The Nucleophosmin-Anaplastic Lymphoma Kinase Fusion Protein Induces c-Myc Expression in Pediatric Anaplastic Large Cell Lymphomas

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The majority of pediatric anaplastic large cell lymphomas (ALCLs) carry the t(2;5)(p23;q35) chromosomal translocation that juxtaposes the dimerization domain of nucleophosmin with anaplastic lymphoma kinase (ALK). The nucleophosmin-ALK fusion induces constitutive, ligand-independent activation of the ALK tyrosine kinase leading to aberrant activation of cellular signaling pathways. To study the early consequences of ectopic ALK activation, a GyrB-ALK fusion was constructed that allowed regulated dimerization with the addition of coumermycin. Expression of the fusion protein caused a coumermycin-dependent increase in cellular tyrosine phosphorylation and c-Myc immunoreactivity, which was paralleled by a rise in c-myc RNA. To assess the clinical relevance of this observation, c-Myc expression was determined in pediatric ALK-positive and -negative lymphomas. Co-expression of c-Myc and ALK was seen in tumor cells in 15 of 15 (100%) ALK-positive ALCL samples, whereas no expression of either ALK or c-Myc was seen in six of six cases of ALK-negative T-cell lymphoma. C-Myc may be a downstream target of ALK signaling and its expression a defining characteristic of ALK-positive ALCLs. (Am J Pathol 2002, 161:875–883)

Lymphoma is the third most common cancer in children and adolescents in the United States, representing 13% of newly diagnosed malignancies in the pediatric age group. Although significant progress has been made in the treatment and outcome of childhood lymphoma, many children still succumb to disease, or suffer acute and long-term toxicity from contemporary multiagent chemotherapy. A subtype of pediatric anaplastic large cell lymphoma (ALCL), anaplastic lymphoma kinase (ALK)-positive lymphoma (ALK+ lymphoma), may serve as an ideal model for the study of the molecular mechanisms of malignant transformation. ALK+ lymphoma is characterized by a t(2;5)(p23;q35) chromosomal translocation that creates a chimeric fusion protein consisting of the amino-terminal portion of the nucleolar phosphoprotein, nucleophosmin (NPM), and the cytoplasmic domain of the receptor tyrosine kinase, ALK. ALK is a recently described receptor tyrosine kinase in the insulin receptor subfamily whose expression is normally restricted to the central nervous system. NPM is a ubiquitously expressed homohexameric nucleolar phosphoprotein that shuttles ribosomal proteins between the nucleus and cytoplasm. Essential functions of NPM in the chimera are provision of both a promoter that drives ectopic expression of ALK in lymphoid cells, and a dimerization motif that facilitates trans-phosphorylation and hence activation of signaling.

The t(2;5) translocation is an essential event in the pathogenesis of ALK+ lymphoma as NPM-ALK potently transforms both rat fibroblast and murine lymphoid cell lines, and induces lymphoid tumors in mice. NPM-ALK homodimers autophosphorylate on multiple residues, creating docking sites for several SH2 domain-containing signaling molecules. Formation of this signaling complex leads to changes in gene expression that underlie the malignant phenotype. One goal of our studies is to understand the changes in cellular transcription that the NPM-ALK fusion protein produces. Ligand-induced dimerization is thought to directly induce the activation of receptor tyrosine kinases. Numerous researchers have generated protein fusions that can be conditionally dimerized in the presence of bivalent compounds such as coumermycin, rapamycin, and FK1012. To better define the immediate early effects of ALK activation, we used this technology to generate a conditionally dimerizable kinase construct, designed to mimic the activity of NPM-ALK in vivo. Regulated dimerization produced an increase in tyrosine phosphorylation of the fusion protein, as well as additional cellular proteins. Unexpectedly, we observed an increase in c-Myc expression in ALK-positive lymphoma samples, as well as additional cellular proteins.

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protein and RNA expression in response to ALK activation. Supporting the relevance of this finding in vivo, c-Myc was detected in 15 of 15 t(2;5)-positive ALCLs, but in 0 of 6 ALK-negative lymphomas. The data suggest that c-Myc is a specific downstream target of aberrant ALK signaling as well as a novel marker for the detection of ALK+ lymphomas.

**Materials and Methods**

**Construction of Expression Plasmids**

**6 Myc-GyrB-ALK**

For transient expression of myc epitope-tagged GyrB-ALK, the coumermycin-binding domain (codons 2 to 220) of the B subunit of *Escherichia coli* DNA Gyrase (GyrB) was cloned into pCS2+MT in-frame with the entire cytoplasmic domain of ALK.

**Wild-Type and Mutant 6 Myc-NPM-ALK Retroviral Constructs**

The 6-myc epitope tag from the pCS2+MT plasmid vector was inserted into the BamHI and EcoRI sites of the pBABE Puro retroviral vector. Full-length NPM-ALK coding sequence was amplified by polymerase chain reaction (PCR) incorporating 5' and 3' EcoRI restriction sites (underlined) using forward primer, 5' CCGAATTCGATG-GGAAGATTCGATGGAC 3', and reverse primer 5' ATGAAATGTTCAGGGCCCAGGCTGGTTC 3'. Full-length NPM-ALK was subcloned into the EcoRI site of the 6 Myc-pBABE Puro retroviral vector.

The conserved lysine residue (amino acid 210, nucleotide 628) within the kinase domain of the wild-type NPM-ALK retroviral construct was mutated to alanine (GCC) to abrogate kinase activity using the QuikChange in vitro site-directed mutagenesis system (Stratagene, La Jolla, CA) following the manufacturer’s instructions with the addition of 4% dimethyl sulfoxide.

**In Vitro Translation**

*In vitro* translation of 6 Myc-GyrB-ALK was performed with the TNT Quick Coupled Transcription/Translation System with SP6 RNA polymerase (Promega, Madison, WI). Reactions were performed at 30°C for 2 hours. *In vitro*-translated 6 Myc-GyrB-ALK was then treated with or without varying concentrations of coumermycin A1 (Sigma, St. Louis, MO) prepared in dimethyl sulfoxide for an additional 30 minutes at 30°C.

**Cell Culture, DNA Transfection, and Retroviral Infections**

Rat1A fibroblasts (kindly provided by Andrew Thorburn, University of Utah), and the retroviral packaging Phoenix cell line (a gift from Nori Matsunami, University of Utah) were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal calf serum (HyClone, Logan, UT) and 100 U/ml of penicillin and 100 μg/ml of streptomycin (Life Technologies Inc., Gaithersburg, MD). Cells were maintained at 37°C and 5% CO2 in a humidified incubator. Rat1A fibroblasts were seeded in 6-well (35-mm) plates and transiently transfected with 1 μg of 6 Myc-GyrB-ALK expression construct, 8 μl of lipofectamine, and 6 μl of Plus reagent per well following the manufacturer’s instructions (Life Technologies Inc.). Forty-eight hours after transfection, cell cultures were treated with varying concentrations of coumermycin for 30 minutes at 37°C. Cells were then briefly washed with 2 ml of phosphate-buffered saline and lysed in 200 μl of RIPA with protease inhibitors [150 mmol/L NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mmol/L Tris-HCl, pH 7.5, 1.4 mmol/L phenylmethyl sulfonyl fluoride, 1 mmol/L ethylenediaminetetraacetic acid, 1 μg/ml each of leupeptin and pepstatin, 1 mmol/L benzamidine, and 1 mmol/L sodium orthovanadate, pH 10] for 30 minutes on ice. Lysates were clarified by microcentrifugation at 13,000 rpm for 10 minutes at 4°C.

The Phoenix packaging cell line was similarly transfected with the wild-type and mutant 6 Myc-NPM-ALK retroviral constructs. Medium was replaced 24 hours after transfection. Forty-eight hours after transfection the medium was removed, filtered through a 0.45-μm filter, and then polybrene was added to a final concentration of 4 μg/ml. Rat1A fibroblasts were then infected with the viral supernatant. After 48 hours, the medium containing retrovirus was removed and replaced by selection medium containing 5 μg/ml of puromycin.

**Immunoblotting**

Protein expression was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting using standard methods. RC20 anti-phosphotyrosine antibodies conjugated to horseradish peroxidase were purchased from Transduction Laboratories (Lexington, KY). Polyclonal anti-phosphotyrosine antibody was a gift from Dr. Steven Wiley, University of Utah. The 9E10 monoclonal antibody recognizing the myc epitope was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Goat anti-rabbit and anti-mouse secondary antibodies coupled to horseradish peroxidase were purchased from Pierce (Rockford, IL).

**Immunohistochemical Staining**

Immunohistochemical staining for ALK-1 and c-Myc co-expression was performed on paraffin-embedded tissues received from the Children’s Hospital Tissue Network (Columbus, OH) obtained from patients enrolled on the Children’s Cancer Group treatment protocol 5941, which is used currently for the treatment of CD30-positive anaplastic large cell (T cell and null cell) lymphomas in children. Fifteen cases of ALK-positive ALCLs, three cases of ALK-negative T large cell lymphoma, and three cases of T-cell lymphoblastic lymphoma were examined.
Paraffin sections were mounted on Fisherbrand/Plus Superfrost slides, heated at 60°C for 30 minutes, deparaffinized, and rehydrated. ALK-1 (DAKO, Carpinteria, CA) staining was performed first after antigen epitope retrieval (3 minutes in an electric pressure cooker followed by 30 minutes of cooling in Tris buffer, pH 9.5). The slides were stained with ALK-1 (1:25 dilution) using a Ventana automated immunostainer (Ventana, Tucson, AZ) and 50 ng of purified cDNA template. Rat ALCL, (Palo Alto, CA), and 50 ng of purified cDNA template. Rat-alk-1 staining (brown chromagen staining) in the nuclear and cytoplasm. Co-expression of c-Myc was identified by co-staining of nuclear structures with the blue chromagen.

Double-label immunohistochemistry was also performed on formalin-fixed cell blocks from two ALK-positive ALCL cell lines, Karpas 299 and SUDHL-1, as well as a non-ALK-expressing cell line, MAC2A. The cell lines were kindly provided by Dr. Megan Lim (University of Utah) and were originally obtained from the American Tissue Culture Collection (Rockville, MD). All cell lines were maintained in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% heat-inactivated fetal calf serum, 2 mmol/L-glutamine, and 100 U/ml of penicillin/streptomycin (Life Technologies, Inc., Gaithersburg, MD) at 37°C and 5% CO₂. Cells were pelleted and fixed in 10% buffered formalin and embedded in paraffin for cell-block preparations. Staining was performed on sections from the cell blocks as described above using an alkaline-phosphatase blue detection for the c-Myc antibody and diaminobenzidine detection for the ALK-1 antibody.

**Real-Time PCR**

C-myc transcript levels were determined in the wild-type and mutant NPM-ALK-expressing cell lines. Total RNA was isolated from cell lines transduced with wild-type and mutant NPM-ALK retroviruses. First strand cDNA was generated in a 40-μl reverse transcription reaction and was purified using a QIAquick PCR purification kit (Qiagen, Chatsworth, CA). Transcript copy numbers in cell lines were prepared from PCR products that were purified using a QIAquick PCR purification kit (Qiagen, Chatsworth, CA), and diluted. Transcript copy numbers in cell lines were calculated by comparing the fluorescence of PCR products of unknown concentration with the fluorescence of serial dilutions of the external standard. Only fluorescence values measured in the log-linear phase of amplification were used for sample quantification. A best-fit log-linear fluorescence curve was calculated for each sample, and these curves in turn were used to estimate the number of transcripts in individual samples. All samples were run in triplicate, and c-myc expression was normalized to the expression of the housekeeping gene PER2.

**Results**

**Conditional Dimerization of GyrB-ALK Induces Phosphorylation of the Fusion in Vitro**

The GyrB-ALK fusion construct (Figure 1) was generated using a standard PCR cloning strategy, and consisted of the c-myc epitope-tagged E. coli DNA GyrB coumermycin-binding domain fused to the cytoplasmic domain of ALK. The portion of ALK contained in this fusion is identical to that present in NPM-ALK chimera. To test the fusion construct, we expressed chimeric protein in an *in vitro* transcripion/translation reaction. Lysates were treated for 30 minutes with varying concentrations of coumermycin. The proteins were then resolved by SDS-PAGE and visualized by Western blotting with anti-phosphotyrosine and anti-myc antibodies (Figure 2). Figure 2 shows that coumermycin induced tyrosine phosphorylation of a protein present in the Myc-GyrB-ALK fusion. Low levels of kinase tyrosine phosphorylation were observed in the absence of drug (lanes 1 and 2). A marked increase in phosphorylation was seen at 100 nmol/L of coumermycin. This result suggests that a small degree of...
background kinase tyrosine phosphorylation occurs in the absence of drug. As has been seen for other conditional dimerizing systems, higher levels of drug decrease the degree of ALK activation (lanes 4 and 5). This may result from the altered stoichiometry of GyrB to drug.

**Conditional Dimerization of a GyrB-ALK Fusion Induces Phosphorylation in Vivo**

To determine whether conditional dimerization occurred in vivo, we transiently transfected Rat1A cells with the Myc-GyrB-ALK expression construct described above. Cells were then treated for 30 minutes with coumermycin concentrations ranging from 0 to 100 μmol/L. Cellular proteins were then separated by SDS-PAGE and the level of phosphorylation was determined by Western blotting with anti-phosphotyrosine antibodies (Figure 3). As was seen in vitro (Figure 2), basal tyrosine phosphorylation of the ALK fusion was seen in the absence of drug (lane 1). Peak tyrosine phosphorylation occurred at coumermycin concentrations of between 1 to 10 μmol/L. At concentrations more than 10 μmol/L, decreased phosphorylation of GyrB-ALK was seen, most likely because of inhibition of dimer formation with excess drug. The difference in the coumermycin concentration required for maximal tyrosine phosphorylation of GyrB-ALK in vivo and in vitro is likely because of differences in cell permeability, protein expression levels, and/or competing binding reactions. Notably, an increase in tyrosine phosphorylation of additional cellular proteins in the presence of drug (indicated with arrows), paralleled that seen with the ALK fusion.

c-Myc Protein Expression Is Induced by GyrB-ALK Activation

We hypothesized that constitutive ALK tyrosine phosphorylation seen in the absence of drug treatment when GyrB-ALK was highly expressed potentially masked more significant increases in the degree of ALK phosphorylation after exposure to coumermycin. To further explore the mechanisms underlying the constitutive activation of GyrB-ALK, gene dosage was modified during the transient transfections of Myc-GyrB-ALK. Rat1A cells were transiently transfected with between 0 to 12 μg of the Myc-GyrB-ALK expression construct in the absence of coumermycin. Cells were lysed and an equivalent amount of protein from each transfection was resolved by SDS-PAGE and visualized by immunoblotting with anti-phosphotyrosine and anti-myc antibodies. Figure 4 shows that the degree of ALK autophosphorylation increased with increasing fusion protein expression except at the highest level of expression. This result suggests that the degree of constitutive phosphorylation of the construct will be a percentage of maximal induction after coumermycin treatment. Additionally, while performing the gene dosage studies, we observed an increase in the expression level of another protein in our anti-myc antibody immunoblot (Figure 4, open circle). The level of this protein increased with increasing Myc-GyrB-ALK dosage up to 4 μg of DNA, and then its level remained constant. If this protein had been a degradation product of Myc-GyrB-ALK, we would have expected its expression level to remain a constant fraction of the total Myc-GyrB-ALK protein concentration. Because it increased only up to a point and then stabilized after GyrB-ALK tyrosine phosphorylation, we investigated if it was cellular c-Myc detected by the 9E10 antibody.

c-My c RNA Expression Is Induced by NPM-ALK Activation

To validate the observations seen in cell line analyses with the Myc-GyrB-ALK fusion (Figure 4), we examined the expression of c-myc RNA in cells stably expressing both the wild-type and mutant NPM-ALK chimeras by retroviral transduction. Rat1A cells expressing each of...
these constructs are shown in Figure 5. Cells expressing the wild-type kinase showed a transformed phenotype, whereas those expressing the mutant appeared morphologically identical to the parent Rat1A cell line. Total RNA was isolated from each cell line, and quantitative real-time PCR was performed to determine c-myc transcript number. As shown in Table 1, c-myc transcript number was induced 3.4-fold in cells expressing wild-type NPM-ALK compared to cells expressing mutant kinase. This finding confirmed the results observed in cell lines with the GyrB-ALK fusion, and suggests that c-Myc expression is induced by NPM-ALK activation.

c-Myc and ALK Are Co-Expressed in 100% of Patient ALK+ Lymphomas

At the inception of this study we aimed to elucidate downstream targets of NPM-ALK activation in childhood lymphoma. Therefore, we sought to extend our observations in cell line studies to pediatric patient samples. Formalin-fixed, paraffin-embedded lymphoma biopsies from pediatric patients enrolled on Children’s Cancer Group therapeutic trials for ALCL were examined by immunohistochemistry for expression of c-Myc and ALK (Figure 6). Fifteen of 15 (100%) of ALCLs staining positive for ALK also co-expressed c-Myc. In contrast, no non-ALCL specimens (0 of 6) that were ALK-negative had detectable c-Myc by immunohistochemistry.

Immunohistochemical analysis of defined ALK-positive lymphoma cell lines (Karpas 299 and SUDHL-1) and a T-cell lymphoma cell line that is ALK-negative (MAC2A) showed similar results to that seen in the clinical specimens with co-expression of c-Myc and ALK-1 in the Karpas 299 and SUDHL-1 cells and expression of c-Myc only in the MAC2A cells (Figure 6).

Discussion

To investigate downstream signaling pathways, we created a regulatable form of ALK, designed to mimic the

Figure 4. c-Myc protein expression is induced by GyrB-ALK activation. To determine basal level of kinase activation in the absence of coumermycin, Rat1A fibroblasts were transiently transfected with 0 to 12 μg of the Myc-GyrB-ALK expression construct. Cells were then lysed and equivalent amounts of lysate were analyzed by immunoblotting with 9E10 anti-Myc mAb (α Myc) and RC-20 anti-phosphotyrosine (α pTyr) antibodies. With increasing expression, increasing autophosphorylation of Myc-GyrB-ALK was seen. While performing gene dosage studies, we observed an increase in a protein the approximate size of c-Myc on 9E10 immunoblots (open circle).

Figure 5. Morphology of cells expressing NPM-ALK. Rat1A fibroblasts were stably transduced with wild-type 6 Myc-NPM-ALK, or mutant 6 Myc-NPM-ALK. A: Rat1A cells transduced with wild-type 6 Myc-NPM-ALK showed morphological features of transformation. B: Rat1A cells transduced with mutant 6 Myc-NPM-ALK were not transformed.
Table 1. c-myc Transcript Number in Cell Lines Expressing Wild-Type and Mutant NPM-ALK

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<th>Cell Line</th>
<th>Mean c-myc copy number (normalized)</th>
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<tr>
<td>Rat1A cell line</td>
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<tr>
<td>expressing wild-type NPM-ALK</td>
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<tr>
<td>Rat1A cell line</td>
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<tr>
<td>expressing mutant NPM-ALK</td>
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<tr>
<td>Expression ratio: wild-type versus mutant NPM-ALK</td>
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and Hodgkin’s disease (7 of 12 cases). Although in that study ALK expression was not determined, the overall frequency of ALK expression in ALCLs is 83% for pediatric tumors and 31% for adult tumors. Therefore, it is likely some or all of those c-Myc-expressing T-cell lymphomas were in fact ALK+ ALCLs.

The functional consequence of c-Myc expression in ALK+ lymphoma is unclear. c-Myc is a transcription factor that drives expression of a wide range of genes associated with proliferation, including cyclin D1, telomerase, Cdc25A, and ornithine decarboxylase. Our results are consistent with previous studies, which suggest that increased c-Myc protein is not common to all lymphomas, but rather restricted to certain subtypes. Whereas c-Myc expression has been associated with a poor prognosis in adult diffuse large B-cell lymphoma, ALK+ lymphomas have a favorable prognosis. Presumably the favorable prognosis in ALK+ lymphomas arises from the ability of c-Myc to drive cell cycle progression and pro-apoptotic genes.
from the complete pattern of aberrant gene activation, rather than solely from c-Myc expression.

In summary, we have created a regulatable system to identify downstream pathways dysregulated in ALK+ lymphoma. Our analyses in ALK-expressing cell lines, and tumor samples revealed an increase in c-myc mRNA and protein. C-Myc immunoreactivity was specifically increased in ALK+ lymphomas. These findings suggest a specific role for c-Myc in ALK-mediated lymphomagenesis, and may provide a new immunohistochemical marker for this subset of ALCls.

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