β-catenin is constitutively active and increases STAT3 expression/activation in anaplastic lymphoma kinase-positive anaplastic large cell lymphoma

Mona Anand, Raymond Lai, and Pascal Gelebart

Department of Laboratory Medicine and Pathology, Cross Cancer Institute and University of Alberta, Edmonton, Alberta, Canada

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Correspondence: Pascal Gelebart, Rm 1466, Department of Laboratory Medicine and Pathology, Cross Cancer Institute and University of Alberta 11560 University Avenue, Edmonton, Alberta, Canada T6G 1Z2. E-mail: pgelbart@ualberta.ca

Raymond Lai, Rm 2338, Department of Laboratory Medicine and Pathology, Cross Cancer Institute and University of Alberta, 11560 University Avenue, Edmonton, Alberta, Canada T6G 1Z2. E-mail: rlai@ualberta.ca

The online version of this article has a Supplementary Appendix.

ABSTRACT

Background

The role of β-catenin in cancer has been most studied in tumors of epithelial cell origin. The functional status and biological significance of this protein in anaplastic lymphoma kinase (ALK)-positive anaplastic large cell lymphoma is unknown.

Design and Methods

ALK-positive anaplastic large cell lymphoma cell lines and patients' tumor samples were examined for status of β-catenin expression and signaling. The subcellular localization of β-catenin was assessed using immunohistochemistry, sub-cellular fractionation and confocal microscopy, while its transcriptional activity was studied using the TOP*Flash*/FOP*Flash* luciferase reporter assay. To examine the biological significance of β-catenin, short interfering RNA was used to knock-down its expression; the resulting biological effects were studied using trypan-blue exclusion and MTS assay, and the impact on its various downstream targets was assessed using quantitative real-time polymerase chain reaction and western blots.

Results

β-catenin was transcriptionally active in three of three ALK-positive anaplastic large cell lymphoma cell lines, and this finding correlates with the nuclear localization of β-catenin in these cells and the neoplastic cells identified in most of the patients' tumor samples. β-catenin is biologically significant in ALK-positive anaplastic large cell lymphoma, since down-regulation of β-catenin resulted in a significant reduction in their cell growth. Down-regulation of β-catenin led to a marked reduction in both the total protein level and the activated/phosphorylated form of STAT3, another signaling protein previously shown to be important in the pathogenesis of ALK-positive anaplastic large cell lymphoma. In contrast to some of the oncogenic tyrosine kinases, modulation of nucleophosmin-anaplastic lymphoma kinase expression did not result in any detectable change in the protein level, nuclear localization or tyrosine phosphorylation of β-catenin; however, inhibition of nucleophosmin-anaplastic lymphoma kinase expression significantly down-regulated the transcriptional activity of β-catenin.

Conclusions

β-catenin signaling is constitutively active in ALK-positive anaplastic large cell lymphoma and represents a previously unknown mechanism by which the high levels of STAT3 expression and activation in these tumors are sustained. Our results suggest that the interaction between oncogenic tyrosine kinases and various cell signaling proteins may be more complex than previously believed.

Key words: ALK$^+$ALCL, β-catenin, STAT3, NPM-ALK.

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Introduction

Anaplastic lymphoma kinase-positive anaplastic large cell lymphoma (ALK⁺ALCL), a distinct type of non-Hodgkin's lymphoma of T/null-cell lineage, primarily affects children and young adults and constitutes 10-30% of all pediatric lymphomas.[1] Most ALK⁺ALCL tumors carry the t(2;5)(p23;35) cytogenetic abnormality, which places the *ALK* (*anaplastic lymphoma kinase*) gene under the regulation of the *NPM* (*nucleophosmin*) gene promoter. The resulting fusion protein (NPM-ALK) has constitutively active tyrosine kinase activity, which has been shown to be critically important for its transformation ability.[2-4] NPM-ALK is known to bind and activate a host of cell signaling pathways, including those of JAK/STAT3,[3,5] Ras/ERK[6] and PI3K/AKT,[7-8] all of which are known to regulate important cellular functions such as cell cycle progression and cell survival. Of these, the signal transduction and activation of transcription 3 (STAT3) pathway is the best characterized; constitutive activation of STAT3 has been shown to be central to the pathogenesis of ALK⁺ALCL.[9-11]

β-catenin is a multifunctional protein that serves as an adhesion molecule in epithelial cells and a transcriptional factor in the context of the Wnt canonical pathway.[12-13] When the Wnt canonical pathway is inactive, β-catenin is bound to GSK3β in the so-called 'destruction complex' that promotes serine/threonine phosphorylation of β-catenin and facilitates its proteasomal degradation. When the Wnt canonical pathway is activated, GSK3β is inactivated, leading to decreased serine/threonine phosphorylation of β-catenin and enhancing its stabilization. Once accumulated in the cells, β-catenin translocates to the nucleus and forms a complex with the TCF/LEF family of transcription factors to initiate the transcription of various target genes including *cyclin D1*, *c-myc* and *c-jun*.[12,14] β-catenin has been recently shown to play a role in the survival and proliferation of normal murine CD4⁺ T cells.[15] In benign human lymphocytes, the expression level of β-catenin is regulated through a post-translational mechanism. Specifically, this protein is continuously degraded in normal resting peripheral blood lymphocytes,[16] such that β-catenin is undetectable by western blot studies.[16-18] Although deregulated β-catenin signaling has been found in many solid tumors, particularly colonic cancer,[12] relatively few studies have elucidated its role in hematologic malignancies; evidence of β-catenin deregulation has been previously found in subsets of T-cell lymphomas, mantle cell lymphoma and myeloid malignancies.[19-23] Directly relevant to this study, a number of oncogenic tyrosine kinases found in several types of hematologic malignancies, such as BCR-ABL and C-KIT, were found to induce tyrosine phosphorylation and stabilization of β-catenin, and increase its transcriptional activity.[21,23-24]

In view of the fact that oncogenic tyrosine kinases found in hematologic cancers can stabilize/activate β-catenin and increase its transcriptional activity, we questioned whether NPM-ALK, also an oncogenic tyrosine kinase found exclusively in ALK⁺ALCL, exerts similar effects on β-catenin. Thus, in this study, we investigated the functional status and biological significance of β-catenin in this type of cancer.

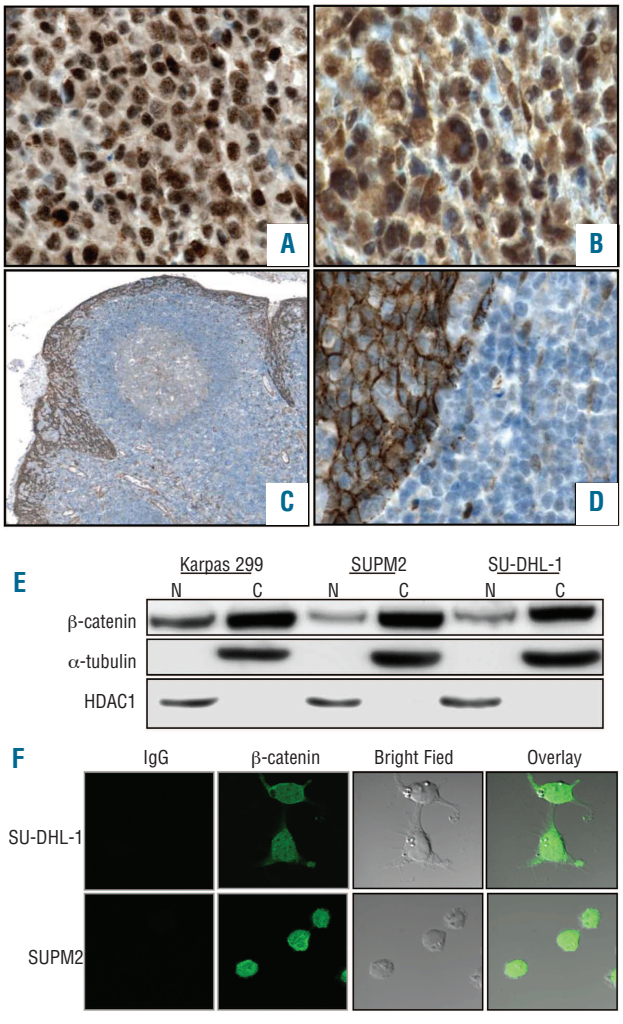

Figure 1. Nuclear localization of β-catenin in ALK⁺ALCL tumors and ALK⁺ALCL cell lines. Immunohistochemistry using a monoclonal anti-β-catenin antibody revealed a distinct nuclear staining pattern in the lymphoma cells of two cases of ALK⁺ALCL (A and B, 1000x magnification). The lymphoma cells of both cases also showed some cytoplasmic staining, with the case illustrated in (B) showing stronger cytoplasmic staining than the case illustrated in (A). Normal tonsillar lymphoid tissues, used as a negative control, showed no detectable staining, whereas bright membranous staining of the normal squamous epithelial lining was identified (C) and (D), 100X and 1000X magnification, respectively). (E) Nuclear-cytoplasmic fractionation and western blot showed the presence of nuclear (N) and cytoplasmic (C) portions of β-catenin in all three ALK⁺ALCL cell lines. β-tubulin and HDAC-1 were used as the controls to assess the efficiency of the cytoplasmic and nuclear fractionation, respectively. (F) Immunofluorescence and confocal microscopy showed the presence of nuclear β-catenin (Alexa fluor 488) in ALK⁺ALCL cell lines when they were stained using the anti-β-catenin antibody; no signal was seen when only the isotype control (IgG) was used.

Design and Methods

Anaplastic lymphoma kinase-positive anaplastic large cell lymphoma cell lines and patients' samples

The three NPM-ALK-expressing ALCL cell lines, Karpas 299, SUPM2, SU-DHL-1, have been described previously.[25] Jurkat, a human T-cell acute lymphoblastic leukemia cell line, was purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 (Gibco, Carlsbad, CA, USA) containing 2 mM of L-glutamine supplemented with 10% fetal bovine

serum (Gibco). GP293 cells were cultured in Dulbecco's modified Eagle's medium (Gibco) containing 4 g/L glucose supplemented with 10% fetal bovine serum. All ALK+ALCL primary tumors were diagnosed at the Cross Cancer Institute and the diagnostic criteria were based on those described in the World Health Organization Classification Scheme.[1] Immunohistochemistry showed both nuclear as well cytoplasmic ALK staining in all cases. The use of these tissues was approved by our Institutional Ethics Committee.

Antibodies, plasmids and drugs

Antibody against ALK (1:500) was bought from Dako, Glostrup, Denmark and β-catenin (clone 14, 1:1000) was purchased from BD Transduction Laboratories, Lexington, KY, USA. Antibodies against β-catenin (whole antiserum, for immunofluorescence and immunoprecipitation studies), HDAC-1 and β-actin (1:10,000) were purchased from Sigma Aldrich (St. Louis, MO, USA). Anti-c-jun (1:1000) was purchased from Cell Signaling (Danvers, MA, USA), while antibodies against total STAT3 and pSTAT3Tyr^{705} (1:1000), α-tubulin (1:1000), phospho-tyrosine (1:2000), phospho-β-catenin^{Y86} and phospho-β-catenin^{Y654} (1:1000) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The plasmid carrying *NPM-ALK* was a kind gift from Dr. S Morris, St. Jude Children's Research Hospital (Memphis, TN, USA) and the *NPM-ALK* construct was cloned into the pCDNA3 vector (Invitrogen, Burlington, Ontario, Canada). The 'kinase-dead' mutant of *NPM-ALK* (210K>R) has been previously described[26] and was a gift from Dr. HM Amin (M.D. Anderson Cancer Center, Houston, TX, USA).

Subcellular protein fractionation and western blot

For subcellular protein fractionation, we employed a kit purchased from Active Motif (Carlsbad, CA, USA) and followed the manufacturer's instructions. Cell lysates for western blots were prepared as follows: cells were washed with phosphate-buffered saline (PBS), and cellular proteins were solubilized using RIPA buffer containing 150 mM NaCl, 1% NP40, 0.5% deoxycholic acid, 0.1% sodium dodecylsulfate (SDS), 50 mM Tris pH8.0 which was supplemented with 40 μg/mL leupeptin, 1 μM pepstatin, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) and 0.1 mM phenylmethylsulfonyl-fluoride (PMSF). The protein concentration of the samples was determined using a BCA Protein assay kit (Pierce, Thermo Fisher Scientific Inc., Rockford, IL, USA). Cell lysates were then electrophoresed on 8% or 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Bio-Rad, Richmond, CA, USA). The membranes were blocked with 5% milk in Tris buffered saline (TBS)-0.1% Tween buffer for 1 h (20 mM Tris-HCL, pH=7.6, 150 mM NaCl) and then incubated with the primary antibodies overnight at 4°C. After three washes with TBS-0.1% Tween, the membranes were incubated with the specific secondary antibody conjugated with horseradish peroxidase (Cedarlane Laboratories, Burlington, Ontario, Canada) for 1 h at room temperature. This was followed by three washes with TBS-0.1% Tween and the protein was detected using a chemiluminescence detection kit (Pierce).

Immunofluorescence and confocal microscopy

Cells were grown on cover slips coated with poly-L-lysine (Sigma Aldrich) in a six-well plate and fixed with 3% paraformaldehyde in PBS (pH 7.4). Cell were rinsed three times with PBS, permeabilized with Triton X100, washed again with PBS, and incubated with 200 μL of anti-β-catenin antibody (1:50, Sigma Aldrich) overnight at room temperature in a humidified chamber. The cover slips were rinsed three times in PBS and incubated with secondary antibody conjugated with Alexa Fluor 488 (Invitrogen) at a 1:250 dilution for 1 h at room temperature. After three rinses in PBS, the cover slips were mounted on a slide using

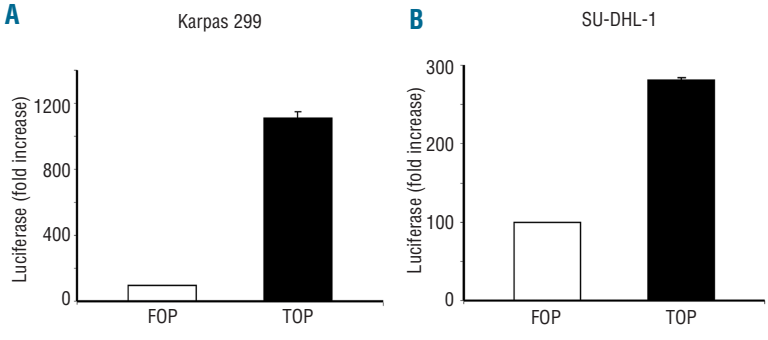

Figure 2. β-catenin is transcriptionally active in ALK+ALCL cells. Double luciferase reporter assays were performed to demonstrate the transcriptional activity of nuclear β-catenin. Increased firefly luciferase activity was detected in cells transfected with TOP*Flash* (vector with intact TCF binding sites) compared to cells transfected with FOP*Flash* (vector with mutated TCF binding sites). The increase was approximately 11-fold for Karpas 299 cells and 3-fold for SU-DHL-1 cells (*P*<0.05, Student's t-test). The firefly luciferase activity was normalized with a control reporter vector (*Renilla* luciferase). Triplicate experiments were performed, and results from a representative experiment are shown.

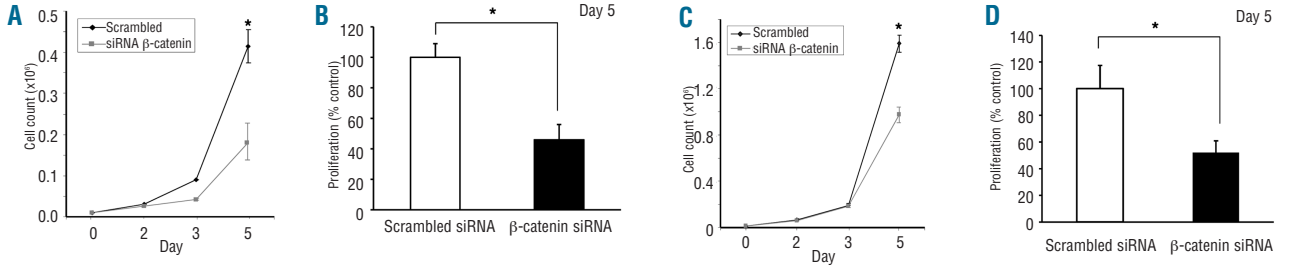

Figure 3. Blockade of β-catenin using siRNA resulted in a significant reduction in the growth of ALK+ALCL cells. Karpas 299 and SUPM2 cells were transfected with siRNA specific for β-catenin (200 pmole/1x10^6 cells) or the scrambled siRNA control and plated in a six-well plate at a density of 10,000-20,000 cells/mL. Viable cells were counted using trypan blue on days 2, 3 and 5. Treatment with β-catenin-specific siRNA led to a significant reduction in cell growth on day 5 in Karpas 299 and SUPM2 cells (*P*<0.008 and *P*<0.003, respectively)(A and C). Triplicate experiments were performed, and results from a representative experiment are shown. Similar experiments were performed using the MTS assay, and a significant reduction in cell growth in both cell lines was observed on day 5 (*P*<0.05) (B and D).

the mounting media (Dako). Cells were visualized with a Zeiss LSM 510 confocal microscope at the Core Cell Imaging Facility, Cross Cancer Institute.

TOP/FOP luciferase reporter assay

To assess the transcriptional activity of β-catenin in ALK+ALCL, we employed the TOP/FOP reporter system using the dual-luciferase kit (Dual-Glo™ Luciferase Assay System, Promega, Madison, WI, USA). ALK+ALCL cells (10×10^6/500 μL RPMI) were transiently transfected with 1 μg of constitutively active vector encoding *Renilla* luciferase (Promega) and 10 μg of β-catenin-responsive firefly luciferase reporter plasmid Top*Flash* (Millipore, Billerica, MA, USA) or the negative control Fop*Flash* (Millipore) using the Electro square electroporator BTX ECM 800 (225V, 8.5 ms, 3 pulses) (Holliston, MA, USA). Cells were harvested after 24 h in culture and both firefly and *Renilla* luciferase activity was measured in duplicate/triplicate according to the manufacturer's instructions. The firefly luciferase activity was normalized against the *Renilla* luciferase activity and fold increase in TOP*Flash* activity compared to FOP*Flash* is reported. To assess the function of NPM-ALK on β-catenin transcriptional activity, Karpas 299 cells were co-transfected with Top*Flash* or Fop*Flash* and short interfering RNA (siRNA) ALK using the BTX electroporator (225V, 8.5 ms, 3 pulses). Cells were harvested after 48 h for luciferase measurements.

Co-immunoprecipitation and immunoprecipitation

Cells were washed with cold PBS and lysed using Cell Lytic Buffer M (Sigma) supplemented with protease inhibitor (Nacalai Inc, San Diego, CA, USA), a phosphatase inhibitor cocktail (Calbiochem, EMD Biosciences, Darmstadt, Germany) and 0.1 mM PMSF (Sigma). After incubating on ice for 30 min, the lysate was centrifuged at 15000g for 15 min. Two micrograms of the primary antibody were added to 500 μg of protein lysate and rotated overnight at 4°C. Control samples with the primary antibody omitted were also included in each test. Fifty microliters of protein A/G Sepharose beads (Santa Cruz Biotechnology) were added to both the test and control lysates and rocked for 2 h at 4°C. The beads were then washed three times with cold PBS, followed by one wash with cold cell lysis buffer. The bound protein was eluted from the beads in 20 μL of SDS protein loading buffer by boiling for 5 min at 100°C and then processed for western blotting. Lysis and washing steps were modified for the immunoprecipitation of the core protein only. To avoid precipitation of the binding partners, the cells were lysed in RIPA buffer and the beads were washed three times with PBS followed by one wash with RIPA buffer.

Immunohistochemistry

Immunohistochemistry was performed using standard techniques.[20] Briefly, formalin-fixed, paraffin-embedded tissue sections or tissue micro-arrays of 4 μM thickness were deparaffinized and rehydrated. Heat-induced epitope retrieval was performed using citrate buffer (pH 6.0) and pressure cooked in a microwave for 20

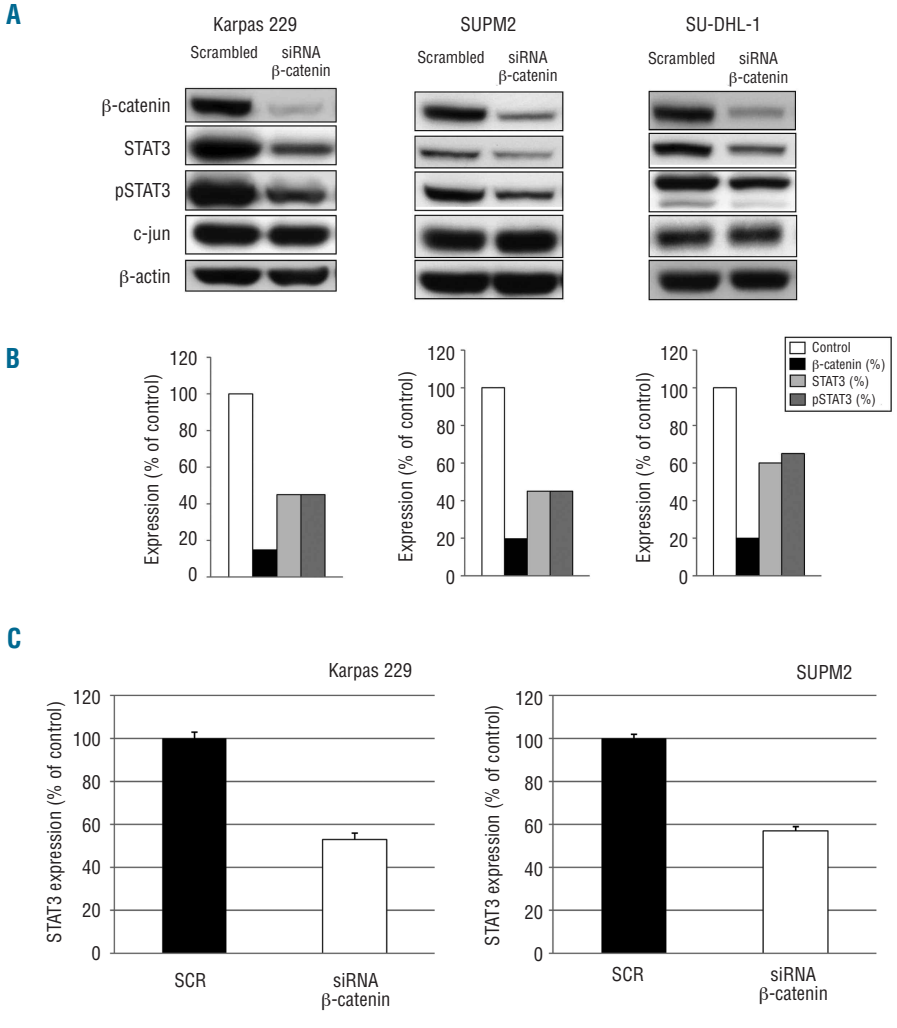

Figure 4. Downregulation of β-catenin using siRNA resulted in a substantial reduction in STAT3 and pSTAT3 in ALK+ALCL cell lines. ALK+ALCL cells were transfected with β-catenin-specific siRNA (200 pmole/$1x10^6$ cells) or the scrambled siRNA control and cell lysates were made 24 h after transfection. Approximately 80% or more reduction in β-catenin expression was seen in all three cell lines (A). This was accompanied by reductions in STAT3 and pSTAT3 levels in these cell lines. No appreciable change in *c-jun*, another β-catenin target gene, was seen on western blots. The densitometry results of the β-catenin, pSTAT3 and STAT3 levels normalized against the β-actin loading control were also shown (B). Real-time polymerase chain reaction experiments were performed to monitor *STAT3* mRNA expression after β-catenin down-regulation. Karpas 299 and SUPM2 cells showed a significant decrease in *STAT3* mRNA transcripts upon treatment with siRNA β-catenin (C and D, respectively)(*P*=0.0173, *P*=0.0003, Student's t-test). The results were normalized to the housekeeping gene (*GAPDH*). Triplicate experiments were performed, and results from a representative experiment are shown.

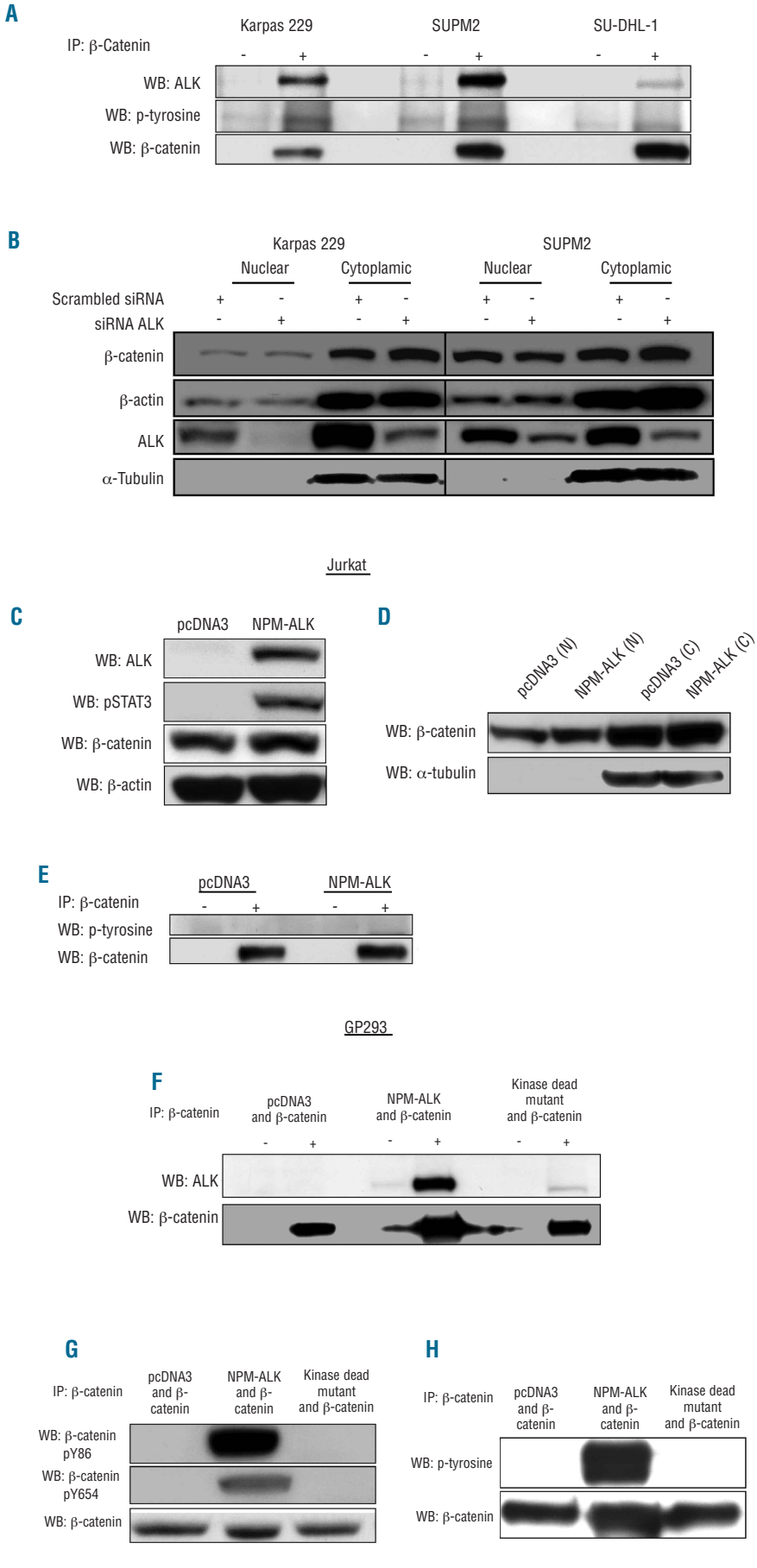

Figure 5. The physical and functional interaction between NPM-ALK and β-catenin. (A) In all three ALK+ALCL cell lines, we observed co-immunoprecipitation of β-catenin and NPM-ALK. However, the immunoprecipitated β-catenin from these cells showed only barely detected phosphorylated tyrosine when the blots were probed with an anti-p-tyrosine antibody. '+' denotes experiments in which anti-β-catenin antibody was used for immunoprecipitation and '-' denotes experiments in which the anti-β-catenin antibody was omitted. (B) ALK-specific siRNA (200 pmole/1x10^6 cells) was used to down-regulate NPM-ALK in two ALK+ALCL cell lines (Karpas 299 and SUPM2). Nuclear-cytoplasmic fractionation and western blot showed no substantial change in either the nuclear or cytoplasmic portion of β-catenin in cells treated with siRNA ALK, as compared to cells treated with the scrambled control. β-actin was used as the loading control and β-tubulin was used to confirm the efficiency of sub-cellular fractionation. (C) Jurkat cells were transfected with a vector encoding *NPM-ALK* or the empty vector (pcDNA3). Lysates made 24 h after transfection showed an increased level of pSTAT3 in cells transfected with *NPM-ALK* but no detectable increase in the total β-catenin levels nor its nuclear localization, as compared to the empty vector control. (D) Immunoprecipitation of β-catenin using a polyclonal anti-β-catenin antibody from Jurkat cells was carried out 24 h after transfection with *NPM-ALK* or the empty vector (pcDNA3) and probed with an anti-p-tyrosine antibody. Endogenous β-catenin was found to be unphosphorylated in Jurkat cells transfected with pcDNA3 and no induction of tyrosine phosphorylation of β-catenin was noted in cells transfected with *NPM-ALK*. (E) A control (-) with the primary antibody omitted was included to exclude the possibility of non-specific binding of protein to agarose beads. (F) GP293 cells were co-transfected with β-catenin and a vector encoding *NPM-ALK* or the control vectors (kinase-dead mutant of NPM-ALK or the empty vector, pcDNA3). Co-immunoprecipitation experiments carried out 24 h after gene transfection showed that NPM-ALK, but not the kinase-dead mutant was bound to the exogenous β-catenin in these cells. (G) Immunoprecipitation of β-catenin using a polyclonal anti-β-catenin antibody from GP293 cells was carried out as described above (A) and probed with antibodies recognizing the total phospho-tyrosine antibody. Total cell lysates made from the above experiment were also probed with specific anti-phospho-tyrosine β-catenin antibody. (H) Both total and specific p-tyrosine β-catenin were up-regulated in GP293 cells transfected with *NPM-ALK* and β-catenin.

min. The endogenous peroxidase activity was blocked using 0.3% H_2O_2 in methanol for 5 min. Tissue sections were then incubated with mouse monoclonal anti-β-catenin antibody (BD Transduction Laboratories, 1:50) overnight at 4°C in a humidified chamber. After two washes with PBS, tissue slides were incubated with biotinylated-linked universal secondary antibody and subsequently with streptavidin–horseradish peroxidase complex according to the manufacturer's instructions (LSAB+ system, Dako). Tissue sections were incubated with 3,3'-diaminobenzidine/H_2O_2 (Dako) for color development and counter-stained with hematoxylin. Tumor cells showing definitive β-catenin nuclear staining were regarded as positive. Tumors with isolated cytoplasmic staining/membrane staining were considered negative. In these experiments isotype-matched purified IgG served as a negative control.

Short interfering RNA and transfections

siRNA (SMARTpool) for ALK and scrambled siRNA (SMARTpool) were purchased from Dharmacon (Lafayette, CO, USA). Two unrelated β-catenin-specific siRNA purchased from Sigma were pooled and transient transfections of ALK$^+$ALCL cells (5×10^6 cells) was performed using the Electro square electroporator BTX ECM 800 (225V, 8.5 ms, 3 pulses). Two hundred picomole of pooled siRNA or scrambled control were used per million ALK$^+$ALCL cells. The efficiency of target gene inhibition was assessed using western blotting.

Assessment of cell growth

ALK$^+$ALCL cells transfected with β-catenin-specific siRNA or scrambled control were plated at a density of 10,000/mL or 20,000/mL and cultured for 5 days. Cells were counted on days 2, 3 and 5 using trypan blue (Sigma) and the results are expressed as total number of viable cells. Seven replicates of the MTS assay (Promega) were done according to the manufacturer's instructions. The absorbance was recorded by a BioRad spectrophotometer at day 5 of cell culture.

Results

β-catenin is expressed and localized to the nucleus in anaplastic lymphoma kinase-positive anaplastic large cell lymphoma cell lines and tumors

Using immunohistochemistry applied to paraffin-embedded tissues, the expression of β-catenin was examined in a cohort of ALK$^+$ALCL tumors (n=12). The results are illustrated in Figure 1. We found 10 cases (83%) showing a definitive nuclear expression pattern. While cytoplasmic staining of β-catenin was frequently seen, the intensity was variable among cases (Figure 1A,B). Tonsillar lymphoid tissue, used as a negative control, showed no appreciable staining; in contrast, squamous epithelial cells showed bright membranous staining (Figure 1C,D).

In contrast to normal peripheral blood T cells that had barely detectable β-catenin (*Online Supplementary Figure S1*), ALK$^+$ALCL cells showed a relatively high level of β-catenin protein. As shown in Figure 1E, subcellular fractionation studies were performed using three ALK$^+$ALCL cell lines: Karpas 299, SUPM2 and SU-DHL-1. Although a relatively high level of β-catenin was present in the cytosol, β-catenin could also be clearly detected in the nuclear fraction of these cells. α-tubulin (a cytoplasmic protein) and HDAC1 (a nuclear protein), served as markers to assess the efficiency of subcellular fractionation and, as expected, these were detected only in the cytoplasmic and nuclear fractions, respectively. To confirm the nuclear localization of β-catenin, we employed confocal microscopy, which clearly revealed the nuclear localization of β-catenin in SU-DHL-1 and SUPM2 cells (Figure 1F).

β-catenin is transcriptionally active in anaplastic lymphoma kinase-positive anaplastic large cell lymphoma cell lines

To demonstrate directly that β-catenin is transcriptionally active in ALK$^+$ALCL cells, we used the TOP*Flash*/FOP*Flash* luciferase reporter system. As shown in Figure 2A,B the luciferase activity in ALK$^+$ALCL cells transfected with the TOP*Flash* reporter vector was significantly higher than that in cells transfected with the negative control FOP*Flash* reporter vector ($P<0.05$, Student's t-test). Both experiments were performed in triplicate, and results of representative experiments are shown.

β-catenin inactivation reduces cell growth in anaplastic lymphoma kinase-positive anaplastic large cell lymphoma cells

To assess the biological importance of β-catenin in ALK$^+$ALCL cells, we assessed whether the knock-down of β-catenin using siRNA affects cell growth. As shown in Figure 3A,D, with the transfection of siRNA specific for β-catenin, there was a dramatic reduction of expression of β-catenin protein (approximately 80% at 24 h) in Karpas 299 and SUPM2 cells. The numbers of viable Karpas 299 and SUPM2 cells, as determined by trypan blue exclusion for up to 5 days, were significantly decreased when β-catenin was down-regulated ($P<0.05$, Student's t-test)(Figure 3B,E). Similar results were obtained using the MTS assay, as shown in Figure 3C,F ($P<0.05$, Student's t-test).

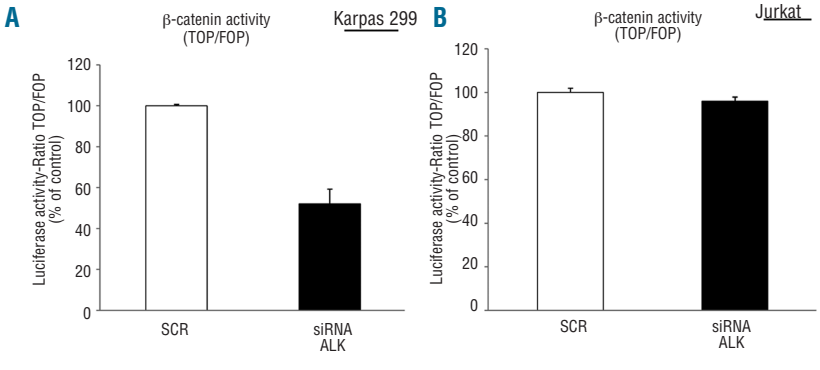

Figure 6. NPM-ALK significantly up-regulates the transcriptional activity of β-catenin. ALK-specific siRNA (200 pmole/1 x10^6 cells) was used to downregulate NPM-ALK in ALK$^+$ALCL Karpas 299 cells. Jurkat cells were used as a control to rule out the possibility of an off-target effect of ALK-specific siRNA and hence treated similarly. After 48 h, the transcriptional activity of nuclear β-catenin was measured by a TOP*Flash*/FOP*Flash* luciferase reporter assay. The transcriptional activity of nuclear β-catenin was decreased by almost 50% after NPM-ALK knockdown in Karpas 299 cells (A) but remained unchanged in Jurkat cells (B). Triplicate experiments were performed and results from a representative experiment are shown.

β-catenin down-regulation decreases the expression of STAT3 and pSTAT3 levels

Since *STAT3* has been reported to be a β-catenin target gene in esophageal squamous cell carcinoma cells,[27] we hypothesized that the mechanism underlying the cell growth-promoting effects of β-catenin in ALK+ALCL may be mediated via STAT3, a signaling protein previously shown to be highly activated and important in the pathogenesis of ALK+ALCL.[3,9,11] In keeping with this hypothesis, transfection of pooled siRNA specific for β-catenin resulted in a substantial (in the range of 40-60%) down-regulation of the total STAT3 protein level in all three cell lines. We also observed a down-regulation of pSTAT3, the level of reduction of which approximated that of STAT3 in all three cell lines (Figure 4A,B). In contrast, the expression level of c-jun was not appreciably changed. To rule out the possibility of an off-target effect for this pooled siRNA, we repeated the experiments using the two individual and unrelated siRNA species in the pool separately, and we showed similar results regarding the changes in the STAT3 and pSTAT3 expression (*Online Supplementary Figure S2*). The regulation of STAT3 expression level by β-catenin appeared to be transcriptional, as the *STAT3* mRNA levels assessed using real-time polymerase chain reaction were significantly decreased in Karpas 299 and SUPM2 cells (Figure 4C). Of note, the decrease in *STAT3* mRNA was comparable to that of the protein (i.e. approximately 40-50%).

The physical and functional interaction between nucleophosmin-anaplastic lymphoma kinase and β-catenin

As mentioned above, a number of oncogenic tyrosine kinases have been previously shown to bind to and activate β-catenin by increasing its tyrosine phosphorylation, stability and nuclear translocation. Thus, we asked whether NPM-ALK, an oncogenic tyrosine kinase playing a central role in ALK+ALCL, also exerts similar effects on β-catenin. In ALK+ALCL cell lines, we found that NPM-ALK was pulled down with β-catenin in all three ALK+ALCL cell lines by co-immunoprecipitation (Figure 5A). However, probing with an anti-phosphotyrosine antibody revealed only a barely detectable level of tyrosine phosphorylation of β-catenin, compared to the negative controls. When we knocked down NPM-ALK expression in Karpas 299 and SUPM2 cells using siRNA, we did not find any detectable change in the protein level or subcellular localization of β-catenin (Figure 5B). In keeping with these results, when we transiently transfected Jurkat cells with *NPM-ALK*, we found that ectopic NPM-ALK expression resulted in a dramatic up-regulation of pSTAT3 (a commonly used surrogate marker of the tyrosine kinase activity of NPM-ALK) but no detectable increase in the total protein level, nuclear translocation or tyrosine phosphorylation of β-catenin in Jurkat cells (Figure 5C-E). Similar results were obtained with GP293 cells, an embryonic kidney cell line that expresses a relatively high level of β-catenin. The lack of increased tyrosine phosphorylation of β-catenin correlated with the observation that these two molecules did not co-immunoprecipitate with each other in GP293 cells (*data not shown*). Interestingly, when we co-transfected *β-catenin* and *NPM-ALK* in GP293 cells, we observed co-immunoprecipitation of these two proteins (Figure 5F); in addition, the introduced β-catenin was pulled down and heavily tyrosine phosphorylated by NPM-ALK but not by the 'kinase-dead' mutant of NPM-ALK as shown by probing with total phospho-tyrosine antibody and confirmed by phospho-specific antibodies (β-catenin p^{Y86} and β-catenin p^{Y654}) which specifically recognize tyrosine phopshorylation of residues Y86 and Y654 of β-catenin (Figure 5G-H).

Nucleophosmin-anaplastic lymphoma kinase significantly modulates the transcriptional activity of β-catenin in anaplastic lymphoma kinase-positive anaplastic large cell lymphoma cells

Although NPM-ALK did not induce any appreciable change in the protein level and nuclear translocation of β-catenin, we investigated whether NPM-ALK modulates the transcriptional activity of β-catenin. Using the TOP*Flash*/FOP*Flash* luciferase reporter system, we assessed the transcriptional activity of β-catenin in the presence or absence of ALK siRNA. To rule out the possibility of an off-target effect of siRNA, Jurkat cells, which have been previously shown to have β-catenin expression and activation,[17] were treated similarly with ALK-specific siRNA. We observed a significant decrease in the transcriptional activity of β-catenin when NPM-ALK expression was down-regulated in Karpas 299 cells (Figure 6A); in contrast, this remained unchanged in Jurkat cells treated with ALK-specific siRNA (Figure 6B). Experiments were performed in triplicate, and the results from a representative experiment are shown in Figure 6.

Discussion

β-catenin, the critical effector of the Wnt pathway, regulates a number of key processes during development including proliferation, differentiation and cell fate determination, as well as tissue homeostasis in adults. β-catenin is normally localized to the cell adhesion junctions in epithelial cells and its abnormal cytplasmic/nuclear stabilization drives uncontrolled transcription of target genes (including *c-jun*, *cyclin D1*, *c-myc*, *survivin*, and *MMP-7*) regulating cell proliferation, survival and cell adhesion.[12] In view of its biological importance, it is not surprisingly that deregulation of β-catenin has been linked to the pathogenesis of a number of human cancers, particularly those with an epithelial cell origin.[28] Illegitimate activation of β-catenin has also been reported in several types of hematopoietic cancers.[19,29-31] Nevertheless, the functional status and biological role of β-catenin have never been investigated in ALK+ALCL. In this study, we found that β-catenin is transcriptionally active in ALK+ALCL cells, and this conclusion is supported by its nuclear localization and the results from the TOP/FOP luciferase assay. As discussed, this is contrast with the situation in normal peripheral blood T cells, in which β-catenin is continuously degraded such that it is undetectable by western blot studies.[16-18] Our data also support the concept that β-catenin is biologically significant in ALK+ALCL, as down-regulation of this protein using siRNA significantly reduced the growth of ALK+ALCL cells. These results are reminiscent of the observation made recently that β-catenin plays an important and complex role in the biology of normal mouse T cells.[15] Our results also uncovered a cross-talk between β-catenin and STAT3 in ALK+ALCL cells. Specifically, inhibition of β-catenin in ALK+ALCL cells induced a substantial reduction in STAT3 expression and activation. This reduction in STAT3 expression appears to be primarily transcriptional,

since the level of reduction at the mRNA level was similar to that seen at the protein level. This conclusion is in keeping with the previous report that the *STAT3* promoter contains multiple TCF binding sites.[27] Moreover, the observation that the down-regulation of β-catenin resulted in a similar level of decrease in both STAT3 and pSTAT3 suggests that the down-regulation of pSTAT3 is simply related to that of total STAT3 levels. This observation suggests that β-catenin is a regulator of STAT3 activation via its control over the total expression of STAT3. Since there are many known factors contributing to STAT3 activation in ALK⁺ALCL, including stimulation by multiple cytokines[5,32] and various tyrosine kinases,[3,33] as well as a defective negative feedback system,[34-35] it is not too surprising that the total STAT3 protein level is the limiting factor for determining the level of STAT3 phosphorylation/activation. On the other hand, the expression of *c-jun*, another known β-catenin target gene, remained unchanged in these cells. This is most likely related to the fact that modulation of various target genes by various transcriptional factors is often cell-type specific.

The etiology underlying the constitutive activation of β-catenin in ALK⁺ALCL is incompletely understood. In chronic myeloid leukemia, there is evidence that the oncogenic, constitutively active tyrosine kinase BCR-ABL contributes to the stabilization of β-catenin by increasing its tyrosine phosphorylation, which is associated with increased nuclear translocation and transcriptional activity.[23] Given that NPM-ALK is also a constitutively active tyrosine kinase, we investigated whether NPM-ALK can exert similar effects on β-catenin as BCR-ABL does. The results are rather surprising. Despite the physical interaction between NPM-ALK and β-catenin, β-catenin was found to be minimally tyrosine phosphorylated in ALK⁺ALCL cells. Down-regulation of NPM-ALK using siRNA did not result in any detectable change in the expression or nuclear localization of β-catenin. The lack of tyrosine phosphorylation of β-catenin by NPM-ALK was further supported by the transfection experiments using Jurkat cells. Results from the experiments using GP293 cells are interesting. While transfection of NPM-ALK alone into these cells resulted in no evident physical or functional interaction with the endogeneous β-catenin, co-transfection of *NPM-ALK* and *β-catenin* into these cells did result in their physical interaction and a marked increase in tyrosine phosphorylation of β-catenin. Overall, these data seem to indicate that even if NPM-ALK can induce tyrosine phosphorylation and nuclear localization of β-catenin in epithelial cells, this mechanism is likely not effective in ALK⁺ALCL cells. In this regard, it is interesting to note that NPM-ALK can interact with a protein without inducing its tyrosine phosphorylation.[36,37] Overall, our data suggest that the interaction between oncogenic tyrosine kinases and various cellular signaling proteins may be more complex than previously believed.

Despite the fact that NPM-ALK does not seem to alter protein level or nuclear translocation of β-catenin in ALK⁺ALCL cells, our results showed that NPM-ALK regulates the transcriptional activity of β-catenin. The mechanism underlying this regulation is unclear, but it is likely that the effect of NPM-ALK on β-catenin transcriptional activity is indirect. Since the transcriptional activity of β-catenin requires co-factors of the TCF/LEF system, it is possible that NPM-ALK regulates the activity of β-catenin through the modulation of other co-factors or by modulating the activity of other pathways. Further studies to investigate this level of regulation may be warranted.

In conclusion, we demonstrate that β-catenin is constitutively activated in ALK⁺ALCL. Our data also demonstrate that β-catenin is important in the pathogenesis of ALK⁺ALCL, in part by increasing the expression of STAT3. Thus, our study has revealed a novel functional link between two oncogenic signaling proteins in ALK⁺ ALCL. Lastly, our results suggest that the interaction between oncogenic tyrosine kinases and various cell signaling proteins may be more complex than previously believed.

Authorship and Disclosures

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References

1. Delsol G FB, Muller-Hermelink HK, Campo E, Jaffe ES, Gascoyne RD, Stein H, et al. Anaplastic Large Cell Lymphoma (ALCL), ALK-Positive. Lyon: IARC, 2008.
2. Armstrong F, Duplantier MM, Trempat P, Hieblot C, Lamant L, Espinos E, et al. Differential effects of X-ALK fusion proteins on proliferation, transformation, and invasion properties of NIH3T3 cells. Oncogene. 2004;23(36):6071-82.
3. Zamo A, Chiarle R, Piva R, Howes J, Fan Y, Chilosi M, et al. Anaplastic lymphoma kinase (ALK) activates Stat3 and protects hematopoietic cells from cell death. Oncogene. 2002;21(7):1038-47.
4. Amin HM, Lai R. Pathobiology of ALK+ anaplastic large-cell lymphoma. Blood. 2007;110(7):2259-67.
5. Dien Bard J, Gelebart P, Anand M, Zak Z, Hegazy SA, Amin HM, et al. IL-21 contributes to JAK3/STAT3 activation and promotes cell growth in ALK-positive anaplastic large cell lymphoma. Am J Pathol. 2009;175(2):825-34.
6. Turner SD, Yeung D, Hadfield K, Cook SJ, Alexander DR. The NPM-ALK tyrosine kinase mimics TCR signalling pathways, inducing NFAT and AP-1 by RAS-dependent mechanisms. Cell Signal. 2007;19(4):740-7.
7. Bai RY, Ouyang T, Miething C, Morris SW, Peschel C, Duyster J. Nucleophosmin-anaplastic lymphoma kinase associated with anaplastic large-cell lymphoma activates the phosphatidylinositol 3-kinase/Akt antiapoptotic signaling pathway. Blood. 2000;96(13):4319-27.
8. Slupianek A, Nieborowska-Skorska M, Hoser G, Morrione A, Majewski M, Xue L, et al. Role of phosphatidylinositol 3-kinase-Akt pathway in nucleophosmin/anaplastic lymphoma kinase-mediated lymphomagenesis. Cancer Res. 2001;61(5):2194-9.
9. Chiarle R, Simmons WJ, Cai H, Dhall G, Zamo A, Raz R, et al. Stat3 is required for ALK-mediated lymphomagenesis and provides a possible therapeutic target. Nat Med. 2005;11(6):623-9.
10. Khoury JD, Medeiros LJ, Rassidakis GZ, Yared MA, Tsioli P, Leventaki V, et al. Differential expression and clinical significance of tyrosine-phosphorylated STAT3 in ALK+ and ALK- anaplastic large cell lymphoma. Clin Cancer Res. 2003;9(10 Pt 1):3692-9.
11. Amin HM, McDonnell TJ, Ma Y, Lin Q, Fujio Y, Kunisada K, et al. Selective inhibition of STAT3 induces apoptosis and G(1) cell cycle arrest in ALK-positive anaplastic large cell lymphoma. Oncogene. 2004;23(32):5426-34.
12. Clevers H. Wnt/beta-catenin signaling in development and disease. Cell. 2006;127(3):469-80.
13. Gordon MD, Nusse R. Wnt signaling: multiple pathways, multiple receptors, and

multiple transcription factors. J Biol Chem. 2006;281(32):22429-33.
14. Staal FJ, Clevers HC. WNT signalling and haematopoiesis: a WNT-WNT situation. Nat Rev Immunol. 2005;5(1):21-30.
15. Ding Y, Shen S, Lino AC, Curotto de Lafaille MA, Lafaille JJ. Beta-catenin stabilization extends regulatory T cell survival and induces anergy in nonregulatory T cells. Nat Med. 2008;14(2):162-9.
16. Chung EJ LM, Lee S, Hwang S-G, Rubin JS, Trepel JB. Posttranslational control of beta-catenin signaling regulates peripheral blood lymphocyte activation. ASH. Atlanta, Georgia, 2005.
17. Chung EJ, Hwang SG, Nguyen P, Lee S, Kim JS, Kim JW, et al. Regulation of leukemic cell adhesion, proliferation, and survival by beta-catenin. Blood. 2002;100 (3):982-90.
18. Tomita M, Kikuchi A, Akiyama T, Tanaka Y, Mori N. Human T-cell leukemia virus type 1 tax dysregulates beta-catenin signaling. J Virol. 2006;80(21):10497-505.
19. Groen RW, Oud ME, Schilder-Tol EJ, Overdijk MB, ten Berge D, Nusse R, et al. Illegitimate WNT pathway activation by beta-catenin mutation or autocrine stimulation in T-cell malignancies. Cancer Res. 2008;68(17):6969-77.
20. Gelebart P, Anand M, Armanious H, Peters AC, Dien Bard J, Amin HM, et al. Constitutive activation of the Wnt canonical pathway in mantle cell lymphoma. Blood. 2008;112(13):5171-9.
21. Kajiguchi T, Chung EJ, Lee S, Stine A, Kiyoi H, Naoe T, et al. FLT3 regulates beta-catenin tyrosine phosphorylation, nuclear localization, and transcriptional activity in acute myeloid leukemia cells. Leukemia. 2007;21(12):2476-84.
22. Fang D, Hawke D, Zheng Y, Xia Y, Meisenhelder J, Nika H, et al. Phosphorylation of beta-catenin by AKT promotes beta-catenin transcriptional activity. J Biol Chem. 2007;282(15):11221-9.
23. Coluccia AM, Vacca A, Dunach M, Mologni L, Redaelli S, Bustos VH, et al. Bcr-Abl stabilizes beta-catenin in chronic myeloid leukemia through its tyrosine phosphorylation. EMBO J. 2007;26(5): 1456-66.
24. Kajiguchi T, Lee S, Lee MJ, Trepel JB, Neckers L. KIT regulates tyrosine phosphorylation and nuclear localization of beta-catenin in mast cell leukemia. Leuk Res. 2008;32(5):761-70.
25. Pulford K, Lamant L, Morris SW, Butler LH, Wood KM, Stroud D, et al. Detection of anaplastic lymphoma kinase (ALK) and nucleolar protein nucleophosmin (NPM)-ALK proteins in normal and neoplastic cells with the monoclonal antibody ALK1. Blood. 1997;89(4):1394-404.
26. Marzec M, Zhang Q, Goradia A, Raghunath PN, Liu X, Paessler M, et al. Oncogenic kinase NPM/ALK induces through STAT3 expression of immunosuppressive protein CD274 (PD-L1, B7-H1). Proc Natl Acad Sci USA. 2008;105(52): 20852-7.
27. Yan S, Zhou C, Zhang W, Zhang G, Zhao X, Yang S, et al. beta-Catenin/TCF pathway upregulates STAT3 expression in human esophageal squamous cell carcinoma. Cancer Lett. 2008;271(1):85-97.
28. Polakis P. The many ways of Wnt in cancer. Curr Opin Genet Dev. 2007;17(1):45-51.
29. Lu D, Zhao Y, Tawatao R, Cottam HB, Sen M, Leoni LM, et al. Activation of the Wnt signaling pathway in chronic lymphocytic leukemia. Proc Natl Acad Sci USA. 2004;101(9):3118-23.
30. Khan NI, Bradstock KF, Bendall LJ. Activation of Wnt/beta-catenin pathway mediates growth and survival in B-cell progenitor acute lymphoblastic leukaemia. Br J Haematol. 2007;138(3):338-48.
31. Derksen PW, Tjin E, Meijer HP, Klok MD, MacGillavry HD, van Oers MH, et al. Illegitimate WNT signaling promotes proliferation of multiple myeloma cells. Proc Natl Acad Sci USA. 2004;101(16):6122-7.
32. Bard JD, Gelebart P, Anand M, Amin HM, Lai R. Aberrant expression of IL-22 receptor 1 and autocrine IL-22 stimulation contribute to tumorigenicity in ALK+ anaplastic large cell lymphoma. Leukemia. 2008; 22(8):1595-603.
33. Cussac D, Greenland C, Roche S, Bai RY, Duyster J, Morris SW, et al. Nucleophosmin-anaplastic lymphoma kinase of anaplastic large-cell lymphoma recruits, activates, and uses pp60c-src to mediate its mitogenicity. Blood. 2004; 103(4):1464-71.
34. Zhang Q, Raghunath PN, Xue L, Majewski M, Carpentieri DF, Odum N, et al. Multilevel dysregulation of STAT3 activation in anaplastic lymphoma kinase-positive T/null-cell lymphoma. J Immunol. 2002;168(1):466-74.
35. Han Y, Amin HM, Franko B, Frantz C, Shi X, Lai R. Loss of SHP1 enhances JAK3/STAT3 signaling and decreases proteosome degradation of JAK3 and NPM-ALK in ALK+ anaplastic large-cell lymphoma. Blood. 2006;108(8):2796-803.
36. Colomba A, Courilleau D, Ramel D, Billadeau DD, Espinos E, Delsol G, et al. Activation of Rac1 and the exchange factor Vav3 are involved in NPM-ALK signaling in anaplastic large cell lymphomas. Oncogene. 2008;27(19):2728-36.
37. Ambrogio C, Voena C, Manazza AD, Martinengo C, Costa C, Kirchhausen T, et al. The anaplastic lymphoma kinase controls cell shape and growth of anaplastic large cell lymphoma through Cdc42 activation. Cancer Res. 2008;68(21):8899-907.