



Translocations and mutations involving the nucleophosmin (NPM1) gene in lymphomas and leukemias

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ABSTRACT

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Nucleophosmin (NPM) is a ubiquitously expressed nucleolar phosphoprotein which shuttles continuously between the nucleus and cytoplasm. Many findings have revealed a complex scenario of NPM functions and interactions, pointing to proliferative and growth-suppressive roles of this molecule. The gene NPM1 that encodes for nucleophosmin (NPM1) is translocated or mutated in various lymphomas and leukemias, forming fusion proteins (NPM-ALK, NPM-RAR α , NPM-MLF1) or NPM mutant products. Here, we review the structure and functions of NPM, as well as the biological, clinical and pathological features of human hematologic malignancies with NPM1 gene alterations. NPM-ALK identifies a new category of T/Null lymphomas with distinctive molecular and clinico-pathological features, that is going to be included as a novel disease entity (ALK+ anaplastic large cell lymphoma) in the new WHO classification of lymphoid neoplasms. NPM1 mutations occur specifically in about 30% of adult de novo AML and cause aberrant cytoplasmic expression of NPM (hence the term NPMc+ AML). NPMc+ AML associates with normal karyotype, and shows wide morphological spectrum, multilineage involvement, a unique gene expression signature, a high frequency of FLT3-internal tandem duplications, and distinctive clinical and prognostic features. The availability of specific antibodies and molecular techniques for the detection of NPM1 gene alterations has an enormous impact in the biological study diagnosis, prognostic stratification, and monitoring of minimal residual disease of various lymphomas and leukemias. The discovery of NPM1 gene alterations also represents the rationale basis for development of molecular targeted drugs.

Key words: nucleophosmin, NPM mutations, lymphomas, ALK, acute myeloid leukemia, normal karyotype, ARF, antibodies.

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Nucleophosmin (NPM), also known as B23,¹ NO38, and numatrin, is an abundant, highly conserved, ubiquitously expressed nucleolar phosphoprotein which belongs to the nucleoplamin/nucleophosmin family of nuclear chaperones.^{2,3} Many findings have revealed a complex scenario of NPM functions and interactions, pointing to proliferative and growth-suppressive roles of this molecule.⁴ Nucleophosmin is an essential protein, since inactivation of the gene encoding for nucleophosmin (*NPM1*) in the mouse germ line leads to developmental defects that cause embryonic death in mid-gestation.⁵ In humans, accumulating evidence suggests that NPM is directly implicated in the pathogenesis of cancer. NPM is over-expressed in solid tumors of diverse histological origin⁶ or is involved in tumor progression.⁷⁻⁹ In several hematologic malignancies, the *NPM1* locus is lost¹⁰ or translocated leading to the formation of oncogenic fusion proteins.¹¹ Moreover, we recently discovered that *NPM1* is mutated in about one-third of adult patients with acute myeloid leukemia (AML),¹² which makes *NPM1* mutations the most frequent genetic lesions so far identified in *de novo* AML. Here we review the structure and biological functions of the NPM molecule and discuss the role that NPM alterations play in the pathogenesis and clinicopathological behavior of human lymphomas and leukemias, focusing on AML carrying cytoplasmic/mutated NPM (NPMc⁺ AML).^{12,13}

The *NPM1* gene and the structure of the encoded protein

The *NPM1* gene contains 12 exons¹⁴ and in humans maps to chromosome 5q35. The NPM protein exists in two alternatively spliced:¹⁴ B23.1, the prevalent isoform in all tissues,¹⁵ contains 294 amino acids,¹⁶ whereas B23.2, a truncated protein, lacks the last 35 C-terminal amino acids of B23.1 and is expressed at very low levels. The NPM molecule contains distinct domains¹⁷ that account for its multiple biochemical functions (Figure 1A). The N-terminal hydrophobic portion of NPM contains the regions that are responsible for the self-oligomerization and chaperone activities of the molecule¹⁸ (Figure 1A). Under native conditions, both in resting and proliferating cells, over 95% of cellular NPM protein exists as an oligomer.¹⁹ The oligomerization domain of NPM also contains a highly conserved motif that appears critical for mediating ADP-ribosylation factor (ARF) binding *in vivo*.²⁰ Through its N-terminal hydrophobic domain, NPM also exerts a chaperone activity, preventing protein aggregation in the nucleolus, favoring histone and nucleosome assembly,²¹⁻²³ and increasing acetylation-dependent transcriptional activity.²⁴ Recently, NPM has also been found to act as a chaperone for Bax; that is, it induces the conformational changes of Bax that precede this molecule's translocation into mitochondria,²⁵ a key event in the control of the apoptotic pathway. The chaperone activity of NPM also depends upon the first of the two acidic domains¹⁷ (stretches of aspartic and glutamic acids) that are located in the middle

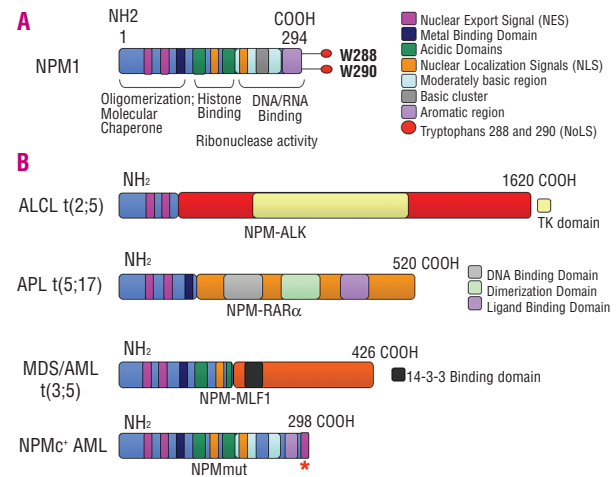


Figure 1. A. Structure and functional domains of wild-type *NPM1*. Starting from the N-terminus, the protein displays two nuclear export signal (NES) motifs (residues 42-49 and 94-102), a metal binding domain, two acidic regions (residues 120-132 and 160-188), a bipartite nuclear localization signal (NLS) motif (residues 152-157 and 190-197), a basic cluster inside a moderately basic region, and an aromatic region at the C-terminus unique to NPM isoform 1 containing the nucleolar localization signal (NLS) with tryptophan residues 288 and 290. **B. Structure and functional domains of *NPM* altered proteins in hematologic malignancies.** The NPM/ALK fusion protein is constituted by the amino-terminal portion of NPM (amino acids 1-116) and the cytoplasmic tail of ALK protein which contains the tyrosine kinase (TK) domain. The NPM/RAR α fusion protein is constituted by the amino-terminal portion of NPM (amino acids 1-147) and the DNA binding, dimerization and ligand binding domain of RAR α protein. A variant fusion protein of 563 amino acids has been described. The NPM/MLF1 fusion protein is constituted by the amino-terminal portion of NPM (amino acids 1-175) and almost the entire MLF1 protein, excluding only the first 16 amino acids at the N-terminus. The 14-3-3 binding domain of MLF1 is indicated. Mutated NPM protein in AML: (NPMmut) mutations at the NPM C-terminus introduce a new NES motif and disrupt tryptophan residues 288 and 290 (or 290 only) at the nucleolar binding domain (asterix). See, for comparison, Figure 1A.

portion of the protein.¹⁶ In fact, the negatively charged acidic cluster on the outside of the NPM oligomer may serve as a binding site for ribosomal basic proteins, possibly minimizing non-specific interactions between ribosomal proteins and rRNA, during ribosome assembly in the nucleoli.¹⁹ The first acidic segment also contains a major protein kinase casein kinase II (CK2) site (Ser 125). Phosphorylation by the abundant CK2 in the nucleolus is involved in NPM chaperoning²⁶ since it reduces the affinity of NPM for substrate, promoting dissociation. Occurring during interphase, phosphorylation may help to regulate the function of NPM in the nucleolus.^{26,27} The segment between the two acidic stretches and part of the C-terminus of NPM accounts for the ribonuclease activity of the protein. The C-terminus also contains a short aromatic stretch with two tryptophans at positions 288 and 290, which are crucial for NPM binding to the nucleolus²⁸ (the so-called nucleolar localization signal). NPM binding to the nucleolus and other subnuclear compartments^{27,29} is also conditioned by several potential cyclin-dependent kinase 1 (CDK1) phosphorylation sites in the C-terminal

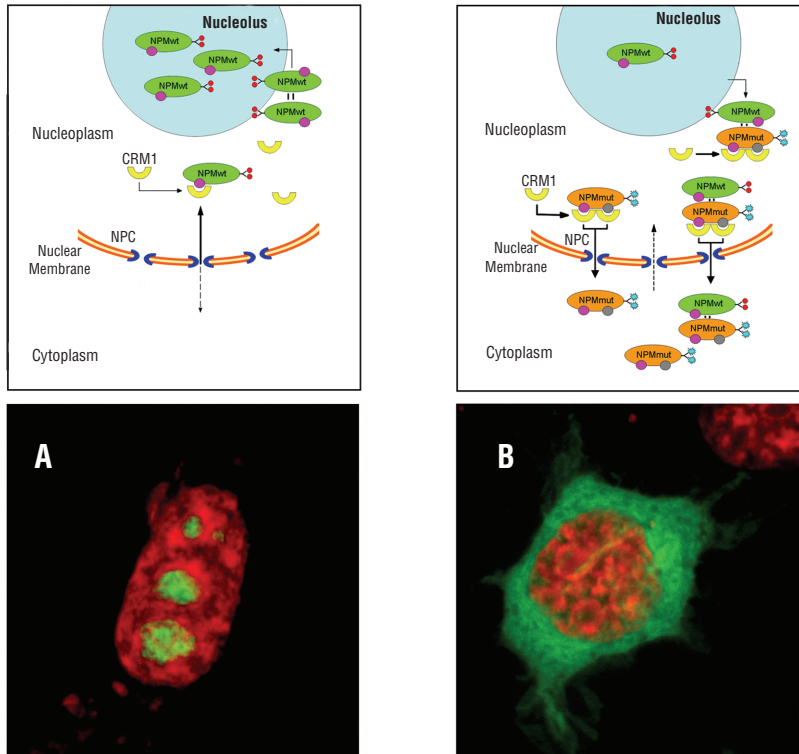


Figure 2. Mechanism of altered nucleocytoplasmic traffic of wild-type and mutant NPM. **A.** Export from the nucleus to the cytoplasm of wild-type NPM is minimal since the wild-type protein contains only weak physiological NES motifs (purple circles). Moreover, the two tryptophans at the C-terminus (red circles) drive wild-type NPM to the nucleolus, where the bulk of protein accumulates. Immunofluorescence analysis (lower panel) clearly shows nucleolar localization of NPM in NIH3T3 mouse fibroblasts transfected with eGFP/wild-type NPM. **B.** NPM mutants accumulate in the cytoplasm (immunofluorescence image in lower panel) since the mutation creates an additional NES motif at the C-terminus (gray circles) which is available for Crm1-mediated nuclear export. Moreover, mutations of the two tryptophans (cyan stars) impair nucleolar localization. Dimerization between mutated and wild-type NPM partially delocalize the latter into cytoplasm. NPC is the Nuclear Pore Complex. The image in the lower panel is a confocal micrograph of NIH3T3 cells transfected with eGFP-NPM mutant A. Nuclei are counterstained red with propidium iodide. Cells were analysed with a Zeiss LSM 510 confocal microscope and reconstructed with Imaris software (Bitplane, Zurich, CH, Switzerland).

region. Phosphorylation induces NPM to shuttle between the nucleolus and nucleoplasm (a common feature of nucleolar components involved in ribosome biogenesis), and appears to reduce the affinity of NPM for other nucleolar components.^{27,30} Finally, the bipartite nuclear localization signal (NLS)¹⁷ and nuclear export signal (NES) motifs^{31,32} of NPM play major roles in regulating the nucleo-cytoplasmic traffic of the protein (*see below*).

NPM expression

NPM is more highly expressed in proliferating cells than in quiescent ones³³ and increases in response to mitogenic stimuli.³⁴ NPM over-expression promotes survival and recovery of hematopoietic stem cells under conditions of stress³⁵ and decreases the sensitivity of human HL-60 leukemic cells to retinoic acid-induced differentiation and apoptosis.³⁶ Conversely, NPM levels drop after retinoic acid-induced differentiation of HL-60 leukemic cells³⁷ and TPA-induced megakaryocytic differentiation of K562 cells.³⁸ Loss of NPM, or inhibition of NPM shuttling, blocks protein translation and arrests the cell-cycle.³² NPM control of cell growth and proliferation is probably the result of several activities which include modulation of ribosome biogenesis as well as interactions with histones, p53 and ARF oncosuppressor proteins (*see below*). The close association of NPM over-expression with increased proliferation concurs with the finding that NPM is a transcriptional target of the *Myc* oncogene.³⁹

The NPM isoforms have different patterns of subcellular distribution: B23.1 is located in the granular component of the nucleolus,¹⁵ which contains maturing pre-ribo-

somal particles;⁴⁰ retention is probably due to its nucleic acid-binding domain interacting with other nucleolar components, possibly RNA.²³ The rare B23.2 isoform mostly localizes in the nucleoplasm.^{30,41}

Although most NPM resides in the nucleolus,^{42,43} this molecule shuttles from the nucleus to cytoplasm.^{44,45} The NLS signal drives NPM from the cytoplasm to the nucleoplasm, where it is translocated to the nucleolus through its nucleolar binding domain, particularly tryptophans 288 and 290.²⁸ NPM remains in nucleoli, even though it contains highly conserved hydrophobic leucine-rich NES motifs within residues 94-102³¹ and 42-49,³² which drive it out of the nucleus. Therefore, in physiological conditions, nuclear import of wild-type NPM predominates over export, thus explaining why most of the wild-type NPM is found in the nucleolus (Figure 2A).

Although the levels of the NPM pools in the nucleoplasm and the cytoplasm are very low, they are crucial for proper execution of the diverse cellular activities of NPM, such as centrosome duplication and ribosome biogenesis.^{31,32} The size of these NPM pools closely depends upon regulation of NPM traffic across the different subcellular compartments. The exchange of NPM between the nucleolus and the nucleoplasm is strictly regulated by the capacity of this molecule to bind more or less efficiently to nucleolar components, which seems in turn to depend upon several variables: the oligomerization state of the molecule; the interaction of NPM with ARF or other nucleolar proteins; the phosphorylation state of CDK1 sites (mutants carrying changes of all four CDK1 sites delocalize in the nucleoplasm);²⁷ and critical tryptophans

at position 288 and 290. Interactions between NPM and other proteins may be involved in nucleoli assembly and disassembly.⁴⁶ Finally, regulation of NPM traffic between the nucleus and cytoplasm mostly occurs through the NLS and NES motifs.^{31,32}

Alterations in the regulation of NPM cellular traffic may contribute to tumorigenesis. Indeed, an abnormal subcellular distribution of fusion and wild-type NPM proteins (*see below*) is a general property of lymphomas and leukemias carrying *NPM1* gene alterations.

The functions of NPM

NPM is a multifunctional protein. Here is a brief description of the main functions of NPM that have been observed to date. NPM is a key player in ribosome biogenesis, supporting cell growth and proliferation. It mediates 5S rRNA nuclear export, interacting with ribosomal protein L5.³² NPM intervenes in processing and/or assembly of ribosomes through its nucleo-cytoplasmic shuttling properties and intrinsic RNase activity,⁴⁷ and its ability to bind nucleic acids,⁴⁸ to process pre-RNA molecules,⁴⁹ and to act as a chaperone,²¹ impeding protein aggregation in the nucleolus during ribosome assembly.⁵⁰ Recently, Pelletier *et al.*⁵¹ demonstrated a mechanistic link between TSC1/mTOR signaling, nucleophosmin-mediated nuclear export of ribosome subunits, protein synthesis levels, and cell growth.

NPM maintains genomic stability^{5,52} by controlling DNA repair mechanisms^{53,54} and centrosome duplication⁵⁵ during mitosis. In resting cells, NPM associates with the unduplicated centrosome and after CDK2-cyclinE-mediated phosphorylation on threonine 95, it dissociates from it,^{31,56} thereby enabling proper chromosome duplication. Following phosphorylation on serine 4 by PLK1 and NEK2A,⁵⁷ NPM re-associates with centrosomes at the mitotic spindle during mitosis.⁵⁸ A recent study points to ROCK II kinase (an effector of Rho small GTPase) being the effector of the CDK2/cyclinE-NPM pathway in the regulation of centrosome duplication.⁵⁹ As NPM inactivation leads to unrestricted centrosome duplication and genomic instability,⁶⁰ NPM appears to protect from centrosome hyper-amplification and from the consequent heightened risk of cellular transformation. This view is supported by the observation that *NPM1*^{-/-} mice develop a myelodysplastic-like syndrome with signs of dyserythropoiesis which is caused by unrestricted centrosome duplication.⁵

NPM is involved in the apoptotic response to stress and oncogenic stimuli,⁶¹ and can modulate the activity and stability of the oncosuppressor protein p53.^{62,63} Notably, nucleolar integrity, NPM and p53 stability are functionally linked.^{62,64,65} Cellular stress-inducing stimuli disrupt nucleolar integrity and re-locate NPM from the nucleolus to the nucleoplasm, leading to p53 activation,^{64,66} and growth arrest. Moreover, nucleoplasmic NPM reinforces p53 stability by binding to, and inhibiting, Mdm2,⁶⁵ a p53 E3-ubiquitin ligase,^{67,68} even though *nucleolar stress* may

activate p53 independently of Mdm2.⁶⁹

NPM stabilizes the oncosuppressor ARF and determines its subcellular localization,⁷⁰ thus contributing to modulating growth-suppressive pathways.⁴ NPM co-localizes with ARF in the nucleolus,⁷¹ forming high molecular weight complexes.⁷² NPM protects ARF from degradation,^{52,73} stabilizing and maintaining a basal ARF level in the nucleolus, probably because ARF assumes a stable structure only when bound to NPM,⁷⁰ thus escaping the rapid proteasome-mediated destruction which many misfolded proteins are subjected to.⁷⁴ Increased ARF levels after oncogenic stress affect NPM polyubiquitination,⁷⁵ promoting NPM degradation and interfering with the NPM nucleo-cytoplasmic shuttling which regulates several functions of NPM. Subnuclear compartmentalization of ARF is important for its regulation.⁷⁶ In response to cellular stresses, NPM and ARF are displaced to the nucleoplasm where competitive binding between Mdm2 and NPM for ARF leads to formation of NPM-Mdm2 and ARF-Mdm2 binary complexes where Mdm2 sequestration strongly activates the p53 pathway.⁷⁷ Thus, the ARF-NPM interaction site is bi-compartmental. In the nucleolus, ARF directly accesses the rRNA processing machinery through its association with NPM, thus preventing export of processed rRNA and inhibiting cell growth.⁷⁸ In the nucleoplasm ARF may regulate the p53 cell cycle pathway through multiple interactions with NPM, Mdm2 and other proteins such as ARF-BP1,⁷⁹ leading to a block of cell-cycle progression. It has been proposed that ARF acts as a *parasitic* peptide on the NPM molecule, since in exploiting this chaperone for its own survival it antagonizes normal NPM activities.⁸⁰ However, the biological consequences of the NPM-ARF interaction still remain poorly defined. NPM seems to fall into a newly discovered category of genes that function both as oncogenes and oncosuppressors.⁴ When NPM expression is aberrantly increased, NPM may function as an oncogene by promoting aberrant cell growth through enhancement of ribosome machinery⁸¹ and, possibly, by inhibiting programmed cell death.⁴ Paradoxically, when NPM expression is reduced and its subcellular distribution altered (as in tumors carrying alterations of the *NPM1* gene), NPM behaves as an oncosuppressor, possibly facilitating tumorigenesis through destabilization and functional impairment of the ARF-tumor suppressor pathway and increased genomic instability.⁴

Lymphomas and leukemias carrying *NPM1* gene translocations

The *NPM1* gene is translocated in CD30⁺ anaplastic large-cell lymphoma and in rare variants of AML¹¹ (Figure 1B). Cells from these tumors contain an oncogenic fusion protein (NPM-ALK, NPM-RAR α or NPM-MLF1) (Figure 1B) and a reduced level of wild-type NPM due to the loss of one functional allele of the *NPM1* gene (hemizygosity).

ALCL expressing NPM-ALK

Anaplastic large cell lymphoma (ALCL) is characterized by a proliferation of tumor cells, usually with anaplastic morphology, which express the CD30 molecule, tend to grow cohesively and invade lymph node sinuses.^{82,83} Due to *ALK* gene translocations, about 60% of ALCL express the anaplastic lymphoma kinase (ALK) protein, hence the term ALK⁺ ALCL.¹¹ About 85% of ALK⁺ ALCL carry the t(2;5)(p23;q35) chromosome translocation,^{11,83} in which the *ALK* gene on chromosome 2 is fused with the *NPM1* gene on chromosome 5.⁸⁴ The chimeric gene encodes for a fusion protein comprising the amino-terminal portion of NPM (containing the oligomerization domain) and the entire cytoplasmic region of ALK (containing the tyrosine kinase domain)⁸⁵ (Figure 1B). The other 15% of ALK⁺ ALCL are molecularly heterogeneous, since the *ALK* gene fuses with many different partners.^{82,83} ALK⁺ ALCL exhibits distinctive molecular, pathological and clinical features⁸³ and, unlike other peripheral T-cell lymphomas, has a good prognosis.⁸⁶ Tumor cells of ALCL with t(2;5) contain the NPM-ALK fusion protein and the wild-type NPM protein. They characteristically express the ALK protein in the cytoplasm and ectopically in the nucleus because, through the NPM oligomerization domain, NPM-ALK forms heterodimers with wild-type NPM, which in turn, via shuttling, import NPM-ALK into nucleoli^{11,87} (Figure 3, top and middle). Due to the presence of the NPM-ALK fusion protein,¹¹ ALCL cells also show aberrant NPM cytoplasmic expression. This can be detected by an antibody against the N-terminus of NPM which is retained in the fusion protein⁸⁷ (Figure 3, bottom). In contrast, wild-type NPM, which is recognized by an antibody against the NPM C-terminus (not retained in NPM-ALK), maintains its nucleolar localization (*not shown*).^{11,87} Through the oligomerization domain of NPM, NPM-ALK can also form homodimers (Figure 3, top panel) that lead to constitutive activation of the ALK tyrosine kinase domain, a major trigger of lymphomagenesis.⁸⁵ This appears a general mechanism that also underlies the formation of homodimers between ALK fusion proteins other than NPM-ALK.⁸⁵ Thus, the NPM moiety of NPM-ALK would appear to play no other role in transformation beyond serving as an oligomerization domain for the chimera.⁸⁸ Since wild-type NPM in ALCL with t(2;5) retains its nucleolar localization,^{11,87} its functions may not be perturbed by the NPM-ALK fusion product. Whether the loss of one functional *NPM1* allele contributes to tumorigenesis⁴ remains an open question.

AML carrying the NPM-RAR α protein

This extremely rare genetic alteration has been so far reported in three children with acute promyelocytic leukemia (APL) carrying the t(5;17) translocation.⁸⁹⁻⁹¹ The t(5;17) causes the *NPM1* gene to fuse with the retinoic acid receptor- α gene (*RAR α*) (Figure 1B). Morphologically these cases look like the typical APL cases carrying the t(15;17)/PML-RAR α rearrangement.⁹¹

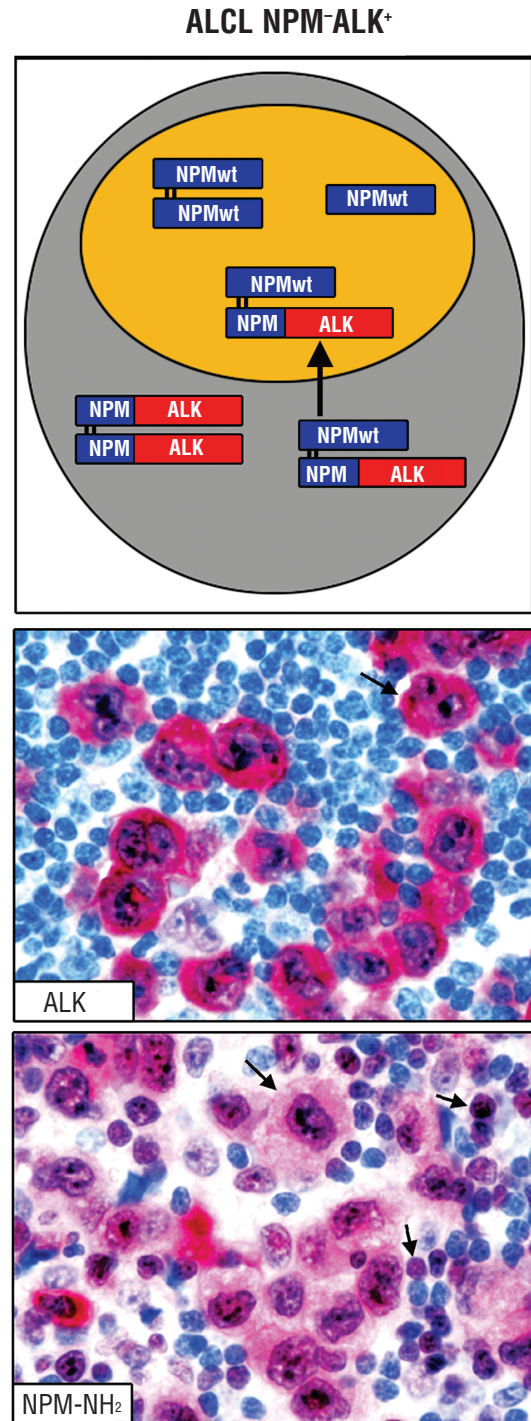


Figure 3. Localization of wild-type NPM and NPM-ALK fusion protein in ALCL with t(2;5). (Top) Schematic representation of wild-type NPM and NPM-ALK fusion proteins in ALCL with t(2,5). Cytoplasmic NPM positivity is due to the presence in the cytoplasm of NPM-ALK. Wild-type NPM can form heterodimers with NPM-ALK causing its dislocation into the nucleus (arrow). Wild-type NPM is nucleus-restricted. (Middle) Immunostaining for ALK protein. ALK is expressed both in the cytoplasm and nucleoli of tumor cells (arrow). Normal residual lymphoid cells are ALK-negative. Alkaline phosphatase anti-alkaline phosphatase (APAAP) technique; x 800. (Bottom) Immunostaining for NPM N-terminus (retained in the fusion protein). NPM shows the expected nuclear localization in addition to aberrant expression in the cytoplasm (long arrow). Normal residual lymphoid cells display nucleus-restricted NPM positivity (short arrows) (APAAP technique; x 800).

Leukemic cells express the NPM-RAR α fusion protein and its reciprocal, as well as wild-type NPM and RAR α from the uninvolved alleles.⁹² In two cases investigated by immunocytochemistry,^{90,91} NPM showed diffuse nuclear positivity which was very unlike the typical wild-type NPM nucleolar staining. Furthermore, after a long latency, NPM-RAR α transgenic mice in which the fusion gene is expressed under the control of a human cathepsin G (*hCG*) minigene, develop monoblastic leukemia with the NPM/RAR α oncoprotein localizing in leukemic cell nucleoli.⁹³ These findings suggest that NPM-RAR α physically interacts with wild-type NPM, either by delocalizing it from the nucleolus or by altering its function in the nucleolus. The NPM-RAR α fusion protein does not appear to interact with PML, nor alter its localization.⁹⁴

Like PML-RAR α , the NPM-RAR α fusion protein modulates retinoid-responsive gene expression,⁹⁵ perturbing the retinoid acid signal pathway and arresting myeloid differentiation at the promyelocyte stage. APL carrying NPM-RAR α shows a good response to differentiation therapy with all-transretinoic acid (ATRA).⁹¹

AML carrying the NPM-MLF1 fusion protein

The rare chromosomal translocation t(3;5)(q25;q35) occurs in <1% of cases of AML and generates a chimeric gene named *NPM-MLF1* (myelodysplasia/myeloid leukemia factor 1).⁹⁶ In the encoded NPM-MLF1 fusion protein, the N-terminal portion of NPM is fused to almost the entire MLF1 protein, excluding only the first 16 amino acids at the N-terminus⁹⁶ (Figure 1B). AML with t(3;5) embraces all French-American-British (FAB) categories but is most frequently M6.⁹⁷

Immunohistochemical hallmarks of leukemic cells carrying NPM-MLF1 are aberrant nuclear MLF1 expression, with a mechanism similar to the t(2;5), and aberrant cytoplasmic NPM positivity.⁹⁸ Cytoplasmic NPM positivity is due to the NPM-MLF1 fusion protein in the cytoplasm and to wild-type NPM which is dislocated, through an unclear mechanism, from the nucleus to the cytoplasm.⁹⁸ As these aberrant staining patterns are frequently observed in myeloid and erythroid cell precursors, at least one myeloid-committed cell appears to be involved.⁹⁸ Interestingly, *HLS7* (the murine gene counterpart to human *MLF1*) influences erythroid/myeloid lineage switching and development of normal hematopoietic cells.⁹⁹ The mechanism underlying the NPM-MLF1 fusion protein induction of malignant transformation remains unknown. Since MLF1 is not usually expressed in normal hematopoietic tissues, NPM-MLF1 may promote ectopic MLF1 expression in hematopoietic cells, thus contributing to myelodysplasia/AML.¹⁰⁰ Interestingly, MLF1 prevents erythro-leukemic cells from undergoing biological and morphological maturation in response to erythropoietin,¹⁰¹ possibly in part through an interaction with the recently identified MLF1IP protein.¹⁰² This finding concurs with the frequent association of t(3;5) AML with the FAB M6 subtype.⁹⁷

Table 1. AML with t(3;5) vs NPMc⁺ AML: common and distinctive features.

Features	AML with t(3;5) ⁹³	NPMc ⁺ AML ¹²
Frequency	< 1% of AML	About 30% of adult AML
Age	Younger	Older
Origin	May be associated with MDS ^o	Usually <i>de novo</i>
Karyotype	t(3;5)(q25;q35)	Normal (85% cases)*
Genetic lesion	<i>NPM/MLF1</i> chimeric gene ^c	<i>NPM</i> exon-12 mutations ^{^^}
MLF1-labeling	Nuclear (aberrant) + cytoplasmic**	None
NPM expression	Nuclear+cytoplasmic	Nuclear+cytoplasmic
Morphology (FAB)	Wide spectrum (mostly M6)	Wide spectrum (mostly M4-M5)
Multilineage involvement	Yes	Yes
CD34 expression	Absent	Absent

^oMDS: myelodysplastic syndrome; ^{*}About 15% of cases show minor cytogenetic abnormalities; ^cNo mutations in the coding region of the *NPM* allele not involved in the translocation. ^{**}May sometimes be only nuclear.

The contribution of NPM to NPM-MLF1-induced leukemogenesis remains to be determined. The interaction between the NPM moiety of the fusion protein and wild-type NPM causes formation of heterodimers (wild-type NPM/NPM-MLF1)^{96,98} and transport of the chimeric protein into the nucleus, which may result in functional alterations.¹⁰³ Reduced levels of wild-type NPM (due to the loss of one *NPM1* functional allele) and its cytoplasmic dislocation may also contribute to leukemogenesis. In fact, NPM^{+/-} heterozygous mice develop a hematologic disorder with features resembling those of human myelodysplastic syndromes.⁴ Despite sharing some features with NPMc⁺ AML (see below), AML patients carrying t(3;5) are younger at presentation, may have suffered from previous myelodysplasia, show no *NPM1* mutations and have a poorer prognosis (Table 1).

AML carrying cytoplasmic/mutated nucleophosmin (NPMc⁺ AML)

In early 2005, we reported that *NPM1* mutations closely associate with AML carrying a normal karyotype¹² (AML-NK). Molecular alterations in AML-NK have always been difficult to study because, although this leukemia subgroup accounts for 40-50% of adult AML,¹⁰⁴⁻¹⁰⁶ it lacks chromosomal rearrangements that direct researchers towards cloning and characterization of oncogenic fusion genes, such as those encoded by the translocations t(15;17), t(8;21) or Inv(16).¹⁰⁷ Attempts to stratify AML-NK using gene microarrays^{108,109} succeeded in associating gene expression patterns with differences in response to treatment, but no specific genetic subgroups emerged. In the difficult setting of AML-NK, our discovery of *NPM1* mutations is yet another example of how simple observations at the microscope¹¹⁰ sometimes provide the key. The clue to the identification of *NPM1* mutations in AML-NK came from our immunohistochemical studies on ALCL with t(2;5).¹¹ Having observed that NPM, instead of being restricted to nucleoli as in normal tissues,¹¹¹ is aberrantly expressed in the cytoplasm⁸⁷ (due to the presence of the

NPM-ALK protein) (Figure 3, bottom) we reasoned that immunohistochemical detection of cytoplasmic NPM in routine biopsy samples might serve as a quick and sensitive method to screen tumors for the presence of *NPM1* gene alterations. Consequently, we extended our immunohistochemical studies to a wide range of human neoplasms, including leukemias, and found the first cases of AML with cytoplasmic NPM expression (NPMc⁺ AML) but no known NPM fusion proteins. Subsequent analysis of a large cohort of AML patients established that cytoplasmic NPM was associated with normal karyotype and other distinctive biological and clinical features.¹² These findings led us to hypothesize that yet unidentified genetic lesion underlies NPMc⁺ AML. As the only distinguishing feature was NPM cytoplasmic dislocation, the *NPM1* gene emerged as the most likely candidate and indeed, gene sequencing revealed mutations at exon 12.¹²

General characteristics of *NPM1* mutations

NPM1 mutations appear to be specific for *de novo* AML.^{12,13} Although mutational studies of *NPM1* in tumors other than AML are scarce, immunohistochemical analysis of NPM in thousands of hematopoietic and extra-hematopoietic neoplasms has consistently revealed nucleus-restricted NPM expression,¹² which indicates no *NPM1* mutations are present.¹¹² Rare reports of *NPM1* mutations in chronic myelomonocytic leukemia^{113,114} are questionable, since most such cases developed AML within 1 year and probably represented early stage M4 or M5 AML with marked monocytic differentiation. Finding *NPM1* mutations in 2/38 (5.2%) myelodysplastic patients¹¹⁵ does not concur with our immunohistochemical studies in 50 patients with various myelodysplastic syndromes who showed nucleus-restricted NPM expression, indicating an unmutated *NPM1* gene (Falini, unpublished results). Thus, whether *NPM1* mutations occur occasionally in myelodysplastic syndromes remains to be clarified in larger series of patients. Although *NPM1* mutations are found in about one-third of adult AML patients, they were detected in only 1/79 human myeloid leukemic cell lines.¹¹⁶ This cell line, called OCI-AML3 was originally generated at the Ontario Cancer Institute, Ontario, Canada,¹¹⁷ from a 57-year old male patient with AML-M4 and an unknown karyotype at diagnosis. The OCI-AML3 cell line exhibits the distinctive features of NPMc⁺ AML, since it carries *NPM1* mutation A and expresses cytoplasmic NPM.¹¹⁶

NPM1 mutations are consistently heterozygous and a wild-type allele is retained. With very few exceptions, i.e. involvement of exon 11¹¹⁸ and exon 9,¹¹⁹ *NPM1* mutations are restricted to exon 12.¹³ About 40 molecular variants of *NPM1* mutations have been described to date in AML patients,¹³ with >95% occurring at nucleotide position 960. The most common mutation (so-called mutation A¹²) duplicates a TCTG tetranucleotide at positions 956 to 959 of the reference sequence (GenBank accession number NM_002520), and accounts for 75%-80% of adult NPMc⁺

AML cases. Mutations B and D are observed in about 10% and 5% of cases, respectively; other mutations are very rare. Independently of their type, all mutation variants generate common alterations at the C-terminus of the NPM leukemic mutants which are responsible for their dislocation into cytoplasm (*see below*). *NPM1* mutations tend to be stable.^{120,121} Loss of mutations at relapse is extremely rare and sometimes associated with a change from a normal to an abnormal karyotype.^{120,121}

Cytoplasmic/mutated NPM closely associates with AML-NK, and it is mutually exclusive with major recurrent genetic abnormalities.^{120,122-127} About 86% of patients with NPMc⁺ AML have a normal karyotype: the other 14% show chromosomal abnormalities that are likely to be secondary.^{12,13} The interaction of *NPM1* mutations with other genetic alterations in AML-NK is interesting. *FLT3*-ITD targets NPMc⁺ AML twice as often as AML-NK carrying nuclear/unmutated NPM.^{12,120,124-127} *NPM1* mutations are likely primary events, preceding the acquisition of *FLT3*-ITD¹²⁷ or other mutations. In contrast, partial tandem duplications within the *MLL* gene (*MLL*-PTD) and cytoplasmic/mutated NPM are usually mutually exclusive.^{12,124,127} In two large studies, *CEBPA*, *KIT* and *NRAS* mutations did not appear to differ between *NPM1*-mutated and *NPM1*-unmutated AML-NK.^{124,125} Mutations of *p53* are rare in NPMc⁺ AML (about 3% of cases),¹²⁰ concurring with their being mainly found in AML with karyotype abnormalities.^{128,129}

Properties of *NPM* leukemic mutant proteins

One of the most distinctive features of NPM mutants is their aberrant localization in the cytoplasm of leukemic cells¹² (Figure 2B). This is causally related to two alterations at the leukemic mutant C-terminus: (i) generation of an additional leucine-rich NES motif;¹³⁰ and (ii) loss of tryptophan residues 288 and 290 (or residue 290 alone) which are crucial for NPM nucleolar localization.²⁸ We recently demonstrated that both alterations are needed to ensure NPM cytoplasmic dislocation¹³¹ (Figure 2B). Observing that rare NPM mutants, despite retaining tryptophan 288, still delocalize in cytoplasm, led us to discover a close correlation between the type of NES motif and C-terminal mutated tryptophans.¹³¹ Mutations of both tryptophans are always found with the very common NES motif, **L-xxx-V-xx-V-x-L**; retention of tryptophan 288 is always associated with rare NES variants in which valine at the second position is replaced by leucine, phenylalanine, cysteine or methionine.¹³¹ One explanation might be that, in order to counterbalance tryptophan 288, which drives mutants to the nucleolus,²⁸ the rare variant NES motifs need to be more efficient than the **L-xxx-V-xx-V-x-L** sequence in binding Crm1 and dislocating NPM to the cytoplasm.

All NPM leukemic mutants interfere with wild-type NPM and oncosuppressor ARF functions by binding and recruiting them into the nucleoplasm and cytoplasm.¹³¹⁻¹³³

Diagnosis of NPMc⁺ AML

NPMc⁺ AML is easily diagnosed by molecular biology techniques¹³⁴⁻¹³⁷ or immunohistochemistry, since detection of aberrant cytoplasmic NPM expression is predictive of *NPM1* mutations.¹¹² Immunohistochemistry is the first choice technique for diagnosing NPMc⁺ AML in the case of a dry-tap or biopsy from extramedullary sites,¹³⁸ and for the analysis of hematopoietic lineage involvement.¹³⁹ Immunohistochemistry must be applied on paraffin sections since fresh material (smears or cytopspins) is not suitable for reliably detecting cytoplasmic dislocation of NPM. Molecular analysis is the first choice assay for monitoring minimal residual disease.¹⁴⁰

Three different types of antibodies are available for the immunohistochemical detection of NPM in tissue sections. Monoclonal antibodies directed against fixative-resistant epitopes of NPM^{12,111,131} do not distinguish between mutated and wild-type NPM and stain NPMc⁺ AML cells in the nucleus (mostly containing wild-type NPM) and in the cytoplasm (Figure 4A) which contains the NPM mutant protein and wild-type NPM (recruited by the mutant, as shown in Figure 2B). Despite lack of specificity for NPM mutants, these antibodies are the most widely used, reliable reagents for the immunohistochemical diagnosis of NPMc⁺ AML.¹¹² Polyclonal antibodies recognizing NPM mutants but not wild-type NPM^{116,131,139} stain only the cytoplasm of NPMc⁺ AML cells, indicating that mutants are mostly cytoplasmic (Figure 4, middle). Due to their high specificity for NPM mutants, these antibodies are useful when studying lineage involvement in NPMc⁺ AML,¹³⁹ but because of antigenic denaturation, are suitable for diagnostic purposes in only 50-60% of samples.¹³⁹ Future efforts should be devoted to generating antibodies against NPM mutants that can be more widely applied. Finally, a monoclonal antibody recognizing wild-type NPM but not NPM mutants^{131,139} stains NPMc⁺ AML cells in the nucleus and cytoplasm (*not shown*), providing evidence that wild-type NPM is dislocated into the cytoplasm. This antibody is of little diagnostic relevance. The best control for assessing specificity of NPM cytoplasmic positivity is immunostaining with an antibody against C23/nucleolin, another abundant shuttling nucleolar protein,⁴⁴ which in NPMc⁺ AML must have nucleus-restricted localization¹² (Figure 4C).

Since cytoplasmic NPM is fully predictive of *NPM1* mutations,¹¹² immunohistochemistry could be used as a first-line screening to rationalize cytogenetic and molecular studies in AML. A potential approach is shown in Figure 5.

Pathological, phenotypic and genotypic features of NPMc⁺ AML

Although associated with a wide morphological spectrum, NPMc⁺ AML is mostly of M4 and M5 categories.^{12,124,125} Interestingly, up to 90% of AML-M5b carry cytoplasmic/mutated NPM;^{12,124} gene expression profiling of AML⁴¹ also revealed a cluster dominated by monocytic

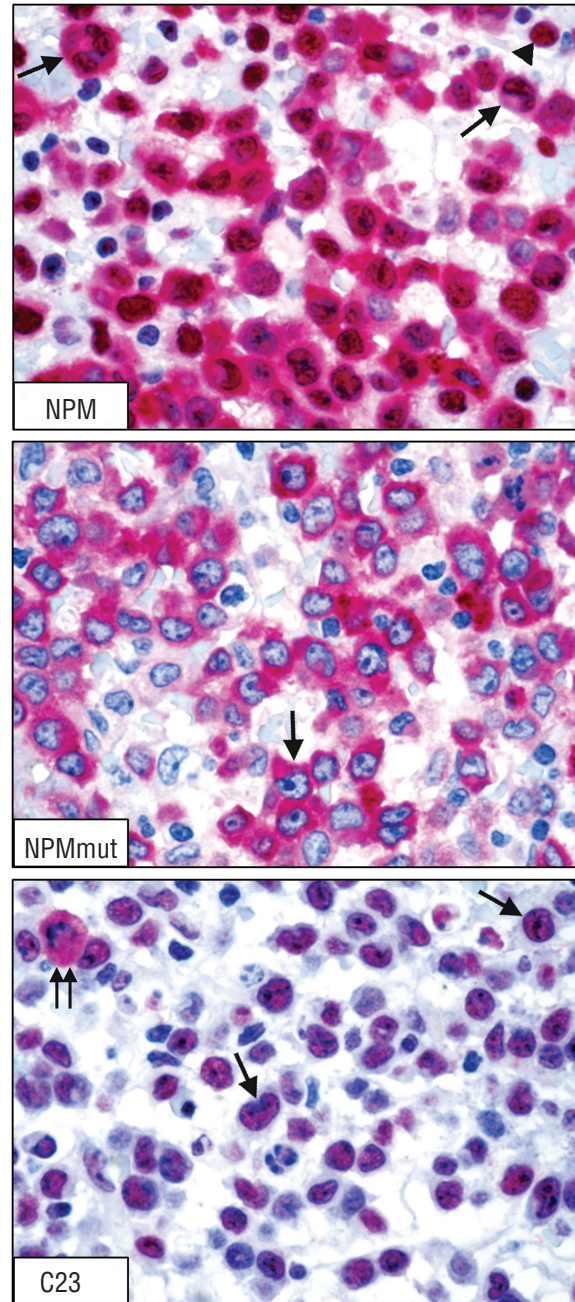


Figure 4. Aberrant cytoplasmic expression of NPM in NPMc⁺ AML. (Top) Nuclear plus cytoplasmic expression of NPM in leukemic blasts (arrows). Normal residual hematopoietic cells show nucleus-restricted NPM (arrowhead). Bone marrow biopsy paraffin section stained with monoclonal antibody 376 that recognizes wild-type and mutated NPM (APAAP technique; hematoxylin counterstaining; $\times 1,000$). (Middle) Cytoplasmic-restricted expression of mutant NPM protein in NPMc⁺ AML (arrow). Bone marrow biopsy paraffin section stained with a polyclonal antibody (Sil-A) that recognizes mutated but not wild-type NPM (APAAP technique; hematoxylin counterstaining; $\times 1,000$). (Bottom) Nucleus-restricted expression of C23/nucleolin in NPMc⁺ AML (arrows). Double arrows indicated the expected NPM cytoplasmic positivity in a mitotic figure. Bone marrow biopsy paraffin section stained with a specific anti-C23 monoclonal antibody (APAAP technique; hematoxylin counterstaining; $\times 1,000$).

leukemias and a high frequency of *NPM1* mutations. A high frequency of *NPM1* mutations has been reported in

AML with prominent nuclear invaginations (*cup-like* nuclei).¹⁴² NPMc⁺ AML shows frequent multilineage involvement.¹³⁹ At immunohistochemistry, over 95% of NPMc⁺ AML cases are negative for CD34,¹² as was subsequently confirmed by gene expression profiling¹⁴³ which also showed *CD133/PROM1* gene down-regulation. Another striking feature of the NPMc⁺ AML gene-expression signature is activation of numerous members of the homeodomain-containing family of transcription factors, including *HOX* and *TALE* genes,^{126,143} some of which are oncogenes implicated in hematopoietic development and leukemogenesis.^{144,145} Interestingly, NPMc⁺ AML cases with a normal karyotype and those carrying minor chromosomal abnormalities show the same molecular signature,¹⁴³ providing evidence that NPMc⁺ AML is a distinct subgroup of leukemia regardless of karyotype, and that the minor chromosomal abnormalities which occasionally accompany *NPM1* mutations are secondary events (*see above*).

Clinical features of NPMc⁺ AML and response to therapy

The incidence of *NPM1* mutations increases with age,^{12,120,124,127} accounting for 2.1%-6.5% of childhood AML,^{121,122,146} (9%-26.9% of pediatric AML-NK)^{121,122,146} but 25%-35%^{120,121,123-127} of adult AML (45.7%-63.8% of adult AML-NK).^{120,121,123-127} These findings suggest that the molecular pathogenesis of AML-NK may be different in adults and children. Notably, there is also evidence for a variability of *NPM1* mutation types according to patient age, with uncommon non-typical (i.e. non-type A) mutations being most prevalent in children and younger adults.¹⁴⁷ Three large clinical studies^{124,125,127} found that *NPM1* mutations are significantly more frequent in females.

Cases of NPMc⁺ AML-NK are associated with higher blast counts and more extramedullary involvement (mainly gingival hyperplasia and lymphadenopathy) than cases of AML-NK without *NPM1* mutations.^{125,127} Platelet counts are significantly higher in NPMc⁺ than in NPMc⁻ AML-NK.^{125,127} Concurring with this clinical observation is the enhanced ability of K562 cells for megakaryocytic differentiation after transfection with a C-terminal NPM mutant.³⁸ Most investigators have found that cytoplasmic/mutated NPM predicts a good response to induction therapy.^{12,120,124,127} In contrast, Döhner *et al.*¹²⁵ observed the best complete remission rate in the *NPM1*-mutated/*FLT3*-ITD negative group, whilst AML patients carrying both mutations showed poor responses.

NPM1 mutations without concomitant *FLT3*-ITD identify a subgroup of AML-NK patients with a favorable prognosis^{124-127,148} and has been associated with an approximately 60% probability of survival at 5 years in younger patients.¹²⁵ In a donor versus no-donor comparison, the good prognostic group of *NPM1*-mutated/*FLT3* ITD-negative patients did not benefit from allogeneic stem cell transplantation,¹²⁵ suggesting that such treatment should not be recommended for NPMc⁺ AML in first complete remission. These results underline the value of molecular

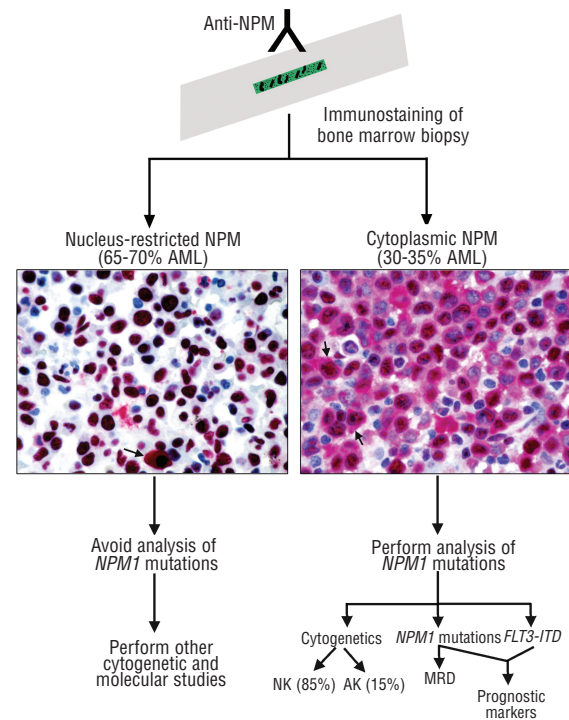


Figure 5. NPM-immunostaining-based approach to molecular and cytogenetic analysis of AML. AML patients can be subdivided by the results of immunohistochemistry on paraffin sections into two subgroups, i.e. those expressing nucleus-restricted NPM (NPMc⁻) (left) or cytoplasmic NPM (NPMc⁺) (right). The arrow in the NPMc⁻ sample indicates the expected cytoplasmic positivity of a mitotic figure. The arrows in the NPMc⁺ sample indicate leukemic cells with aberrant cytoplasmic expression of NPM. NPM mutational analysis can be avoided in NPMc⁻ AML but should be performed in NPMc⁺ cases, since molecular information about *NPM1* status in NPMc⁺ AML can serve as a prognostic factor (in association with *FLT3*-ITD) and is critical for monitoring minimal residual disease. NK: normal karyotype; AK: abnormal karyotype (other than recurrent cytogenetic abnormalities); MRD: minimal residual disease.

genetic screening in improving risk stratification of patients with AML-NK.¹⁴⁹ Screening is facilitated by recently developed assays that simultaneously detect *NPM1*, *FLT3*-ITD and *CEBPA* mutations,^{150,151} all of which have a prognostic impact in AML-NK.^{124-127,152}

Unsolved issues

Several questions remain unanswered on the role of NPM in the development of hematopoietic malignancies and on the clinical significance of *NPM1* gene alterations. Although the ALK, RAR α and MLL1 moieties are believed to dominate in promoting tumorigenesis in lymphomas and leukemias carrying NPM fusion proteins, there is evidence that NPM moieties are not only innocent bystanders. This view is supported by the finding that leukemias developing in transgenic mice expressing either PML/RAR α , PLZF/RAR α , or NPM/RAR α show different phenotypes.⁹³ Since NPM behaves as an oncosuppressor,⁴ a reduction in wild-type NPM may also contribute to tumorigenesis, due to ineffective ARF stabilization with consequent inactivation of the ARF growth-inhibitory

function. However, dose reduction of NPM at the nucleolar level may differ among tumor types, e.g. lower levels in AML carrying t(3;5) in which one allele is altered and the wild-type NPM is dislocated into the cytoplasm⁸⁸ than in ALCL with t(2;5), in which one allele is altered but the location of wild-type NPM is apparently unaffected by the translocation.⁸⁷

The molecular mechanism underlying the development of NPMc⁺ AML is unclear. The ability of NPM leukemic mutants to recruit the oncosuppressor p19^{ARF} in the cytoplasm and interfere with its stability and activities^{132,133} may contribute to the development of AML. However, p19^{ARF} is only one potential target of NPM mutants. In NPMc⁺ AML, the perturbed cellular traffic of the wild-type and mutant NPM proteins could influence the nuclear/nucleolar import and functions of other, as yet unidentified, critical molecules.¹⁵³⁻¹⁵⁵ The close associations of FLT3-ITD with NPM1 mutations in clinical practice^{12,124-127} suggests that these genetic lesions may co-operate to promote leukemic transformation. There is also experimental evidence that the NPM leukemic mutants (but not wild-type NPM) co-operate with adenovirus E1A to transform primary MEF in soft agar.¹⁵⁶ NPMc⁺/E1A⁺ MEF cells also show high efficiency at forming foci in low-density seeding assays, comparable to that of RasV12⁺/E1A⁺ MEF cells.¹⁵⁶

Interestingly, the gene expression profile of NPMc⁺ AML is characterized by up-regulation of genes involved in stem cell maintenance (such as HOX genes).^{126,143} NPMc⁺ AML also shows Notch1-ligand JAG1 up-regulation and CDKN2C/p18-INK4C repression,¹⁴³ both of which are associated with hematopoietic stem cell expansion.^{157,158} Accordingly, NPM leukemic mutants may confer self-renewal properties on hematopoietic targets.

Studies on NPM abnormalities in leukemias may lead to updating of the World Health Organization (WHO) classification of AML.¹⁵⁹ The term primary AML not otherwise characterized,¹⁶⁰ which is defined according to slightly mod-

ified FAB criteria, encompasses about 60% of AML cases, including all AML-NK and, therefore, NPMc⁺ AML. We propose that, because of its distinctive genetic, biological and clinical features, NPMc⁺ AML and NPMc⁺ myeloid sarcoma¹⁶¹ should be included as a separate AML entity in the upcoming version of WHO classification.

Multicenter studies are warranted to assess the favorable prognostic value of NPM1 mutations (in the absence of FLT3-ITD) in various clinical settings and to provide answers to a series of questions. Does the prognostic value of mutated NPM1 also apply to the poor prognostic category of AML patients over 60 years old? Should NPM1-mutated/FLT3-ITD negative AML-NK patients be exempted from allogeneic stem cell transplantation as first-line therapy?^{162,163} Is the NPM1 mutation a better molecular marker than FLT3-ITD¹⁶⁴ or Wilms' tumor gene (WT1)¹⁶⁵ for monitoring minimal residual disease in AML-NK? Will quantification of NPM1-mutated copies¹⁴⁰ predict early relapse and patients with long-term survival? Recent retrospective studies indicate that this may be the case,¹⁶⁶ but large prospective clinical trials are required to confirm this exciting finding. Finally, better understanding of the mechanism underlying leukemogenesis in NPMc⁺ AML may eventually lead to the development of more specific anti-leukemic therapies.

Authors' Contributions

BF had the original idea which, inspired by his immunohistochemical studies, led first to the identification of ALK⁺ anaplastic large cell lymphomas as a distinct clinico-pathologic entity, and subsequently to the discovery of AML carrying cytoplasmic mutated NPM (NPMc⁺ AML); IN and NB clarified the molecular mechanism of altered NPM transport in NPMc⁺ AML; MPM, AL performed the biochemical analysis of NPMc⁺ AML; CM, PG: involved in the study of NPM1 mutations; FM, MFM: carried out the clinical analysis of NPMc⁺ AML patients.

Conflict of Interest

BF and CM applied for a patent on clinical use of NPM mutants. All other authors declare no conflict of interest.

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