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The leukemic oncoprotein NPM1-RARA inhibits TP53 activity

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Abstract

The variant acute promyelocytic leukemia (APL) translocation t(5;17)(q35;q21) fuses the N-terminus of nucleophosmin (NPM1) to the retinoic acid receptor alpha (RARA). We found that ectopic NPM1-RARA expression decreased TP53 protein levels in target cells. NPM1-RARA impaired TP53-dependent transcription. Cells expressing NPM1-RARA were more resistant to apoptotic stimuli. This work identifies the TP53 tumor suppressor as a novel target through which NPM1-RARA impacts leukemogenesis, and confirms the importance of impairment of TP53 in establishment of the APL phenotype.

Keywords

Acute Promyelocytic Leukemia; NPM1-RARA; TP53; apoptosis; nucleophosmin

INTRODUCTION

Acute promyelocytic leukemia (APL) is characterized by myeloid maturational arrest at the promyelocytic stage [1]. The majority of APL cases are characterized by t(15;17)(q24;q21), a balanced chromosomal translocation which fuses the genes encoding the tumor suppressor PML and the retinoic acid receptor alpha (RARA). The resulting PML-RARA fusion protein perturbs myeloid maturation through a number of mechanisms, serving as a dominant-negative for the transcriptional activation properties of RARA, as a dominant negative for PML (a regulator of senescence and apoptotic pathways), and potentially as a rogue transcriptional activator[1].

In an effort to understand the contribution of these different mechanisms towards development of APL, we have been studying variant translocations with similar phenotype, though different genotype from PML-RARA APL. Though rare, these “experiments of nature” serve as important tools with which to dissect the pathways underlying leukemogenesis[2]. To date nine variant translocations have been characterized, all of which share the same C-terminal domains of RARA as PML-RARA. The N-terminal sequences of

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POTENTIAL CONFLICTS OF INTEREST
None.

the fusions involve ZBTB16 (PLZF)[3] in t(11;17)(q23;q21); NPM1[4] in t(5;17)(q35;q21); NUMA1[5] in t(11;17)(q13;q21); STAT5B[6] in t(17;17)(q21;q21); PRKAR1A[7] in t(17;17)(q21;q24); BCOR[8] int(X;17)(p11;q21); FIP1L1[9] in t(4;17)(q12;q21); NABP1 (OBFC2A)[10] in der(2)t(2;17)(q32;q21); and TBL1XR1 (TBLR1)[11] in t(3;17)(q26;q21).

We have focused our studies on t(5;17)(q35;q21), which stands out as the second most common variant after t(11;17)(q23;q21) [2] and which shares a similar phenotype with t(15;17) [12], and responds similarly to the differentiating effects of all-trans retinoic acid [13]. The t(5;17) translocation fuses the same C-terminal sequences of RARA to the N-terminal 117 amino acids of nucleophosmin (NPM1) [4]. NPM1 is a nucleolar phosphoprotein, with diverse functionalities [14,15]. NPM1 (also known as B23, numatrin, and NO38) is a molecular chaperone originally identified through its role in ribosome biogenesis and nucleocytoplasmic transport. NPM1 has been found to bind to centrosomes, where it serves as a substrate for phosphorylation by CDK2/cyclin E, which dissociates NPM1 from centrosomes to initiate centrosomal duplication. NPM1 serves as a histone chaperonin, and may have activities in chromatin remodeling. NPM1 binds and stabilizes several cellular proteins, including retinoblastoma protein, TP53, MDM2, BAX, CDKN2A (ARF), YY1, and MYC (for review, see Colombo et al. [14]).

Like PML-RARA, ectopic expression of NPM1-RARA produces an APL-like phenotype in vitro and in vivo [16-18]. We have previously shown that NPM1-RARA is expressed primarily in the nucleus [19,20], and interacts with co-activator and co-repressor complexes in a ligand-dependent fashion [21]. It binds to DNA both as homodimers and heterodimers with RXR, and similar to PML-RARA, its activity as a transcriptional regulator varies by cell-type and target promoter [4,21]. We have recently demonstrated that NPM1-RAR binds to the tumor necrosis factor receptor type 1-associated DEATH domain protein (TRADD), to impact TNF-induced activation of the extrinsic apoptotic pathway [19,22].

In this study we test the effects of NPM1-RARA expression on TP53-mediated apoptosis. We find that NPM1-RARA downmodulates TP53 levels and transcriptional activity, to impact TP53 apoptotic function.

MATERIALS AND METHODS

Tissue culture

COS7 and U937 cell lines were obtained from the ATCC (Manassas, VA). COS7 were grown in DMEM (Mediatech, Herndon, VA) supplemented with 10% Fetal Bovine Serum (Invitrogen, Carlsbad, CA) and 2 mM L-Glutamine, and 100U/ml Penicillin and 100 micrograms/ml Streptomycin. U937 cells were grown in RPMI 1640 Mediatech, Herndon, VA), with the same supplements. COS7 cells were transfected with DNA by CaPO₄ co-precipitation, as previously described [21]. Generation and characterization of NPM1-RARA expressing U937 subclones have been described elsewhere [23].

Plasmids and Reagents

Cloning of NPM1-RARA cDNA and creation of the pLTN1-NPM1-RARA expression plasmid has been previously described [4]. The TP53 luciferase reporter plasmid containing

a 2.37 kb region of the p21 promoter that encodes the TP53 response element, as well as the TP53 expression plasmid encoding the open reading frame of TP53 in pCDNA, were gifts from R. Steinman (University of Pittsburgh). Antibodies against TP53 were purchased from Bethyl Laboratories (Montgomery, TX), and caspase 3 from Assay Designs (Ann Arbor, MI).

Luciferase Assays

Cells were lysed 24 hours after transfection, and luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. A β -galactosidase expression vector was used as transfection control.

Assay of apoptosis

Etoposide was purchased from Sigma-Aldrich (St. Louis, MO). Cleavage of procaspase-3 was determined by immunoblot, using an anti-caspase 3 antibody (Santa Cruz Biotechnology). To assess release of cytochrome c, cells were treated with digitonin as described [24], and the cytoplasmic and mitochondrial fractions separated by centrifugation, before immunoblotting with an antibody against cytochrome c (Santa Cruz Biotechnology).

RESULTS

NPM1-RARA expression decreases TP53 protein levels

Etoposide treatment of cells leads to a rapid increase in TP53 levels[25]. In order to determine whether constitutive expression of NPM1-RARA impacted TP53 response to chemotherapy, we treated U937 cells, or U937 clones stably expressing NPM1-RARA, with etoposide for 20 hours. TP53 expression was assessed by immunoblotting. We found a decrease in TP53 levels in the NPM1-RARA-expressing clones, compared with parental U937 cells, or a control clone transfected with vector alone (Figure 1). This indicates that NPM1-RARA blocks etoposide-induced increase in TP53 levels.

NPM1-RARA impairs TP53-dependent transcription

To test the effects of NPM1-RARA expression on TP53 transcriptional activity, we co-transfected COS7 cells with a TP53-dependent-promoter reporter construct along with expression plasmids encoding NPM1, TP53, and either NPM1-RARA, or carrier DNA. We found that expression of NPM1-RARA decreased TP53-dependent luciferase activity (Figure 2), indicating that NPM1-RARA suppresses TP53 activity at the transcriptional level

NPM1-RARA impedes etoposide-induced cell death

TP53 activity is necessary for etoposide-mediated apoptosis[25]. To determine the functional consequences of NPM1-RARA's destabilizing effects on TP53, we treated U937 clones expressing NPM1-RARA with 20 μ M etoposide. We assessed apoptosis by two assays: activation of caspases as reflected by cleavage of procaspase 3, and depolarization of mitochondria as reflected by release of cytochrome c from the mitochondria. Figure 3A indicates that etoposide did not induce degradation of procaspase-3 in the NPM1-RARA expressing clones, compared to the U937 parental line or the control clone. Similarly,

NPM1-RARA expressing clones exhibited less cytochrome c release from mitochondria after challenge with etoposide (Figure 3B). These results indicate resistance of the NPM1-RARA expressing clones to TP53-dependent etoposide-induced apoptosis.

DISCUSSION

NPM1-RAR inhibits TP53 levels, TP53-dependent transcription and TP53-mediated apoptosis. Our results indicate that NPM1-RARA impedes chemotherapy-induced activation of apoptosis.

Impairment of TP53 activity has also been shown for PML-RARA, which modulates TP53 through binding and recruitment of histone deacetylase to alter acetylation, and thereby the stability, of TP53[26]. Though wild type NPM1 has been shown to interact directly with TP53, it does so via the NPM1 C-terminal domains [27,28] that are not present in the NPM1-RARA fusion protein. Direct interaction between NPM1-RARA and TP53 is thus precluded: indeed, we did not identify TP53 in a proteomic screen for NPM1-RARA interacting proteins (data not shown).

Our results are consistent with several potential mechanisms. First, NPM1-RARA could bind to NPM1 in a dominant-negative fashion to prevent NPM1 binding and stabilization of TP53. Second, NPM1-RARA could impede NPM1 binding to MDM2[29], leading to increased activity of the MDM2 TP53 ubiquitin protein ligase, resulting in enhanced degradation of TP53. Further, NPM1 has been shown to interact with and stabilize CDK2NA (P14ARF) within the nucleolus[29]; though not yet studied, one could speculate that NPM1-RARA could directly or indirectly impact CDK2NA to alter TP53 stability.

We propose that the mechanism by which NPM1-RARA effects TP53 activity differs from the mechanism through which PML-RARA alters TP53 function, in which formation of a PMLRARA/TP53 complex modulates acetylation of TP53[26]. Though we[21] and others[30] have demonstrated NPM1-RARA interaction with a histone deacetylase containing co-repressor complex analogous to PML-RARA, we did not observe formation of a tripartite NPM1-RARA/NPM1/TP53 complex (data not shown).

It is important to note that the same regions of N-terminal NPM1 that are expressed in the NPM1-RARA fusion are also expressed in NPM1-ALK, a product of the t(2;5)(p23;q35) translocation in anaplastic lymphoma[31], the t(3;5)(q25.1;q34) MLF1-NPM1 fusion seen in myelodysplastic syndrome and acute myeloid leukemia (AML), as well as the NPM1_c mutations seen in normal-cytogenetic AML[32]. It is intriguing to speculate that the defective NPM1 in these malignancies could also alter TP53 activity.

It should be noted that the decrease in TP53 transcriptional activity (Figure 2), is likely to be more pronounced in primary t(5;17) APL cells, in which NPM1-RARA is expressed from the strong NPM1 promoter, resulting in equimolar levels to NPM1[4].

TP53 is commonly mutated in AML, but rarely in APL. PML-RAR inhibits TP53, and it has recently been shown that restoration of normal TP53 activity is required for ablation of APL leukemia initiating cells[33] Though it is likely that the mechanism by which PML-RARA

and NPM1-RARA impede TP53 activity differ, the fact that both negatively impact TP53 activity supports the notion that TP53 is functionally, if not structurally, impaired in APL.

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Figure 1. NPM1-RARA suppresses TP53 levels

Parental U937 cells, a vector-transfected control clone, or three NPM1-RARA-expressing U937 clones, were exposed to 20 μ molar etoposide for 20 hours. Immunoblots were probed with antibodies against TP53 and the C-terminus of RARA.

Figure 2. NPM1-RARA impedes transcription of a TP53 reporter

COS7 cells were transiently transfected with a TP53 luciferase reporter construct NPM1 and TP53 expression plasmids, along with either pBluescript carrier DNA as control, or NPM1-RARA. Luciferase activity was assayed after 24 hours. Shown are the mean and standard error of three replicates.

Figure 3A. NPM1-RARA suppresses etoposide-induced cleavage of procaspase 3

Parental U937 cells, a vector-transfected control clone, or two NPM1-RARA-expressing U937 clones, were exposed to 20 μ molar etoposide for 20 hours. Immunoblots were probed with anti-caspase antibody, capable of detecting uncleaved procaspase 3 and the cleaved activated product.

Figure 3B. NPM1-RARA suppresses etoposide-induced release of cytochrome c

Parental U937 cells, a vector-transfected control clone, or two NPM1-RARA-expressing U937 clones, were exposed to 40 μ molar etoposide for 3 hours. Immunoblots of the cytoplasmic and mitochondrial fractions were probed with antibodies against cytochrome c or RARA or COX IV (as mitochondrial loading control) and actin (as cytoplasmic loading control).