The Expression of CD30 in Anaplastic Large Cell Lymphoma Is Regulated by Nucleophosmin-Anaplastic Lymphoma Kinase–Mediated JunB Level in a Cell Type–Specific Manner

Faye Yuan-Yi Hsu, Patrick B. Johnston, Kathleen A. Burke, and Yi Zhao

Departments of Biochemistry and Medicine, Norris Cancer Center, University of Southern California Keck School of Medicine, Los Angeles, California and Division of Hematology, Mayo Clinic College of Medicine, Rochester, Minnesota

Abstract

Chromosomal translocation t(2;5) and the resulting fusion protein nucleophosmin-anaplastic lymphoma kinase (NPM-ALK) are detected in 50% to 70% of anaplastic large cell lymphoma (ALCL), which is a T/null cell non–Hodgkin’s lymphoma showing anaplastic morphology with cell surface expression of CD30. Because aberrant CD30 expression was also observed in the T-cell lymphoma derived from lineage-specific NPM-ALK transgenic mice, we tested the hypothesis that there might be a functional relationship between the two neoplastic-related proteins: NPM-ALK and CD30. In this study, we used the RNA interference method to modulate NPM-ALK protein expression in ALCL-derived, t(2;5)-positive Karpas 299 cells. We observed decreased CD30 expression when NPM-ALK was repressed. Further analysis suggested that JunB functioned as the mediator of NPM-ALK-derived CD30 transcriptional regulation. The NPM-ALK-repressed cells, which had low CD30 expression, were characterized with lower cell proliferation compared with cells in the control group, suggesting that altered CD30 expression may correlate to NPM-ALK-mediated tumor cell growth inhibition. Combination of NPM-ALK repression and CD30 ligand leads to significantly increased tumor cell growth inhibition compared with one method alone, suggesting its potential application for ALCL-specific cancer treatment.

Introduction

Anaplastic large cell lymphoma (ALCL) is a subtype of the T/null cell–derived non–Hodgkin’s lymphoma with cell surface expression of Ki-1/CD30 (1). About 70% of systemic ALCLs are associated with detectable anaplastic lymphoma kinase (ALK), which is a receptor tyrosine kinase protein normally only restrictively expressed in the neural system in adults (2). Among the ALK-positive patients, the fusion of nucleophosmin (NPM) and ALK, NPM-ALK, created by a balanced reciprocal chromosomal translocation t(2;5)(p23;q35), is most common (3). The chromosomal translocation produces the self-activated tyrosine kinase by fusing the ALK kinase domain with the oligomerization domain of NPM and its promoter, which constitutively drives the fusion gene transcription. NPM-ALK confers the tumorigenic effect by stimulating proliferative or survival signaling pathways in ALCLs via phosphatidylinositol 3-kinase (4, 5), signal transducers and activators of transcription 3 (6), and Src kinase (7). In addition to those signal proteins, there are numerous other NPM-ALK interaction molecules identified by tandem mass spectroscopy without characterized biological effects (8).

CD30 is a member of the tumor necrosis factor receptor (TNFR) superfamily, as defined by its sequence homology, and is a characteristic cell surface receptor for either mitogen or viral infection activated lymphocytes and neoplastic cells of both Hodgkin’s lymphoma and non–Hodgkin’s lymphoma (9). The restricted expression pattern of CD30 results from the elements in its complex promoter. These elements are as follows: a downstream promoter element located at positions +24 to +39 that is responsible for transcription start site selection, a constitutive core promoter region at −38 to −241 that contains three Sp-1 binding sites and two ETS binding sites, and CCAT repeats containing microsatellite sequences (MSS) at −336 to −1.2 kb, which primarily plays the repressive role to prevent CD30 expression in most cell types and resting peripheral blood cells (10). One of the Sp-1 binding sites in MSS was identified to be a JunB binding site in the tumor cells of Hodgkin’s disease, Hodgkin and Reed-Sternberg (H-RS) cells, which can lead to derepression of the MSS-mediated negative regulation and result in high CD30 expression (11).

Intriguingly, CD30 plays pleiotropic biological functions via stimulating different signaling pathways in different cell types. Unlike other TNFR family members, such as Fas or TNFR-1, CD30 does not possess the death domains that trigger caspase cascades in apoptotic processes. In H-RS cells, overexpressed CD30 can transmit survival and proliferative signals through nuclear factor-κB (NF-κB) via the binding to TNFR-associated factor (TRAF) family members in a ligand-independent manner (12). The role of CD30 in ALCL is completely different from that in H-RS cells. Instead of facilitating cell growth, the stimulation of CD30 causes cell cycle arrest and induces apoptotic cell death in ALCL cells (12–14). This phenotype may be as a result of the presence of NPM-ALK protein, which binds to TRAF proteins and abrogates CD30-mediated cell proliferation via NF-κB pathways (15).

To our knowledge today, it remains unclear if there is functional correlation between NPM-ALK and CD30 expression in ALCL cells. Is NPM-ALK involved in the regulation of CD30 gene expression? Will the suppression of NMP-ALK affect the CD30 gene expression? Which transcription factor(s) is(are) involved in this regulation? And what is the biological effect of the CD30 overexpression? The current study was our attempt to answer these questions. Using RNA interference to inhibit NPM-ALK expression in ALCL-derived cells, we found that the expression of CD30 is down-regulated via transcription factor JunB. Furthermore, NPM-ALK repression-mediated CD30 down-regulation is accompanied by a dramatic reduction in the proliferation activity in ALCL cells. The combination of small interfering RNA (siRNA) with CD30 ligand resulted in an enhanced cell growth inhibition.
Materials and Methods

**Cell culture, reagents, and transfection.** ALCL cell line Karpas 299 was kindly supplied by Dr. Alan Epstein (University of Southern California, Los Angeles, CA), SR cells were purchased from the American Type Culture Collection (Manassas, VA), and Jurkat cells were provided by Dr. Bret Ball (University of Southern California); these cell lines were cultured at 37°C in RPMI1640 supplemented with 10% fetal bovine serum (FBS) and 2 mmol/L L-glutamine. 293T cells were cultured at 37°C in DMEM with 10% FBS. Enhanced green fluorescent protein (GFP)–targeting siRNA (siGFP) was used as control, and its sequence is 5'-AAGCTGACCCTGAAGTTCATC-3'. NPM-ALK-targeting siRNA is named siNPM-ALK3, and its sequence is 5'-CAGCACTTAGTAGTGTACCGC-3'. Both siGFP and siNPM-ALK3 were ordered from Dharmacon Research (Lafayette, CO). Both siRNAs were annealed immediately before use. A cytomegalovirus promoter-driven JunB expression clone was purchased from OriGene Technologies, Inc. (Rockville, MD). CD30 reporter construct pGL3-CD30cMS is a gift from Dr. Ryouichi Horie (The University of Tokyo, Tokyo, Japan). NPM-ALK expression vector was a gift from Dr. Steven Morris (St. Jude Children's Research Hospital, Memphis, TN). Recombinant human CD30 ligand was purchased from R&D Systems (Minneapolis, MN). Fluorescence-conjugated antibody was purchased from BD PharMingen (San Jose, CA) for the flow cytometer analysis of the surface expression of CD30.

All transfections were done by LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) following the product protocol for the modification for siRNA transfection that 100 nmol/L siRNA was used for each reaction. In control experiments, pGEM was used instead of JunB cDNA. Fresh medium was added at 4 hours after transfection for 293T cells; 17 hours for Karpas 299 cells.

**Immunofluorescence assay.** Cells (3 x 10^5 to 5 x 10^5) were centrifuged and washed by PBS once followed by incubating with fluorescein-conjugated antibody (FITC-anti-CD30) in 1% FBS for 20 minutes in the dark for surface staining, and the cells were washed by PBS again. For bromodeoxyuridine (BrdUrd) analysis or cell cycle analysis, these surface CD30-labeled cells were subjected to further staining as described in the following sections.

**Western blot analysis.** For Western blot analysis, cells were washed by PBS and then resuspended in lysis buffer [10 mmol/L HEPES-KOH (pH 7.8), 10 mmol/L KCl, 1.5 mmol/L MgCl2, 20% glycerol, 0.5% NP40] with protease inhibitor Complete purchased from Roche Diagnostics Corp. (Indianapolis, IN). Cells were put on ice for 30 minutes followed by vortexing 15 to 20 seconds. The lysed cells were then centrifuged at 12,000 x g, 4°C for 10 minutes, and supernatants were collected. Protein samples were resolved by PAGE and blotted onto Immobilon-P membrane (Millipore, Billerica, MA) for antibody detection. For NPM-ALK protein, a monoclonal antibody against the ALK COOH-terminal domain was purchased from Immunotech (Fullerton, CA); anti-β-actin monoclonal antibody was purchased from Sigma-Aldrich (St. Louis, MO) and antibody against CD30 and anti-JunB was purchased from Santa Cruz Biotechnology (San Diego, CA). Visualization of bound antibodies was accomplished by a chemiluminescence assay using the Enhanced Chemiluminescence Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ).

**Luciferase reporter assay.** The CD30 reporter construct pGL-CD30cMS and the activator protein-1 (AP-1) binding site mutant construct pGL-CD30cMSmAwere gifts from Dr. Ryouichi Horie. The reporter activity was analyzed using the Luciferase Assay System (Promega, Madison, MI). Cell lysates were prepared at 48 hours after transfection of the reporter construct using passive lysis buffer (Promega). In brief, 3 x 10^6 cells were resuspended and lysed in 100 μL passive lysis buffer and mixed gently for 15 minutes at room temperature followed by 15 seconds of vortexing. Cell lysate (20 μL) was then added into the luminometer tube containing 100 μL luciferase substrate solution (Luciferase Assay Reagent) immediately before

Figure 1. Surface CD30 is decreased in ALCL cells with repressed NPM-ALK expression. Karpas 299 cells transfected with siNPM-ALK3 or control siGFP were subjected to either flow cytometry for measuring surface CD30 level (A) or Western blot analysis for measuring total NPM-ALK protein (B). A, FITC-conjugated monoclonal antibody against CD30 was used for surface staining. Western blot analysis of NPM-ALK was done by using monoclonal antibody specific to the COOH-terminal domain of ALK. Right, blot of β-actin is used as the loading control for each sample. Results were representative of four independent experiments.
reading using TD20/20 Luminometer (Turner Designs, Sunnyvale, CA) with a 10-second integration reading time after 2-second measurement delay.

**Cell proliferation analysis.** Cell cycle profiles of treated or control cell samples were determined by fluorescence-activated cell sorting (FACS) analysis. Cells were washed with PBS, fixed in ice-chilled 80% ethanol for at least 2 hours at 4°C, and washed and resuspended in PBS buffer containing RNase A (25 μg/mL) at 37°C. After incubating at 37°C for 15 minutes, the cells were centrifuged and resuspended in 100 μg/mL propidium iodide (Sigma-Aldrich) for 15 minutes at room temperature before analysis. ModFit software (Variety Software House, Topsham, ME) was used to estimate the population in each phase of the cell cycles. Proper gating of viable cells with normal forward and side scatters was used to eliminate those cells damaged from the handling process.

Active cell proliferation was monitored by BrdUrd incorporation using the BrdUrd Flow kit (BD PharMingen). A final concentration of 1 μmol/L BrdUrd was added in the cultured cells for 30 to 40 minutes before staining. The cells were pelleted, and the surface CD30 was stained using FITC-conjugated anti-CD30 antibody (BD PharMingen) for 20 minutes on ice. After centrifugation and washing, the cells were resuspended in BD Cytoperm buffer for permeabilization and fixation. Cells were then treated with the following reagents according to the manufacturer’s protocol: BD Cytoperm Plus buffer, BD Cytofix/Cytoperm buffer, and DNase to expose BrdUrd epitopes. Then, the samples were stained with phycoerythrin-conjugated anti-BrdUrd antibody followed by FACS analysis.

**Cell viability assay.** Cell viability assays were determined by CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega). In a 96-well cell culture plate, 20 μL of the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) solution were added to 100 μL of culture medium for a background reading. After incubating 2 to 3 hours at 37°C, the plate was read. The measurement is the absorbance at 490 nm (A490) using an EMax precision microplate reader (Molecular Devices Corp., Sunnyvale, CA). Cell viabilities are presented as a comparison to the viability of siGFP-treated controls.

**Cell apoptosis assay.** Cell apoptosis was analyzed by the TACS Annexin V-FITC Apoptosis Detection kit (R&D Systems). Cells (3 × 10⁵) were resuspended in the premixed reaction solution containing 1× binding buffer, propidium iodide (100 μg/mL), and FITC-conjugated Annexin V (250 ng/mL). Labeled cells were analyzed by FACS, and apoptotic cells were defined as propidium iodide–negative and FITC-positive cells.

**Results**

**CD30 surface expression is down-regulated in NPM-ALK-repressed ALCL cells.** To evaluate if CD30 expression was affected by suppression of NPM-ALK in the (2;5)-positive ALCL-derived cells, CD30 surface expression was monitored by FACS analysis at different times after transfection with NPM-ALK-targeted siRNA (siNPM-ALK3). As shown in Fig. 1A, significant CD30 down-regulation was observed in the siNPM-ALK3-treated cells when compared with the siGFP-treated cells, in which the CD30 expression was unaffected. The down-regulation of CD30 expression started from day 2 after transfection (23% of cells) and reached the maximum at days 3 and 4 after transfection (54.8% and 45.6% of cells underwent CD30 down-regulation, respectively). The down-regulation of CD30 expression in the Karpas 299 cells was correlated to the level of NPM-ALK gene silencing, which, as shown in Western blot analysis (Fig. 1B), also reached the nadir point at days 3 and 4 after transfection. Although 90% to 95% of cells were transfected, we cannot completely shut down the NPM-ALK protein expression due to its high expression and long half-life of 48 hours. This result showed for the first time a relationship between NPM-ALK and the CD30 expression, suggesting that the overexpression of CD30 in ALCL might be the consequence of the presence of NPM-ALK fusion protein.

**NPM-ALK regulates CD30 transcription.** We next wanted to determine if the observed decrease in surface CD30 reflected a change in the gene expression resulting from the silencing of the NPM-ALK gene. First, we investigated if the change in total CD30 protein correlated to the change in NPM-ALK gene expression. At days 3 and 4 after transfection of siNPM-ALK3 or siGFP (the time points showing the maximum gene silencing of NPM-ALK), Karpas 299 cells were analyzed by Western blot to determine the CD30 protein levels. As shown in Fig. 2A (top), the CD30 protein level was dramatically down-regulated in siNPM-ALK3-treated cells but not in siGFP-treated cells. The broadened CD30 band is likely due to its extensive glycosylation. β-Actin was used as internal loading control (Fig. 2A, bottom). Using immunofluorescence assays, there was no noticeable difference in the CD30 cellular distribution in siGFP control and siNPM-ALK3-treated Karpas 299 cells (data not shown), suggesting that the decreased CD30 on cell surface resulted from the decrease of total CD30 protein.

Next, we investigated the potential mechanism of CD30 regulation by the NPM-ALK fusion protein. Because NPM-ALK is a constitutive kinase that triggers a wide variety of downstream factors, many of which are involved in gene regulation, we hypothesized that NPM-ALK regulates CD30 at the gene transcription level. Using pGL3-CD30cMS, a construct with a luciferase reporter gene driven by the CD30 promoter, we determined if silencing the NPM-ALK gene affected CD30 gene regulation. Following siNPM-ALK3 and control siGFP treatment, pGL3-CD30cMS was transfected into Karpas 299 cells and the cell lysate was harvested to determine the luciferase activity. The results showed an 8- to 10-fold reduction in luciferase activity in the NPM-ALK-repressed cells compared with the siGFP controls (Fig. 2B), suggesting that NPM-ALK regulates CD30 gene expression via transcriptional regulation.

![Figure 2. NPM-ALK regulates CD30 gene expression at the level of transcription in ALCL-derived cells. Cell lysates from Karpas 299 cells were collected at days 3 and 4 after transfection, and Western blot analyses were done using monoclonal antibody against CD30. A blot of β-actin is used as the loading control for each sample. A CD30 gene expression reporter, pGL3-CD30cMS, was transfected into the siNPM-ALK3- or siGFP-treated Karpas 299 cells at day 2 after siRNA transfection. B, luciferase assays were conducted using equal cell numbers from each sample at 48 hours after reporter transfection to monitor the CD30 promoter activity in the cells.](https://www.aacrjournals.org)
The expression of JunB is regulated by NPM-ALK. To identify the transcription factors, which were regulated by NPM-ALK and which in turn regulate the expression of CD30, we investigated JunB expression in our NPM-ALK silencing system. JunB was reported to play a critical role in activating CD30 expression in H-RS cells (11). The dynamic change of JunB protein level was monitored in Karpas 299 cells treated with siNPM-ALK3 and control siGFP. As shown in Fig. 3A, starting from day 2 after siNPM-ALK3 treatment, JunB expression declined and reached its nadir at day 3. The time frame of the JunB protein down-regulation correlated to the down-regulation of NPM-ALK from siNPM-ALK3 treatment, which reached the maximum level of inhibition at day 3 after siNPM-ALK3 transfection (Fig. 1B). These data suggest that the JunB down-regulation might result from the NPM-ALK siRNA-mediated NPM-ALK repression.

To confirm that NPM-ALK regulates JunB expression, we determined if NPM-ALK could induce the expression of JunB in HEK293T cells, which endogenously express neither JunB nor NPM-ALK protein. As shown in Fig. 3B, transfection of the NPM-ALK expression plasmid induced the expression of JunB in a dose-dependent manner. These results indicate that JunB, which may be a key factor responsible for CD30 overexpression in ALCLs as it was in H-RS cells, is a downstream factor being regulated by NPM-ALK. Because JunB and c-Jun share the same binding site under certain circumstance, c-Jun expression was determined by Western blot analysis. There was no difference in control and in NPM-ALK-reduced samples; hence, c-Jun is therefore excluded as a possible trans-factor for CD30 regulation (data not shown).

Next, we did real-time reverse transcription-PCR (RT-PCR) to determine if NPM-ALK induced a change in JunB gene expression in 293T cells. As shown in Fig. 3C, there is a significant increase in JunB mRNA levels, suggesting that the NPM-ALK-induced increase in JunB is most likely at the transcriptional level.

JunB-mediated transactivation of CD30 expression is in a cell type-specific manner. To investigate the function of JunB in regulating CD30 expression, a JunB cDNA expression plasmid and pGL3-CD30cMS reporter construct were cotransfected into Karpas 299 or NPM-ALK-negative cells. Forty-eight hours after transfection, luciferase activity was determined. Interestingly, different cells showed different responses. In Karpas 299 cells, with exogenously introduced JunB, the CD30 promoter activity was dramatically enhanced 10- to 20-fold compared with controls, in which empty pGEM was used instead of the JunB expression plasmid (Fig. 4A, middle). Luciferase activity was determined if NPM-ALK could induce the expression of JunB in 293T cells, which are NPM-ALK negative, JunB-mediated transactivation of the CD30 promoter was not observed (Fig. 4B). These results imply that, in addition to JunB, other cellular factor(s) is involved in the strict regulation of CD30 expression. Consistent with this observation, CD30 expression was undetectable in
HEK293T cells when JunB expression was induced by NPM-ALK (data not shown). To confirm our observation of JunB transactivation of the CD30 promoter, we did the reporter assay using the construct with an AP-1 binding site mutation (pGL-CD30cMSmA). As shown in Fig. 4C, in contrast to the results using the wild-type CD30 reporter construct (Fig. 4A), the reporter activity of the AP-1 binding site mutant (pGL-CD30cMSmA) showed no significant change in the presence of JunB. This result suggests that JunB enhances the expression of CD30 protein by binding to the AP-1 site in the upstream promoter region.

**Cell proliferation is repressed in NPM-ALK repression-associated low CD30 expression cells.** NPM-ALK gene silencing resulted in two subgroups of cells with either low or high CD30 expression, whereas siGFP treatment had no effect on CD30 expression, which remained high (Fig. 1A). To identify a change in biological phenotype correlated to the change in CD30 expression, we analyzed the cell proliferation and cell cycle profiles of cells with different levels of CD30 expression. Cell proliferation activity was analyzed using BrdUrd staining. Four days after siNPM-ALK3 or siGFP transfection, Karpas 299 cells were treated with BrdUrd and then stained with anti-CD30 and anti-BrdUrd antibodies. Using FACS, the cells with low or high CD30 expression were gated as shown in Fig. 5A and further analyzed for BrdUrd staining. As shown in Fig. 5B, significantly fewer BrdUrd-positive cells were present in the CD30 low population in comparison with the CD30 high population (9.1 ± 1.1% versus 22.0 ± 4.3%, respectively; \( P < 0.05 \)), suggesting that the cell proliferation was repressed in the CD30 low population induced by NPM-ALK repression.

Next, we did cell cycle analysis in cells with high or low CD30 expression. In the siNPM-ALK3-transfected cells, the high CD30 cells showed a similar cell cycle profile as the siGFP-transfected cells. Both showed ~45% of cells in the G0-G1 phase (47.8 ± 7% and 42.5 ± 3.9%, respectively; \( P = 0.24 \)). The CD30 expression levels were also similar in siGFP-transfected cells and the high CD30 cells in siNPM-ALK3-transfected cells. However, the low CD30 cells showed a different cell cycle profile from the high CD30 cells (Fig. 5C and D). Compared with high CD30 population, significantly more cells in low CD30 population were in G0-G1 phase (77.8 ± 8.8% versus 47.8 ± 7.0%; \( P < 0.005 \)) and fewer cells were in the S phase (11.2 ± 6.1% versus 23.5 ± 6.9%; \( P < 0.05 \)) and G2 phase (8.3 ± 3.9% versus 20.9 ± 7.1%; \( P < 0.05 \); Fig. 5C and D). These indicated that more cells in the low CD30 expression population had cell cycle arrest in the G0-G1 phase and that cell proliferation was inhibited.

**Combination of NPM-ALK silencing and CD30 stimulation enhances tumor cell growth inhibition.** Both CD30 and NPM-ALK are neoplastic cell-specific proteins. Stimulation of CD30 or silencing of NPM-ALK leads to suppression of cell proliferation. We reasoned that, by combining both approaches, we might be able to achieve a better cancer cell-specific growth inhibition. To test this hypothesis, different doses of CD30 ligand were added to Karpas 299 cells 3 days after siNPM-ALK3 transfection or siGFP transfection. Cell viability and apoptosis were determined 20 hours after adding CD30 ligand and compared with cells treated with siNPM-ALK3 or CD30 ligand alone. Consistent with our previous results (16), siNPM-ALK3-mediated NPM-ALK repression diminished the cell viability to ~60% [bovine serum albumin (BSA) treatment; Fig. 6A]. CD30 ligand alone resulted in cell viability loss in a dose-dependent manner (siGFP treatment; Fig. 6A, black columns). The combination of siNPM-ALK3 and CD30 ligand produced a greater cell viability loss than either treatment alone (Fig. 6A, white columns). We observed a similar trend in the cell apoptosis assays (Fig. 6B): the combination of NPM-ALK repression with siNPM-ALK3 and CD30 ligand resulted in higher apoptosis rate. Interestingly, the combination treatment with siNPM-ALK3 and low-dose CD30 ligand treatment (0.1 µg/mL; Fig. 6B, white columns) achieved similar levels of significant cell growth inhibition as the high dose of CD30 ligand alone (1.2 µg/mL; Fig. 6B, black columns). With different ALCL cell line, SR cells, we obtained similar results that the combination of siNPM-ALK with CD30 ligand can induce ALCL cell apoptosis and decrease the cell viability as shown in Fig. 6C and D. All these results suggest that simultaneously targeting both NPM-ALK and CD30 may be more effective as a lymphoma-specific therapeutic strategy.

**Discussion**

The goal of this study is to investigate the functional correlation between NPM-ALK protein and CD30 protein. Although both NPM-ALK and CD30 are highly expressed in most ALCL and both play critical roles in regulating cancer formation and proliferation, the relationship between NPM-ALK and CD30 in the tumorigenesis process is unknown: they might directly interact with each other or
the coexpression of both proteins may be a coincident event. In our previous study (16), NPM-ALK siRNA resulted in growth inhibition of ALCL cells. In this study, we used the same methodology to study the functional correlation of NPM-ALK protein and CD30.

First, we found that CD30 expression on the cell surface is significantly down-regulated when Karpas 299 cells were treated with siNPM-ALK3 and the decrease in CD30 expression is correlated to the decrease in NPM-ALK expression (Fig. 1). Further studies indicated that the decrease of CD30 expression on the cell surface resulted from the down-regulation of total CD30 protein after treatment with siNPM-ALK3 (Fig. 2A). Using a CD30 reporter system, we found that introduction of siNPM-ALK3 resulted in a dramatic decrease in CD30 promoter activity (Fig. 2B). These data lead us to further explore the mechanism of the down-regulation of CD30 expression in the presence of siNPM-ALK3. As a cytoplasmic protein, NPM-ALK is unlikely to interact directly with the CD30 promoter. We started to identify the transcription factors that might interact directly with the CD30 gene and regulates its expression. JunB was one of the candidate proteins regulating CD30 expression in ALCL cells.

JunB belongs to the Jun protein family. It regulates target gene expression by forming the AP-1 complex and then binding to the AP-1 sites in the promoter region. Like the other proteins in Jun protein family, JunB regulates the expression of proteins involved in cell proliferation and survival. However, unlike c-Jun, which primarily facilitates tumor formation, the function of JunB is pleiotropic in tumorigenesis: it can be either a positive or a negative regulator in different circumstances (17). Recently, JunB was detected in a variety of CD30-positive neoplastic cells, including ALCL, Hodgkin’s lymphoma, cutaneous ALCL, and diffuse large B-cell lymphoma (18). It has been reported that, in H-RS cells, JunB is the critical regulator responsible for the transactivation of CD30 expression via binding to the AP-1 site located in the MSS region in the CD30 promoter (11). Our results indicated that JunB was a downstream factor of the NPM-ALK-mediated effect in ALCL. As shown in Fig. 3A, the inhibition of NPM-ALK fusion protein expression in Karpas 299 cells leads to the down-regulation of JunB protein. On the other hand, the introduction of NPM-ALK protein to 293T cells resulted in the dose-dependent increase in JunB protein as shown in Fig. 3B. Real-time RT-PCR data showed the dramatic increase of JunB gene expression after the transfection of NPM-ALK cDNA in 293T cells, indicating that NPM-ALK protein regulates the expression of JunB. These results suggested that NPM-ALK positively regulates the expression of JunB protein. Using NPM-ALK transgenic mice, Turner et al. (19) reported that c-Jun NH2-terminal kinase (JNK) is activated by NPM-ALK. It is possible that JNK may also play a role in activating the JunB protein.

Next, we assessed if JunB regulated the expression of CD30. Using a CD30 gene expression reporter system, we observed that the introduction of JunB protein can significantly increase CD30 transcription activities in Karpas 299 cells (P < 0.001; Fig. 4A). However, we could not observe the phenomena in 293T cells, which are and non-ALCL cell lines (Fig. 4B). These results indicate that JunB alone is not sufficient to stimulate CD30 expression and other factor(s) is required. The additional factor(s) could be supplied by the infection of EBV or other unidentified factors. It has been reported that 60% to 80% of Hodgkin’s lymphomas are associated with EBV infection (12). EBV enhances the CD30 expression by two possible mechanisms: first, it integrates upstream of the CD30 promoter region or the viral LMP-1 protein up-regulates a CD30 transactivator, Sp-1. However, the incidence of EBV infection in ALCL is very rare, suggesting that some unidentified factor(s) might play roles.

After characterizing the mechanism of NPM-ALK-mediated CD30 regulation, the next question is “what are the biological consequences of CD30 down-regulation caused by NPM-ALK repression?” The observed tumor cell growth inhibition caused by NPM-ALK repression was assessed by determining both cell proliferation and apoptosis levels (16). In this study, low CD30 cells showed the G0/G1 cell cycle arrest phenotype. However, it is not yet clear if the decreased cell proliferation is the biological consequence of CD30 down-regulation induced by NPM-ALK repression or it is an independent event from the decreased CD30 expression. NPM-ALK associates with dozens of cellular proteins (8), several of which have been found to have potent roles in regulating cell growth and survival. It is possible that the modulated CD30 expression directly contributes to the cell proliferation regulation. On the other hand, CD30 modulation may be simply a bystander effect of NPM-ALK and does not contribute to lymphomagenesis.
It is known that the stimulation of CD30 has pleiotropic effects in different cell types, it can enhance the cell growth of Hodgkin’s disease cells but not the ALCL cells. Although we currently do not know the function of CD30 overexpression in ALCL, the fact that the combination of CD30 ligand and NPM-ALK repression can further inhibit cell growth is an interesting discovery that may have certain clinical application.

Targeting endogenous NPM-ALK expression causes significant growth inhibition primarily in ALCL-derived, t(2;5)-positive cells. We have further shown that the combination of NPM-ALK repression and chemotherapy (doxorubicin) enhanced the tumor cell growth inhibition (16). In this study, we report the significantly enhanced inhibition of tumor cell growth when NPM-ALK and CD30 ligand are applied together. Because the high and specific expression of both CD30 and NPM-ALK is present in ALCL cells, these results provide a potential new strategy to treat ALCL patients by simultaneously targeting both proteins with less adverse effects than chemotherapy.

Although NPM-ALK can be detected in 50% to 70% ALCL, there are ALCL subtypes with various ALK fusion protein expression, and some of the ALCLs are ALK negative. All reported ALK fusions in ALCL contain the similar structural feature of an NH2-terminal oligomerization domain fused to the COOH-terminal kinase domain of ALK (3). For the different types of ALK fusion proteins, JunB may be activated by a common mechanism mediated by ALK activity, and then, CD30 expression is induced by elevated JunB expression. The lack of CD30 expression in some ALK-positive tumor cells may result from the lack of the required cellular factor(s).

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