lysates were precleared and after centrifugation incubated at 4 °C for 2 h with ALK1-protein G. Immunocomplexes were washed three times with the lysis buffer and then boiled in sample buffer. After separation on SDS-polyacryamide gel, proteins were transferred onto nitrocellulose membranes blotted with anti-phosphotyrosine (4G10) monoclonal antibody, the anti-tropomyosin antibody, and the anti pp60Src rabbit polyclonal antibody (sc-19 from Santa Cruz).

2.5. Immunostaining, confocal microscopy analyses and sub-cellular fractionation

Immunocytochemistry was performed as previously described[1] using antibodies against actin (AC-15 Sigma), ALK

(ALK-1 Dakocytomation) and FITC-conjugated antibody against mouse IgG (dilution 1:50) (Sigma).

Nuclei were labelled with chromomycin A3 (0.1 mg/ml) and visualised using a Zeiss LSM510 confocal microscope.

To visualise the association of X-ALK fusion proteins with the cytoskeleton, Western blot with ALK1 antibody (Dakocytomation) was performed after sub-cellular fractionation as previously described.[3]

3. Results and discussion

We have previously shown that X-ALK expressing NIH3T3 clones (described in Ref. [1]) displayed differential biological effects regarding their *in vitro* migratory capacity and ability to cross an endothelial cell mono-layer.[1] Interestingly, *TPM3-ALK*-transfected cells displayed the highest migratory and invasive capacities. Since basement membrane degradation and trans-endothelial cell migration are preliminary and critical steps associated with tumour invasion and metastasis, we chose to investigate the metastatic potential of cells expressing various forms of X-ALK fusion proteins. For this purpose, we used an *in vivo* experimental metastasis assay to measure the lung colonizing capacity of X-ALK expressing cells (Fig. 1). This experimental approach allows the analysis of the development and quantification of lung metastases, taking advantage of the shorter and more predictable experimental time compared to spontaneous metastases obtained through subcutaneous injection. Indeed, in a previous study, transfected cells were grafted subcutaneously in athymic nude mice and metastasis in other organs was investigated but could not be detected due to the short period of observation.[1] The tumours were not left to develop for more than 12 days because mice developed large tumours and began to die from day 14 after subcutaneous injection of X-ALK expressing cells.[1]

Western blotting analysis performed on cell extracts enabled us to select clones expressing comparable levels of ALK fusion proteins with their expected molecular weights:

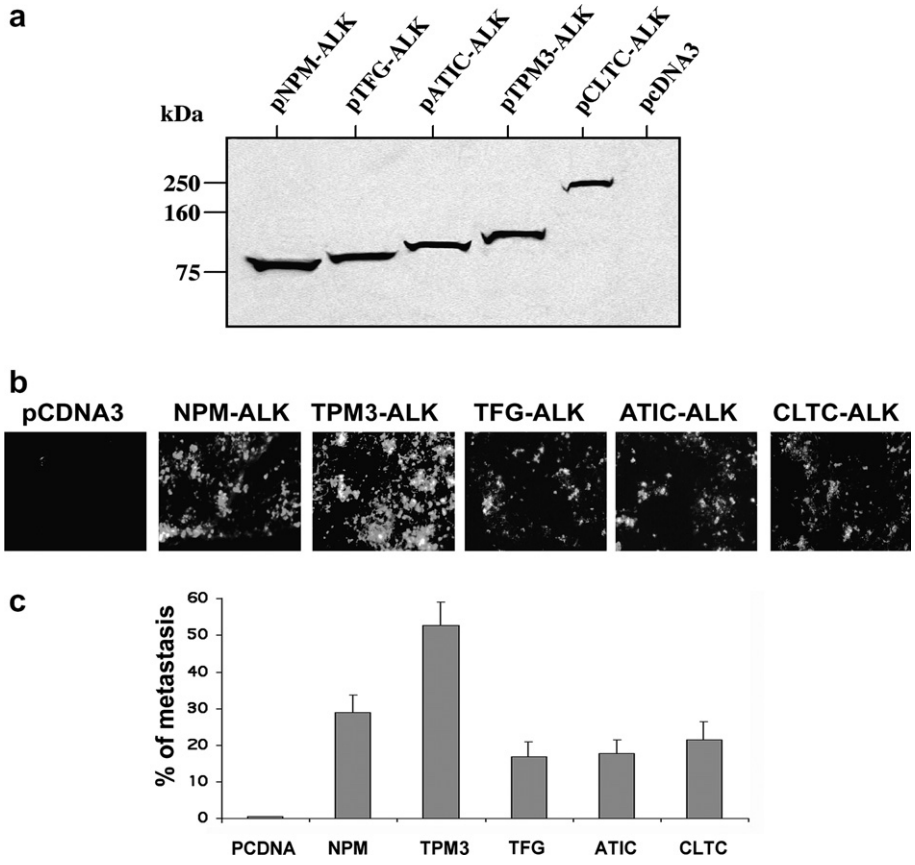

Fig. 1 – Experimental lung metastasis assay. (a) Western blot analysis of ALK expression in total cell extracts from NIH3T3 cells stably transfected with *NPM-ALK*, *TFG-ALK*, *ATIC-ALK*, *TPM3-ALK* and *CLTC-ALK* cDNA, respectively. [1] pCDNA3 represents empty vector transfected cells (negative control). Molecular weights are indicated. Detection (b) and quantification (c) of lung metastases after PKH26-GL labelled cell injection. 1×10^6 cells labelled with PKH26-GL red fluorescent cell linker kit (Sigma) were intravenously injected in the retro-orbital plexus of nude mice.[2] Ten days after cell inoculation, lungs metastases were scored under a Leica fluorescence microscope using a standard filter setup for TRITC. Quantification of metastases was performed using the LEICAQWin software. The values shown represent the mean ± SEM of the percentage of labelled NIH3T3 transfected cells present in the lung sections in two independent experiments. Six athymic nude mice were used for each X-ALK expressing cell in each independent experiment and 12 frozen sections of each lung were quantified for each mouse (each value represent the fluorescence quantification of 144 lung sections).

i.e. 80 kDa (NPM-ALK), 85 kDa (TFG-ALK), 96 kDa (ATIC-ALK), 104 kDa (TPM3-ALK), and 250 kDa (CLTC-ALK) (Fig. 1a).

To examine the metastatic potential of each X-ALK clone, cells were labelled with the fluorescent membrane cell linker PKH26-GL to facilitate their detection*in vivo*. We verified that control pCDNA3 and *X-ALK* transfected cells, both labelled and unlabelled, exhibited similar growth properties *in vitro* (data not shown), indicating that PKH26-GL labelling did not affect cell viability or growth properties. As shown in Fig. 1b and c, mice injected with control cells produced no pulmonary metastasis whereas mice injected with the X-ALK expressing cells developed lung metastases. Since the TPM3-ALK mice died 12 days after injection due to respiratory distress, all results were obtained 10 days after injection. TPM3-ALK cells produced the most lung metastases, approximately twice as many as NPM-ALK expressing cells. The ATIC-ALK, TFG-ALK, CLTC-ALK cells gave rise to roughly equal levels of metastases, all of which were lower than NPM-ALK cells (Fig. 1b and c). The higher metastatic potential of TPM3-ALK cells is in agreement with previous results showing that TPM3-ALK-transfected cells display the highest migratory capacity of the X-ALK fusions, demonstrated both in matrigel invasion assays and assays that measure the *in vitro* migratory capacities of cells crossing monolayers of endothelial cells.[1]

Such a difference might be related to differential proteolytic enzyme secretion, expression of adhesion molecules or an alteration of the actin cytoskeleton. A combination of all three mechanisms could be responsible for the highest invasive capacities of TPM3-ALK expressing cells, but the last hypothesis is in agreement with our previous results that showed that the TPM3-ALK expression level correlates with the capacity of cells to cross a reconstituted basement-membrane *in vitro* (BioCoat Matrigel Invasion assay).[1] Indeed, cytoskeletal components are known to regulate biophysical characteristics, cellular signalling and metastatic potential. In addition, tropomyosins are essential to stabilise actin filaments by protecting them from the action of severing proteins.[22,23] Decreased expression levels of tropomyosins have been found in fibroblasts transformed by various oncogenes, prostate cancer, breast cancer and in metastatic melanoma cells.[24] In addition, inducible NPM-ALK expression has been demonstrated to both modify the cytoskeleton and alter the migration pattern of NIH3T3 cells.[25] A recent proteomic study of anaplastic lymphoma cell lines showed that several proteins involved in the organisation of the cytoskeleton are differentially expressed in ALK-positive cell lines derived from t(2,5) patients versus ALK-negative cell lines.[26] Therefore, we investigated the cytoskeletal organisation of X-ALK expressing cells.

We first analysed the expression level of tropomyosin in each ALK expressing clone by Western blot. As shown in Fig. 2a, all X-ALK expressing cells show a marginal reduction in tropomyosin expression. The reduction of tropomyosin expression in TPM3-ALK expressing cells, which is slightly more pronounced than the other X-ALK fusions, is interesting due to the fact that the formation of heterodimers consisting of endogenous TPM3 and TPM3-ALK could also decrease the availability of tropomyosin for interaction with the actin cytoskeleton. To determine whether or not the formation of such

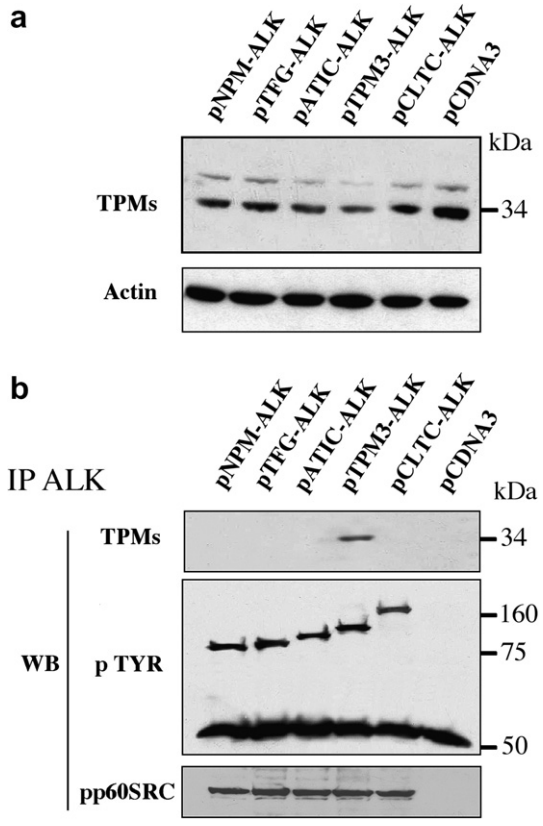

Fig. 2 – Wild type tropomyosin expression analysis and interaction of TPM3-ALK fusion protein with endogenous tropomyosin. (a) Western blot analysis of tropomyosins expression level in total cell extracts of NIH3T3 clones stably transfected with cDNAs encoding the NPM-ALK, TGF-ALK, ATIC-ALK, TPM-ALK, CLTC-ALK proteins, and in empty vector transfected cells (pCDNA3). The amount of ß-actin was used as a control for the homogeneity of loading. Molecular weight markers are shown. (b) Interaction of each ALK fusion protein with endogenous tropomyosin was determined by co-immunoprecipitation. Lysates from NIH3T3 cells expressing the X-ALK variant proteins were immunoprecipitated with ALK1 antibody. Immune complexes were subjected to SDS-PAGE and detected with anti-tropomyosin antibody (upper panel), anti-phosphotyrosine antibody (4G10) (middle panel), and the anti pp60Src rabbit polyclonal antibody (sc-19 from Santa Cruz) (lower panel). Wild type tropomyosin was only found associated to TPM3-AKL, while pp60Src co-precipitate with the five X-ALK fusion proteins.

heterodimers occurs, we performed co-immunoprecipitation experiments with ALK antibody and immunoblotting with anti-tropomyosin antibody. We showed that TPM3-ALK but not the other fusion proteins interact with endogenous tropomyosin (Fig. 2b, upper panel). Immunoblotting with an anti-phosphotyrosine antibody (4G10) led to the detection of roughly equal amounts of the various phosphorylated X-ALK proteins indicating that in these cells, each X-ALK variant kinase activity is comparably activated (middle panel) thus excluding the effect of differential kinase activation on the metastatic capacities. Moreover, as expected, Src was

shown to be efficiently co-immunoprecipitated with all the five X-ALK fusion proteins (Fig. 2b, lower panel).

We verified the subcellular distribution of NPM-ALK and TPM3-ALK fusion proteins in transfected NIH3T3 cells by confocal microscopy with an anti-ALK antibody. In agreement with the staining pattern observed in inflammatory myofibroblastic tumours and in anaplastic large cell lymphomas, NPM-ALK expressing NIH3T3 cells show both cytoplasmic and nuclear/nucleolar staining while TPM3-ALK staining is restricted to the cytoplasm (Fig. 3a).

To investigate the association of TPM3-ALK with the cytoskeleton, we next determined the presence of TPM3- and NPM-ALK fusions in fractionated cytoplasmic extracts. TPM3-ALK, but not NPM-ALK, was found in the fraction containing the cytoskeletal components (Fig. 3b). Taken together with the decrease in the availability of wild type TPM3, this finding may explain the morphological differences observed between TMP3-ALK and other X-ALK expressing cells, which appear spindle-shaped (Fig. 3c). To further contrast the organisation of the actin cytoskeleton between TPM3- and NPM-ALK expressing cells, we used confocal microscopy with actin antibodies. Fig. 3c shows that the cytoskeletal architecture is strikingly altered in TPM3-ALK cells with a disruption of stress fibres, which could contribute to the transformed phenotype and increased cell motility of TPM3-ALK expressing cells.[1] The same correlation between tropomyosin expression and cell motility has been demonstrated with Ras-transformed fibroblasts, where the normalisation of tropomyosin expression abrogates the increase in cell motility.[24] A critical role for tropomyosin family members in tumour invasion and metastasis has been highlighted by several studies which indicate that high-grade breast, prostate, bladder and brain tumours express significantly lower levels of tropomyosins compared to normal tissues.[27–30]

It has been conclusively demonstrated that translocations involving *ALK* generate X-ALK fusion proteins that are capable of oligomerisation through the ALK partner.[4] This newly acquired multimerisation capacity leads to an activation of the ALK kinase activity. The data presented here suggest that additional more diverse X-specific characteristics can also be endowed on the fusion proteins, which depend upon the functions of the X-partner. Such characteristics include the degree of transformation, cellular morphology and level of tumourigenesis *in vivo*.[1] Having first uncovered specific TPM3-ALK characteristics using a transfected cell line, we are now capable of investigating if such characteristics are also present in various clinical samples that contain different X-ALK translocations.

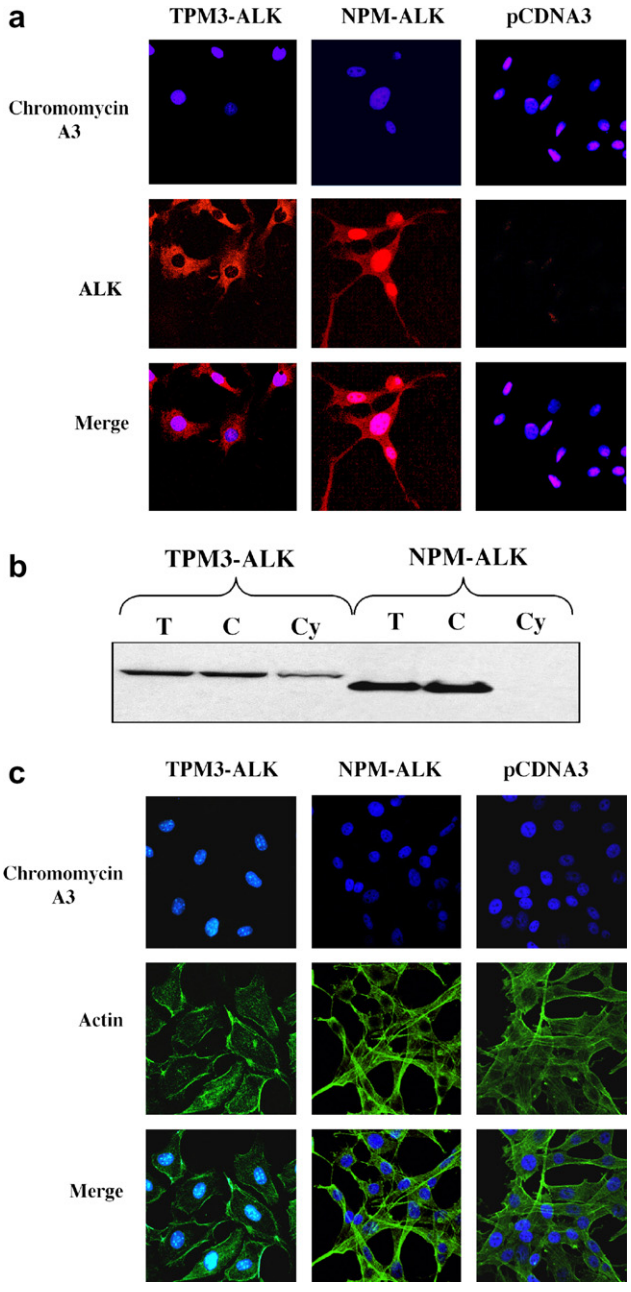

Fig. 3 – Association of TPM3-ALK with the cytoskeleton components. (a) ALK-staining pattern in NIH3T3 cells expressing the TPM3 and NPM-ALK variant proteins are similar to those observed in biopsy specimens from patients. In NIH3T3 transfected with NPM-ALK construct, ALK staining is cytoplasmic, nuclear, and nucleolar whereas in cells expressing TPM3-ALK, the staining is restricted to the cytoplasm. (b) Subcellular distribution analysis of TPM3-ALK and NPM-ALK fusion proteins in NIH3T3 tranfected cells by Western blotting on total lysate (T), cytoplasmic fraction (C) and the cytoskeleton bound (Cy) (i.e. the Triton X-100-insoluble fraction). To visualise the association of X-ALK fusion proteins with the cytoskeleton Western blot with ALK1 antibody (Dakocytomation) was performed after sub-cellular fractionation as previously described.[3] TPM3-ALK, but not NPM-ALK, was clearly found to be associated with the cytoskeleton fraction. (c) Confocal microscopy showing the different actin-staining patterns in TPM3-ALK NPM-ALK and pCDNA3 NIH3T3 transfected cells. Immunocytochemistry was performed as previously described[1] using antibodies against actin (AC-15 Sigma) and FITC-conjugated antibody against mouse IgG (dilution 1:50) (Sigma). Nuclei were labelled with chromomycin A3 (0.1 mg/ml) and visualised using a Zeiss LSM510 confocal microscope.

We must determine the clinical relevance of our results with respect to the different biological behaviours of TPM3- as well as other X-ALK-positive tumours. Towards this end, it would be interesting to repeat these experiments with cellular models developed from stably transfected lymphocytes, such as Baf-3 cells, or from established ALCL cell lines that express TPM3-ALK and compare their metastatic potential to ALC cell line expressing NPM-ALK already existing such as SUDHL-1 or KARPAS 299. Upon demonstration of a clinical relevance for the down-regulation of tropomoyosins and modification of cytoskeletal architecture in TPM3-ALK tumours, we can proceed to develop these cellular models that can be used to not only further investigate the biological relevance of TPM3-ALK's association with the cytoskeleton but also test therapeutic options that could be designed to abrogate such interactions.

Conflict of interest statement

None declared.

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