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Acute myeloid leukemia with mutated nucleophosmin (*NPM1*): is it a distinct entity?

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After the discovery of *NPM1*-mutated acute myeloid leukemia (AML) in 2005 and its subsequent inclusion as a provisional entity in the 2008 World Health Organization classification of myeloid neoplasms, several controversial issues remained to be clarified. It was unclear whether the *NPM1* mutation was a primary genetic lesion and whether additional chromosomal aberrations and multilineage dysplasia had any impact on the biologic and prognostic features of *NPM1*-mutated AML. Moreover, it was uncertain how to classify AML patients who were double-mutated for *NPM1* and *CEBPA*. Recent studies have shown that: (1) the *NPM1* mutant perturbs hemopoiesis in experimental models; (2) leukemic stem cells from *NPM1*-mutated AML patients carry the mutation; and (3) the *NPM1* mutation is usually mutually exclusive of biallelic *CEPBA* mutations. Moreover, the biologic and clinical features of *NPM1*-mutated AML do not seem to be significantly influenced by concomitant chromosomal aberrations or multilineage dysplasia. Altogether, these pieces of evidence point to *NPM1*-mutated AML as a founder genetic event that defines a distinct leukemia entity accounting for approximately one-third of all AML. (*Blood*. 2011;117(4):1109-1120)

Introduction

The remarkable molecular heterogeneity of acute myeloid leukemia (AML)[1] has made a genetic-based classification essential for accurate diagnosis, prognostic stratification, monitoring minimal residual disease, and developing targeted therapies. The category of "AML with recurrent genetic abnormalities," which includes the genetically best defined myeloid neoplasms, underwent major changes in the 2008 World Health Organization (WHO) classification.[2] The 4 molecularly distinct entities that had been described in the 2001 WHO classification were expanded to include AML with t(6;9), AML with inv(3) or t(3;3), and AML (megakaryoblastic) with; t(1;22) and 2 provisional entities: AML with mutated *CEBPA* and AML with mutated nucleophosmin (*NPM1*) (Table 1). The latter accounts for approximately one-third of all AMLs[3] and has distinct genetic, pathologic, immunophenotypic, and clinical characteristics.[4,5] The WHO synonym for AML with mutated *NPM1*, NPMc$^+$ AML (c$^+$ indicates "cytoplasmic positive"),[3] focuses on its most distinguishing functional feature, that is, aberrant expression of nucleophosmin in the cytoplasm of leukemic cells.[6] This unique immunohistochemical pattern, which led in 2005 to the discovery of *NPM1* mutations in AML,[3] is an excellent surrogate marker for molecular studies because it is fully predictive of *NPM1* mutations.[7,8]

The present review is an update of the distinct genetic and clinical features of AML with mutated *NPM1*.

AML with mutated *NPM1* shows distinct genetic features

Several pieces of evidence suggest the *NPM1* mutation is a founder genetic alteration (Table 2) in AML.

With the exception of rare cases of myelodysplastic syndrome (MDS)/myeloproliferative neoplasms[9] that require further confirmation, the *NPM1* mutation or its immunohistochemical surrogate (cytoplasmic nucleophosmin) appears to be restricted to AML[3,10] and is usually expressed in the whole leukemic population. It has a recurrence rate of approximately 30% in AML and is mutually exclusive of other AML recurrent genetic abnormalities.[3,11] As expected for a founder genetic lesion, the *NPM1* mutation is stable over the course of disease.[12,13] Notably, it has been detected in AML at relapse, even many years after the initial diagnosis,[14] in patients experiencing more than one relapse and in relapses occurring in extramedullary sites.[15] Although loss of *NPM1* mutation has been sporadically observed in *NPM1*-mutated AML,[16] no extensive investigations were performed to exclude secondary, clonally unrelated, AML.[17] Because many groups currently use *NPM1* mutation as a tool to evaluate minimal residual disease, further data on the stability of *NPM1* mutations should be soon available. Finally, when AML with mutated *NPM1* carries a concomitant *FLT3*-ITD (~ 40% of cases),[3] the *NPM1* mutation appears to precede *FLT3*-ITD.[18,19]

As expected for a founder genetic lesion, the *NPM1* mutation defines a subgroup of AML with a distinct gene expression profile (including down-regulation of *CD34* and up-regulation of *HOX* genes)[20-22] and microRNA signature[22-24] (including up-regulation of *miR-10a* and *miR-10b*). Sequencing of the whole genome from 2 cases of AML with normal karyotype (AML-NK) at 91%[25] and 98% resolution,[26] respectively, did not reveal any recurrent lesion, other than the *NPM1* mutation, which showed features of a primary genetic hit. Indeed, in one case,[25] the *NPM1* and *FLT3* genes were involved, whereas the other patient[26] harbored a mutated *NPM1* gene and concomitant *NRAS* and *IDH1* gene mutations. Mutations

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Table 1. WHO classifications of "AML with recurrent genetic abnormalities"

WHO 2001	WHO 2008
AML with t(8;21)(q22;q22), (*AML1/ETO*)	AML with t(8;21)(q22;q22*); RUNX1-RUNX1T1*
AML with inv(16)(p13q22) or t(16;16)(p13;q22), (*CBF*β/*MYH11*)	AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBF*β/*MYH11*
Acute promyelocytic leukemia AML with t(15;17)(q22;q12), (*PML/RAR*α) and variants	Acute promyelocytic leukemia AML with t(15;17)(q22;q12); *PML/RAR*α*
AML with 11q23 (*MLL*) abnormalities	AML with t(9;11)(p22;q23); *MLLT3-MLL*†
	AML with t(6;9)(p23;q34); *DEK-NUP214*
	AML with inv(3)(q21;q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*
	AML (megakaryoblastic) with t(1;22)(p13;q13); *RBM15-MKL1*
	AML with mutated *NPM1* (provisional entity)‡
	AML with mutated *CEBPA* (provisional entity)‡

*The rare variant translocations of *RAR*α with partner genes other than *PML* are recognized separately because they may exhibit atypical APL features, including resistance to all-*trans*-retinoic acid therapy.

†Compared with the 2001 WHO scheme, the category of AML with *MLL* gene abnormalities of 2008 WHO classification only includes AML with *MLLT3-MLL*. Rearrangements of *MLLT3-MLL* should be specified in the diagnosis. Partial tandem duplication of *MLL* should not be placed in this category.

‡Defined as "provisional" to indicate that more study is needed to characterize and establish them as unique entities.

of *FLT3* and *NRAS* in AML are widely recognized as secondary genetic events, which are associated with tumor progression. The impact of *IDH1* mutation[26,27] on the molecular pathogenesis of AML remains to be elucidated. Interestingly, one *NPM1*-mutated/*IDH1*-mutated AML patient was recently reported to have lost *IDH1* mutation at relapse while retaining the *NPM1* mutation, suggesting that at least in this case *IDH1* mutation was probably a secondary event.[28] Studies of additional genomes from AML patients with normal karyotype are warranted to clarify the pathogenetic role of *NPM1* mutation and its relationship with other mutations.

Table 2. Distinctive features of AML with mutated *NPM1* (NPMc$^+$ AML)

Genetic features
NPM1 mutation* is specific for AML, mostly "de novo"
Usually all leukemic cells carry the *NPM1* mutation
Mutually exclusive with other "AML with recurrent genetic abnormalities"
NPM1 mutation is stable (consistently retained at relapse)
NPM1 mutation usually precedes other associated mutations (eg, *FLT3*-ITD)
Unique GEP signature (↓ *CD34* gene; ↑ *HOX* genes)
Distinct microRNA profile
Clinical, pathologic, immunophenotypic, and cytogenetic features
Common in adult AML (~ 30% of cases), less frequent in children (6.5%-8.4%)†
Higher incidence in female‡
Close association with normal karyotype (~ 85% of cases)
~ 15% of cases carry chromosome aberrations, especially +8, del9(q), +4
Wide morphologic spectrum (more often M4 and M5)
Frequent multilineage involvement
Negativity for CD34 (90%-95% of cases)§
Good response to induction therapy
Relatively good prognosis (in the absence of *FLT3*-ITD)

GEP indicates gene expression profiling.

*Or its immunohistologic surrogate (cytoplasmic NPM, NPMc$^+$).

†Lower incidence in Chinese children.

‡In most, but not all, studies.

§Less than 10% CD34$^+$ cells.

Overall, the features of *NPM1*-mutated AML appear to overlap with those of well-recognized primary AML genetic lesions, such as the *AML1-ETO* fusion gene (Table 3). Similar characteristics are also shown by AML carrying double *CEBPA* mutations, but not by AML-NK associated with other mutations (Table 3), because the latter are probably secondary genetic events. As an example, *FLT3*-ITD and *FLT3*-TKD are less stable than *NPM1* mutation, being lost at relapse in approximately 9% and 50% of cases, respectively.[29,30] Instability has been also reported for *NRAS*[31] and *WT1*[32] mutations. Consequently, if recurrence and the other distinctive features shown in Tables 2 and 3 are to be considered the main criteria for judging the relevance of an individual genetic alteration for pathogenesis, the *NPM1* mutation appears the most probable candidate as the primary, driving genetic lesion in approximately 60% of AML-NK. This view is further supported by recent evidence showing the NPM1 mutant perturbs hemopoiesis in experimental models and is expressed in the leukemic stem cells from patients with *NPM1*-mutated AML (discussed in the next 2 sections).

Besides the primary genetic event, secondary cooperating mutations are thought to play a major role in leukemogenesis.[33] Recurrent genetic lesions that probably cooperate with the *NPM1* mutation include chromosomal aberrations (in ~ 15% of cases)[3] and mutations, such as those affecting the *FLT3*-ITD, *FLT3*-D835, *NRAS*, *IDH1*, and *TET2* genes (in ~ 60% of cases). Hypothetical steps of leukemic transformation in *NPM1*-mutated AML are shown in Figure 1.

How does mutated NPM1 protein promote leukemia?

The *NPM1* gene encodes for a protein that, although nucleolar at steady state,[6] shuttles between nucleus and cytoplasm.[34] Acting as a molecular chaperone to establish multiple protein-protein interactions, NPM1 is involved in critical cell functions,[35] such as control of ribosome formation and export, stabilization of the oncosuppressor p14^{Arf} protein in the nucleolus, and regulation of centrosome duplication. Although the *NPM1* gene was strongly implicated in cancer pathogenesis,[35] how the NPM1 mutant protein promotes leukemia remains elusive. Because the *NPM1* mutation always results in aberrant cytoplasmic dislocation of the mutant protein,[36,37] this event appears critical for leukemogenesis.[6,38] Increased nucleophosmin export into cytoplasm probably perturbs multiple cellular pathways by "loss of function" (NPM1 nucleolar interactors are delocalized by the mutant into leukemic cell cytoplasm) and/or "gain of function" (the hypershuttling NPM1 mutant works in a deregulated fashion). Moreover, the NPM1 mutant could have neomorphic features (eg, capability to interact with new protein partners in the cytoplasm).[4,6]

NPM1 mutant-mediated cytoplasmic delocalization of nuclear proteins[6] was implicated in knocking-down the oncosuppressor Arf[39,40] and activating the c-MYC oncogene.[41] In addition, the function of wild-type nucleophosmin in *NPM1*-mutated AML cells is profoundly affected by its reduction at the nucleolar physiologic site. Reduction of wild-type NPM1 in the nucleolus is the result of both heterozygosity and dislocation into cytoplasm through forming heterodimers with NPM1 mutant.[6] In the *Npm* knockout mouse, *Npm* inactivation led to genomic instability which, in turn, promoted in vitro and in vivo cancer susceptibility. *Npm* heterozygous cells were more susceptible to oncogenic transformation and

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Table 3. Features of mutations most frequently associated with AML carrying a normal karyotype (AML-NK) compared with a primary genetic lesion [t(8;21)]

Feature	Primary genetic event in AML* [eg, t(8;21)]	*NPM1*	*CEBPA*	*FLT3* ITD	*FLT3* TKD	*NRAS*	*WT1*	*MLL*-PTD	*IDH1*
Recurrence	Yes	50%-60%	5%-10%	30%	10%-15%	10%-12%	7%-10%	5%-10%	~ 15%
Distinct GEP	Yes	Yes	Yes‡	No	No	NA	Yes	No	No
Distinct microRNA profile	Yes	Yes	Yes	Yes	NA	NA	NA	NA	No
Specificity for AML	Yes	Yes	Yes	Yes§	Yes§	No	No	Yes	No
Mutually exclusive†	Yes	Yes	Yes	No	No	No	No	Yes	Yes‖
Timing of the event	Early	Early	Early	Usually late¶	Usually late¶	Usually late	NA	Early	NA
% mutated cells within the leukemic population	All	All	All	It may occur in a subclone	It may occur in a subclone	It may occur in a subclone	NA	All	All
Loss at relapse	No	No	No	Possible	Possible	Possible	Possible	No	Rarely[28]

GEP indicates gene expression profiling; and NA, not available data.
*Refers to typical features of an "AML with recurrent genetic abnormality" (WHO 2008) that is used for comparison.
†With other recurrent genetic abnormalities.
‡Refers to biallelic *CEBPA*-mutated cases.
§Rarely occurs in ALL.
‖Occasionally seen in AML carrying recurrent cytogenetic abnormalities and complex karyotype.
¶In *NPM1*/*FLT3*-ITD double-mutated cases, *NPM1* mutation appears to precede *FLT3*-ITD.

Npm$^{+/-}$ mice developed spontaneous tumors, especially myeloid malignancies,[42] indicating how NPM1 acts as haploinsufficient tumor suppressor in vivo.

The NPM1 mutant may also exert its transforming properties through gain of function in cytoplasm. Interestingly, the NPM1 mutant bound and inhibited caspase 6 and 8 signaling in leukemic

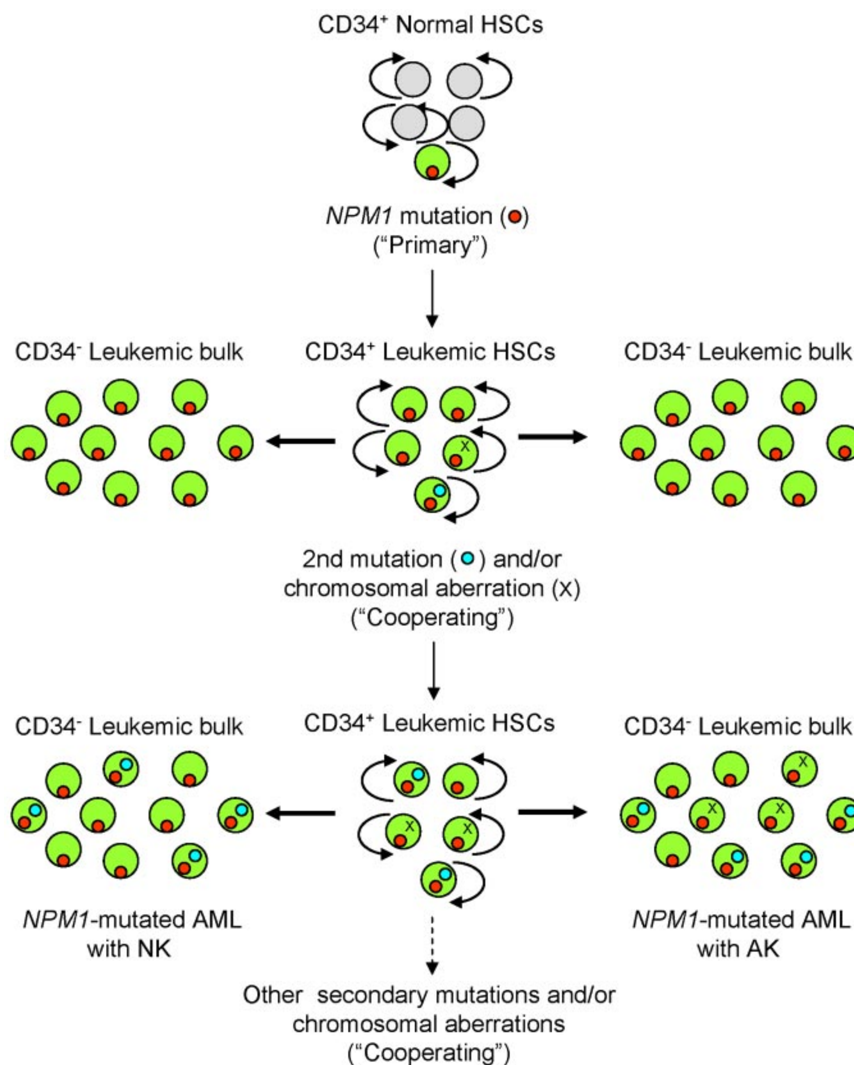

Figure 1. Hypothetical steps of genetic evolution in *NPM1*-mutated AML. In this scheme, the CD34^{+} hematopoietic stem cell (HSC) compartment (whether normal or leukemic) is shown in the central column, whereas its more differentiated CD34-negative progeny is shown in the right and left columns. The primary, driving *NPM1* mutation (red dot) in an HSC causes transformation that leads to the "leukemic phenotype." Other mutations (light blue dots), such as *FLT3*-ITD, occur later in clonal evolution. Leukemic cells in approximately 15% of *NPM1*-mutated AML can also acquire a chromosomal abnormality (X), whereas in 85% of cases they maintain a normal karyotype. Both later mutations and chromosomal abnormalities are usually expressed in a leukemic cell subclone whose size may vary from one patient to another. For simplicity, occurrence of the second mutation and a chromosomal abnormality in the same cells is not shown. According to the 2-hit hypothesis, only 2 mutations are indicated, but additional mutations may be involved. Light gray circles represent normal HSC and multipotent progenitors; and green circles indicate the CD34^{+} normal hemopoietic progenitor compartment where primary *NPM1* mutation (red dot) and secondary mutations (blue dot) and/or chromosomal aberrations (X) occur, giving rise to the CD34^{-} leukemic bulk population.

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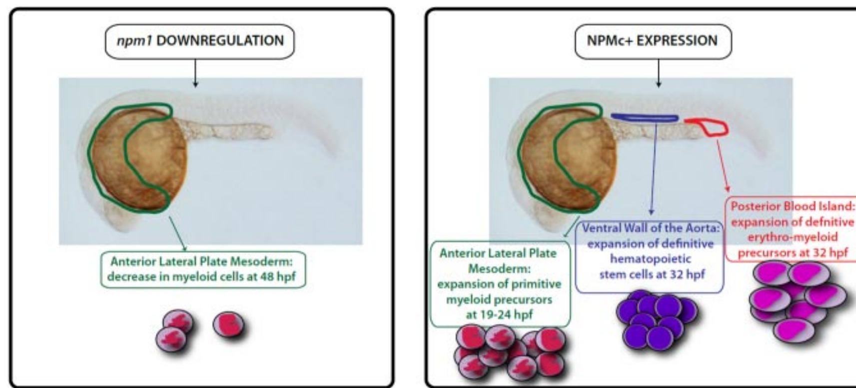

Figure 2. NPM1 mutant in zebrafish model. In zebrafish, where mutant NPM1 was expressed ubiquitously, not only did it cause expansion of primitive myeloid cells but it also resulted in increased numbers of both definitive erythromyeloid progenitors (*gata1*$^+$/*lmo2*^{bright}) and hematopoietic stem cells (*c-myb*$^+$/*cd41*$^+$) in the ventral wall of the aorta.

cell cytoplasm.[43] In the future, functional alterations of other NPM1 interactors are expected to be identified in *NPM1*-mutated AML.

In vitro studies demonstrated the NPM1 mutant promoted oncogenic transformation of primary cells in cooperation with oncogenic E1A.[44] In vivo, the NPM1 mutant impacted directly on myelopoiesis, favoring myeloid proliferation in transgenic mice[45] and in a zebrafish embryonic model.[46] In the transgenic mouse model, the most frequent human *NPM1* mutation (type A) was driven by the myeloid-specific human *MRP8* promoter. NPMc$^+$ transgenic mice developed a nonreactive myeloproliferation with mature GR-1$^+$, Mac-1$^+$ cells accumulating in bone marrow and spleen.[45] In zebrafish, ubiquitous mutant NPM1 not only caused expansion of primitive myeloid cells but also resulted in increased numbers of definitive erythro-myeloid progenitors (*gata1*$^+$/*lmo2*^{bright}) and hematopoietic stem cells (*c-myb*$^+$/*cd41*$^+$) in the aorta ventral wall (Figure 2).

However, in none of these models was the NPM1 mutant alone able to initiate AML. In the mouse model, the inability of enhanced myeloproliferation to progress to spontaneous overt AML may have been determined by either the cell type expressing NPMc$^+$ or by low-level mutant expression in hemopoietic cell cytoplasm, which does not reproduce the features of human *NPM1*-mutated AML exactly. In the zebrafish embryo, follow-up for AML development was not possible because of the transient nature of mutant NPM1 expression. Consequently, to exert its oncogenic effect, NPM1 may need to act under different conditions, such as targeting a specific myeloid precursor and/or achieving a mutant to wild-type expression ratio that is appropriate for cytoplasmic delocalization of both nucleophosmin forms[6,38] and/or being accompanied by a secondary cooperating event.[44] Knockin mice models mimicking human *NPM1*-mutated AML more closely are needed to address these issues.

Origin of *NPM1*-mutated AML

Consistent CD34 negativity in the great majority of *NPM1*-mutated AML cases[3] raises the question of whether a minimal pool of CD34$^+$/CD38$^-$ *NPM1*-mutated progenitors exists. In *NPM1*-mutated AML, we and other investigators[47,48] found that the small fraction of CD34$^+$ hemopoietic progenitors, including CD34$^+$/CD38$^-$ cells, carried the *NPM1* mutation. When transplanted into immunocompromised mice, CD34$^+$ cells generated a leukemia that recapitulated the patient's original disease, morphologically and immunohistochemically (aberrant cytoplasmic NPM1 and CD34 negativity).[47]

The engraftment potential of the CD34$^-$ fraction in *NPM1*-mutated AML appears more controversial. In one study,[47] no or limited engraftment was observed in NOG mice. In contrast, Taussig et al[48] reported a more consistent engraftment of the CD34$^-$ leukemic cells in immunocompromised mice. These findings may reflect some degree of heterogeneity in the leukemic stem cell compartment of *NPM1*-mutated AML.

Despite CD34 negativity, *HOX* genes, which are involved in stem cell maintenance, are consistently up-regulated in *NPM1*-mutated AML.[20-22] However, it remains to be elucidated whether leukemic stem cells in *NPM1*-mutated AML originate from very early progenitors or from committed myeloid precursors, with subsequent reactivation of stem cell self-renewal machinery through *HOX* gene reprogramming.

Relationships between AML with mutated *NPM1* and other myeloid neoplasms

AML with mutated *NPM1* shows distinctive genetic, pathologic, immunophenotypic, and clinical features[4,5] (Table 2) that differentiate it from other myeloid neoplasms in the 2008 WHO classification.

"Other AML with recurrent genetic abnormalities"

AML with mutated *NPM1* is mutually exclusive with other entities listed in the category of "AML with other recurrent genetic abnormalities" according to WHO-2008 (Table 1). Rare AML patients have been reported to carry the *NPM1* mutation and recurrent cytogenetic abnormalities.[18,21] These cases remain controversial because it is unclear whether the genetic alterations occurred in the same, or in different, leukemic cell populations.[11] The significance of the rare association of *NPM1* and *CEBPA* gene mutations in AML is discussed in "AML with mutated *NPM1*: new insights into controversial issues of the 2008 WHO classification."

AML with MD-related changes

The 2008 WHO classification did not recognize a clear demarcation between *NPM1*-mutated AML and AML with myelodysplasia (MD)–related changes. Recent findings suggest they may be 2 distinct entities (this issue is discussed in detail in "AML with mutated *NPM1*: new insights into controversial issues of the 2008 WHO classification").

Therapy-related myeloid neoplasms

Approximately 10% of therapy-related AML are *NPM1*-mutated.[49] However it is still unclear whether therapy-related AML with mutated *NPM1* is a treatment-induced secondary leukemia (such as

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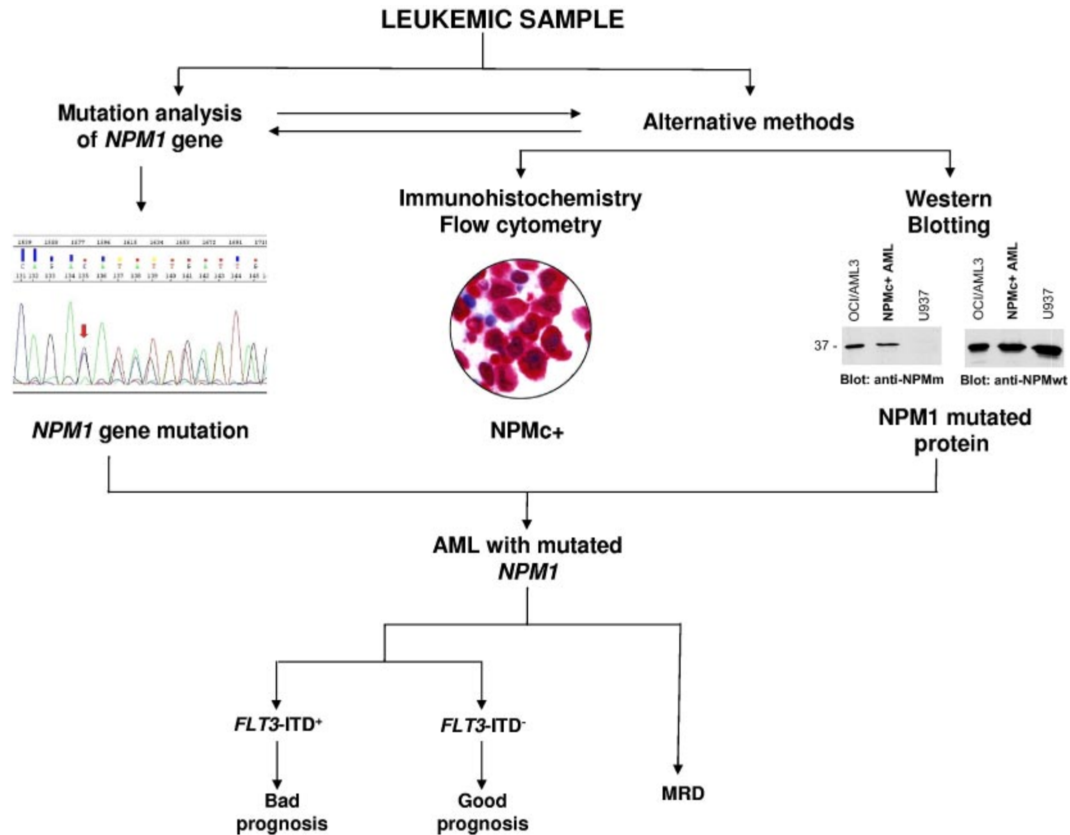

Figure 3. Molecular and alternative methods for diagnosis of *NPM1*-mutated AML. AML with mutated *NPM1* can be diagnosed either by mutational analysis or by alternative methods based on detection of aberrant cytoplasmic expression of nucleophosmin (immunohistochemistry on tissue sections or flow cytometry) or the mutant NPM1 protein with specific antibodies (Western blotting). The 2 approaches are complementary (bidirectional arrows). Evaluation of the *FLT3* status should be carried out in all *NPM1*-mutated AML patients because it is instrumental to identify the subgroup of cases with *NPM1*-mutated/*FLT3*-ITD-negative genotype that has a more favorable prognosis. Primers can be designed that allow monitoring of minimal residual disease (MRD).

occurs with other AML-carrying recurrent cytogenetic abnormalities) or a de novo *NPM1*-mutated AML in patients with a history of therapy.[50]

AML not otherwise specified

This is the least characterized myeloid neoplasm(s) in the 2008 WHO classification. Other entities, including AML with mutated *NPM1*, can be clearly differentiated through their distinctive molecular (when present), morphologic, immunophenotypic, and clinical features.

Myeloid sarcoma

Like other myeloid neoplasms associated with specific recurrent genetic abnormalities, AML with mutated *NPM1* can present as isolated myeloid sarcoma, show concomitant bone marrow and extramedullary involvement, or relapse in extramedullary organs. Skin and lymph nodes are most frequently affected, even though all anatomic sites can be involved.[51] In a large retrospective study in paraffin-embedded samples, approximately 15% of myeloid sarcoma carried cytoplasmic mutated nucleophosmin at immunohistochemistry.[52] As expected, these cases showed overlapping features with *NPM1*-mutated AML, including CD34 negativity and no clinical history of previous myelodysplastic or myeloproliferative neoplasm indicating blastic transformation or evolution.[52]

Myeloid proliferations related to Down syndrome

We had the opportunity to investigate 2 cases of this rare neoplasm and did not find cytoplasmic *NPM1* at immunohistochemistry (B.F., unpublished results, December 2009).

Blastic plasmacytoid dendritic cell neoplasm

NPM1-mutated AML and blastic plasmacytoid dendritic cell neoplasm may sometimes present with similar clinical and pathologic features, including skin involvement and expression of the macrophage-restricted CD68 molecule (monoclonal antibody PG-M1). Recent immunohistochemical findings clearly indicate they are separate disease entities,[53] as *NPM1*-mutated AML consistently shows nucleophosmin expression in the cytoplasm, whereas blastic plasmacytoid dendritic cell neoplasm is characterized by nucleus-restricted nucleophosmin positivity (predictive of *NPM1* gene in germline configuration).[53]

Diagnosis of *NPM1*-mutated AML: the strength of flexibility

One important prerequisite for a disease being included as an entity in the WHO classification is that it can be easily recognized worldwide, according to well-established and reproducible criteria. Fortunately, several molecular assays and surrogate methods are currently available for diagnosing AML with mutated *NPM1*[54] (Figure 3).

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Molecular analysis

Highly specific and sensitive molecular assays are available for detecting *NPM1* mutations.[55] One of the most frequently used at diagnosis is fragment analysis (genescan analysis),[18] which has the advantage of multiplexing with *FLT3*-specific or *CEBPA*-specific assays.[56] It does not, however, discriminate type A *NPM1* mutation from rare variants, and all samples that are positive at fragment analysis have to be sequenced for detailed characterization. On the other hand, melting curve assays, which include mutation-specific probes, are not only useful in screening but also discriminate between type A, B, and D mutations,[57] and sequencing is required only for 5% of patients with rare mutation types. These methods at diagnosis show a sensitivity of approximately 5%.

More sensitive methods have to be applied to detect minimal residual disease, and the mutation sequence at diagnosis needs to be known. The most sensitive are quantitative real-time polymerase chain reaction (PCR) assays with mutation specific primers, which can be applied on DNA[58] as well as on RNA.[57,58] RNA-based quantitative real-time PCR is able to detect 1:100 000 cells. Another alternative is latent nuclear antigen-mediated PCR clamping, which is rapid and has a sensitivity of 1:100 to 1:1000.[59] Although usually carried out on RNA or DNA extracted from peripheral blood or bone marrow leukemic blasts,[55,60] paraffin-embedded samples[52] and plasma[61] are also suitable for analysis.

Approximately 50 molecular variants of *NPM1* mutations have been identified so far.[62] They are almost always at exon 12 but have occasionally been found in other exons.[37] *NPM1* mutations are detected in approximately one-third of adult AML (50%-60% of all AML with normal cytogenetics)[3,4] but only in 6.5% to 8.4% of pediatric AML[63-65]; they were absent in children younger than 3 years.[64] Type A *NPM1* mutation (4 base TCTG insertion) is the most frequent in adults (75%-80% of cases), whereas *NPM1* mutations other than type A predominate in children.[66]

Although gene expression,[20-22] microRNA,[23,24] and methylation[67] profiles identified distinct signatures associated with *NPM1*-mutated AML, these procedures are currently not used for diagnostic or prognostic purposes in the everyday clinical practice.

Detection of cytoplasmic nucleophosmin: a surrogate for molecular analysis

One of the WHO's primary goals is the widespread use of the genetic-based AML classification. As molecular techniques are not always available for diagnosis, especially in developing countries, there is great interest in suitable substitutes. Morphology and immunophenotype (frequent CD34 negativity) cannot be used because *NPM1*-mutated AML encompasses various French-American-British categories, and the absence of CD34 is also observed in other AML genetic subtypes. Appearing to fill the gap for AML with mutated *NPM*1 is a simple, low-cost, and highly specific immunohistochemical assay, which predicts *NPM1* mutations by looking at ectopic nucleophosmin expression in the cytoplasm of leukemic cells[7,8] in bone marrow and in extramedullary sites (myeloid sarcomas; Figure 4). This approach successfully assessed multilineage involvement in bone marrow samples from patients[68] and tracked engraftment of $CD34^+$ *NPM1*-mutated AML cells in immunocompromised mice.[47] Detection of cytoplasmic NPM as surrogate for molecular diagnosis of *NPM1*-mutated AML is reminiscent of identifying acute promyelocytic leukemia with t(15;17) or ALK-positive anaplastic large cell lymphomas, by, respectively, anti-PML (PG-M3)[69] and anti-ALK monoclonal antibodies.[70]

Questions arise about which samples, techniques, and type of anti-NPM antibodies should be used. Aberrant cytoplasmic expression of nucleophosmin is optimally detected in paraffin sections from B5-fixed/ethylenediaminetetraacetic acid-decalcified bone marrow trephines.[7,8] Less reliable results were reported in bone marrow biopsies fixed in formalin and decalcified in formic acid.[71] Preliminary findings from our laboratory suggest discrepancies may result from the decalcifying agent (formic acid) rather than to formalin fixation (B.F., unpublished results, February 2010). Expression of cytoplasmic NPM was difficult to assess by immunocytochemistry in smears,[72] probably because of artifact diffusion among cell compartments and even outside leukemic cells.[54] More recently, flow cytometry was successfully used to detect nucleophosmin accumulation in leukemic cell cytoplasm[73,74] (Figure 4). This assay could serve as a complementary or even as an alternative procedure to bone marrow biopsy immunohistochemistry, allowing rapid measurement of cytoplasmic NPM1 and correlations with other markers in routine immunophenotyping.

Which antibodies should be used to visualize subcellular expression of nucleophosmin? Some anti-NPM antibodies recognize both wild-type and mutated NPM1,[3] whereas others identify only the NPM1 mutant.[68,74] Immunohistochemistry as first line screening for *NPM1*-mutated AML is best achieved using the former because they detect all NPM1 mutated proteins, including those generated by the very rare *NPM1* mutations occurring in exons other than 12. In contrast, reagents that are specific for NPM1 mutant A[74] fail to identify some mutants and may be more suitable for flow cytometric monitoring of minimal residual disease.

Prognostic features of *NPM1*-mutated AML

AML with mutated *NPM1* is highly responsive to induction chemotherapy.[3,4] Approximately 80% of patients achieve complete remission with clearance of leukemic cells as early as 16 days after starting treatment.[75] The exquisite chemosensitivity of *NPM1*-mutated AML is probably related to the aberrant dislocation of nucleophosmin from nucleolus to cytoplasm, but the underlying mechanism through which this occurs remains unknown.

The prognostic significance of *NPM1* mutations was mainly investigated in AML with normal karyotype. In patients younger than 60 years, the outcome is similar to the "good-risk" AML categories carrying t(8;21) or inv(16),[64,76] unless a concomitant *FLT3*-ITD mutation is present.[18,21,57,76,77] This is hardly surprising as *FLT3*-ITD impacts negatively on the prognosis of other AML genetic subtypes, including AML with mutated *CEBPA*.[78] Similarly, the good prognosis of AML with t(8;21)/*RUNX1*/*RUNX1T1*-positive is worsened by the presence of concomitant *Kit*-D816 mutations.[79] As a certain number of patients succumb to their disease, even in the prognostically favorable subgroup of *NPM1*-mutated AML without *FLT3*-ITD, other, as yet unidentified, secondary genetic lesions may cooperate with *NPM1* to induce leukemia and influence prognosis. *NPM1* mutations are frequently associated with *IDH1* mutations, which were recently identified by whole genome sequencing.[26] Some investigators reported that, when concomitant, *IDH1* mutations may adversely impact the favorable prognosis associated with *NPM1*-mutated/*FLT3*-ITD-negative genotype,[27,80] leading to the suggestion that *IDH1* mutation analysis might serve to refine prognostic stratification in *NPM1*-mutated AML cases without *FLT3*-ITD.[27,80] However, these findings were not confirmed in other studies[28,81] where the unfavorable effect on prognosis of *IDH1* mutation was mainly found in AML patients with the unmutated *NPM1* genotype.

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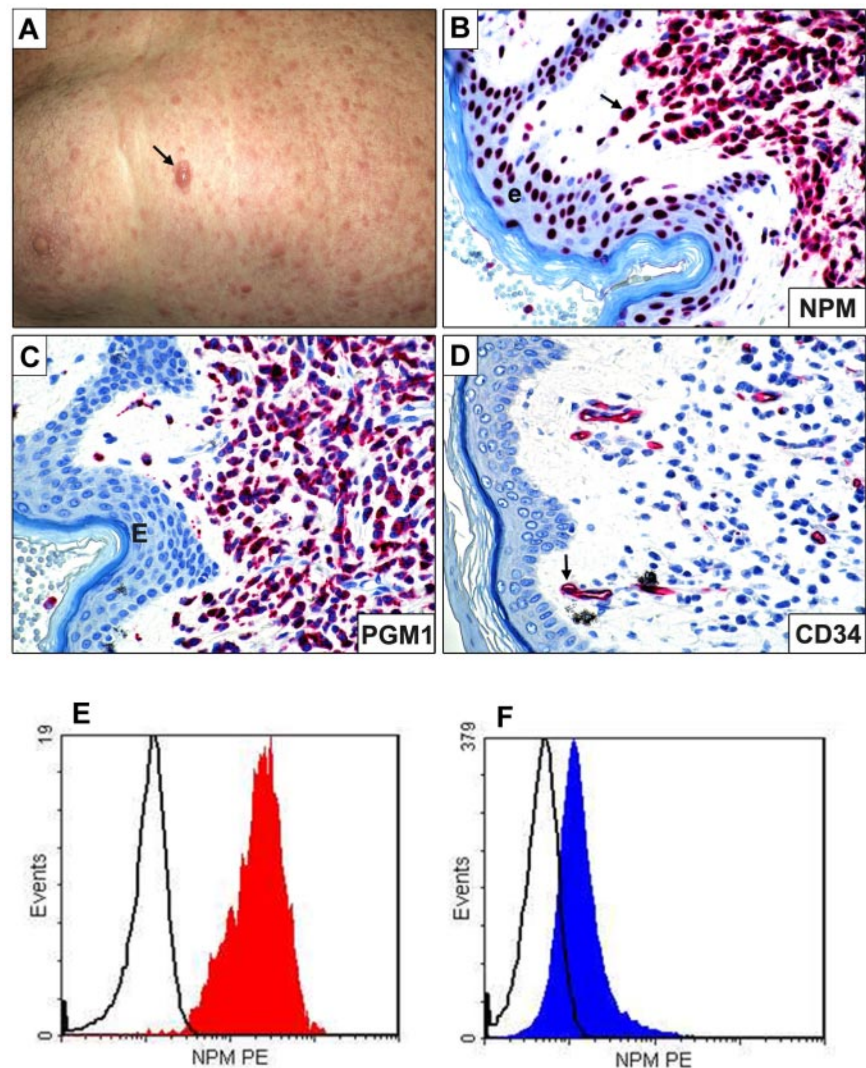

Figure 4. Myeloid sarcoma expressing cytoplasmic NPM1 and flow cytometric detection of cytoplasmic nucleophosmin in AML. (A) Multiple skin lesions; the arrow indicates the largest lesion. (B) Leukemic cells infiltrating the derma show aberrant cytoplasmic expression of NPM (arrow); the cells of epidermis (e) exhibit the expected nucleus-restricted positivity for NPM. (C) Leukemic cells express the histiocyte-restricted form of CD68 (monoclonal antibody PG-M1). (D) Leukemic cells are $CD34^-$; the arrow indicates a $CD34^+$ vessel that serves as internal control. (B-D) Alkaline phosphatase antialkaline phosphatase technique; hematoxylin counterstaining; images were collected using an Olympus B61 microscope and a UPlan FI 100×/1.3 NA oil objective; Camedia 4040, Dp_soft Version 3.2; and Adobe Photoshop 7.0. (E) Flow cytometry analysis of cytoplasmic nucleophosmin in AML. NPM1-mutated AML M5b 48% blasts showing the phenotype: $CD34^-CD13^+CD33^+CD117^+MPO^-CD56^+NPMc^+$. (F) AML M1 with wild-type *NPM1* gene and 93% blasts with phenotype: $CD34^+$ $CD13^+$ $CD33^+$ $CD117^+$ $MPO^+CD56^+NPMc^-$ (bottom left and right; courtesy of Prof Christian Thiede and Dr U. Oelschlaegel, University of Dresden, Dresden, Germany).

Although the prognostic impact of *NPM1* mutations was largely demonstrated for AML patients younger than 60 years, several studies included elderly patients[57] who were recently investigated in depth. In patients older than 60 years, Büchner et al[82] found a 52.1% incidence of *NPM1*-mutated AML-NK compared with 66.4% in patients younger than 60 years ($P = .0189$). The favorable constellation of mutant *NPM1* and normal *FLT3* status was found at comparable frequencies (36.5% and 33.2%) in younger and older patients, equally predicting better survival and longer duration of remission in multivariate analyses. In 909 AML patients who were older than 60 years, Röllig et al[83] revealed that karyotype, age, *NPM1* mutation status, white blood cell count, lactate dehydrogenase, and CD34 expression were independent prognostic markers for overall survival. The authors defined a novel prognostic model and found that *NPM1* mutation status significantly influenced overall survival, whereas *FLT3*-ITD status did not. Finally, in AML-NK patients 70 years of age or older, Becker et al[22] found that, at multivariate analysis, the *NPM1* mutation was the only factor influencing prognosis. Overall survival was approximately 40% if an *NPM1* mutation was present but only 5% in cases carrying an unmutated *NPM1* gene.[22] Taken together, these findings support the value of *NPM1* mutation as a molecular tool for selecting elderly patients for whom aggressive chemotherapy is worth adopting.

As for any type of AML that has attained complete remission, the question is whether the patient should undergo an allogeneic stem cell transplantation, which is so far the most effective treatment modality for AML. Because of its intrinsic risk of morbidity and mortality, this procedure is generally reserved for young AML patients carrying high-risk genetic abnormalities. In contrast, AML patients with relatively good prognosis, such as those carrying t(15;17), t(8;21), or inv(16), are usually not transplanted in first complete remission.[1] This policy was also proposed for AML with mutated *NPM1* in the absence of concurrent *FLT3*-ITD because no apparent benefit seems to derive from allogeneic transplantation in these patients[76] who account for approximately 16% of all newly diagnosed de novo AML younger than 60 years.[1] These cases are currently treated with conventional therapy, with or without autologous stem cell transplantation. Further prospective studies are warranted to confirm these findings.

AML with mutated *NPM1:* new insights into controversial issues of the 2008 WHO classification

In the 2008 WHO classification, *NPM1*-mutated AML was listed as a provisional entity because uncertainties persisted about the

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Table 4. Clonal chromosome abnormalities detected in *NPM1*-mutated AML and other AML with recurrent cytogenetic abnormalities

	AML				
Karyotype	***NPM1* mutation (n = 689)**	**t(8;21) (n = 100)**	**inv(16) (n = 73)**	**t(15;17) (n = 147)**	**11q23/*MLL* (n = 79)**
Additional abnormalities	105/689 (15.2%)	71/100 (71.0%)	24/73 (32.9%)	61/147 (41.5%)	37/80 (46.2%)
−X/−Y	18	48	3	4	1
+4	11	2	0	0	2
−7	3	0	0	0	0
+8	43	5	11	21	15
+13	2	1	1	0	2
+19	0	1	0	0	5
+21	5	0	2	0	7
+22	1	0	13	0	2
del(7q)	1	2	3	4	0
del(9q)	9	17	0	5	1
del(11q)	0	2	0	0	0
ider(17)(q10)t(15;17)	0	0	0	10	0
Other	67	15	9	48	40

This table is an update of the findings reported by Haferlach et al.[84]

biologic significance and prognostic impact of additional chromosomal aberrations and multilineage dysplasia in AML with mutated *NPM1* and how AML patients who were double-mutated for *NPM1* and *CEBPA* should be classified. Recent studies provided insights into these areas.

What is the biologic and clinical significance of chromosomal aberrations in AML with mutated *NPM1*?

Approximately 15% of AML with mutated *NPM1* harbor chromosomal aberrations other than typical recurrent cytogenetic abnormalities.[3] The significance of these chromosomal abnormalities was recently addressed in 631 AML patients with mutated/cytoplasmic NPM1.[84] Chromosomal aberrations were found in 14.7%, with the most frequent anomalies being +8, +4, −Y, del(9q), and +21[84] (Table 4). Several findings suggested these chromosomal aberrations were secondary events.[84] Although less frequent, they were mostly similar to additional chromosome aberrations that are widely regarded as secondary events in AML with t(8;21), inv(16), t(15;17), or 11q23/*MLL*-rearrangements.[84] They were often subclones within the leukemic population with normal karyotype[3] (mosaicism). More importantly, 4 of 31 *NPM1*-mutated AML patients with NK at diagnosis remained *NPM1*-mutated while switching to the following abnormal karyotype at relapse: del(9q) (n = 2), t(2;11) (n = 1), and inv(12) (n = 1).[84] In addition, few *NPM1*-mutated AML with abnormal karyotype at diagnosis showed either clonal regression (change from abnormal to normal karyotype) or switched to a different abnormal karyotype at relapse, while retaining the original *NPM1*-mutated gene status.[84] *NPM1*-mutated AML with normal or abnormal karyotype showed the same gene expression profile and immunophenotype.[84] Finally, in 2 independent clinical trials, the karyotype did not appear to impact on the favorable prognosis (overall and event-free survival) of *NPM1*-mutated/*FLT3*-ITD-negative AML patients.[84] However, another study observed that an abnormal karyotype had a negative impact on event-free survival of *NPM1*-mutated AML.[85] The discrepancy may be the result of the small number of patients analyzed by Micol et al[85] and/or differences in therapy or type of chromosomal aberrations.

The major problem with these studies is that, because of the rarity of chromosomal aberrations in *NPM1*-mutated AML, their prognostic significance has been difficult to assess and has been based on all abnormal karyotypes being grouped together. However, as single abnormalities, they may have distinctly different outcomes. Large meta-analysis studies should help to further clarify this issue.

What is the biologic and clinical significance of myelodysplasia-related changes in AML with mutated *NPM1*?

According to the new WHO classification,[86] a case is diagnosed as AML with MD-related changes, in the presence of one or more of the following: (1) previous, well-documented, history of MDS or MDS/myeloproliferative neoplasm; (2) myelodysplasia-related cytogenetic abnormalities; and (3) multilineage dysplasia (ie, detection of dysplasia in 50% or more of cells in 2 or more myeloid lineages in bone marrow and/or peripheral blood smears). When the 2008 WHO classification was being prepared, the significance of an *NPM1* mutation in the setting of morphologic dysplasia in an AML patient with NK was still unclear.[87] Thus, the new WHO classification presently recommends that cases with overlapping features should be diagnosed as "AML with MD-related changes," additionally annotating the presence of *NPM1* mutation.[86]

A large study on 318 AML patients with mutated *NPM1*[88] provided definitive evidence that multilineage dysplasia, detected in approximately 23% of cases (Figure 5), has no impact on gene expression profile or pathologic, immunophenotypic, clinical, and prognostic features of *NPM1*-mutated AML. These findings indicate that presence of an *NPM1* mutation should predominate over multilineage dysplasia as disease-defining criterion. This is in line with lack of biologic and clinical significance of multilineage dysplasia in other AML genetic subtypes.[89]

NPM1-mutated AML also differs from AML with MD-related changes as it does not usually evolve from previous MDS or MDS/myeloproliferative neoplasm[3] and shows distinctive features that seem to be independent of whether the karyotype is normal or abnormal,[84] further supporting the view that these 2 leukemias are distinct entities (Table 5).

What is the significance of rare AML cases carrying both *NPM1* and *CEBPA* mutations?

A minority (~ 4%) of *NPM1*-mutated AML also carry a *CEBPA* mutation.[90] At the time of preparation of 2008 WHO classification, this fact was thought to be difficult to reconcile with the claim that *NPM1* and *CEBPA* mutations defined distinct AML entities.

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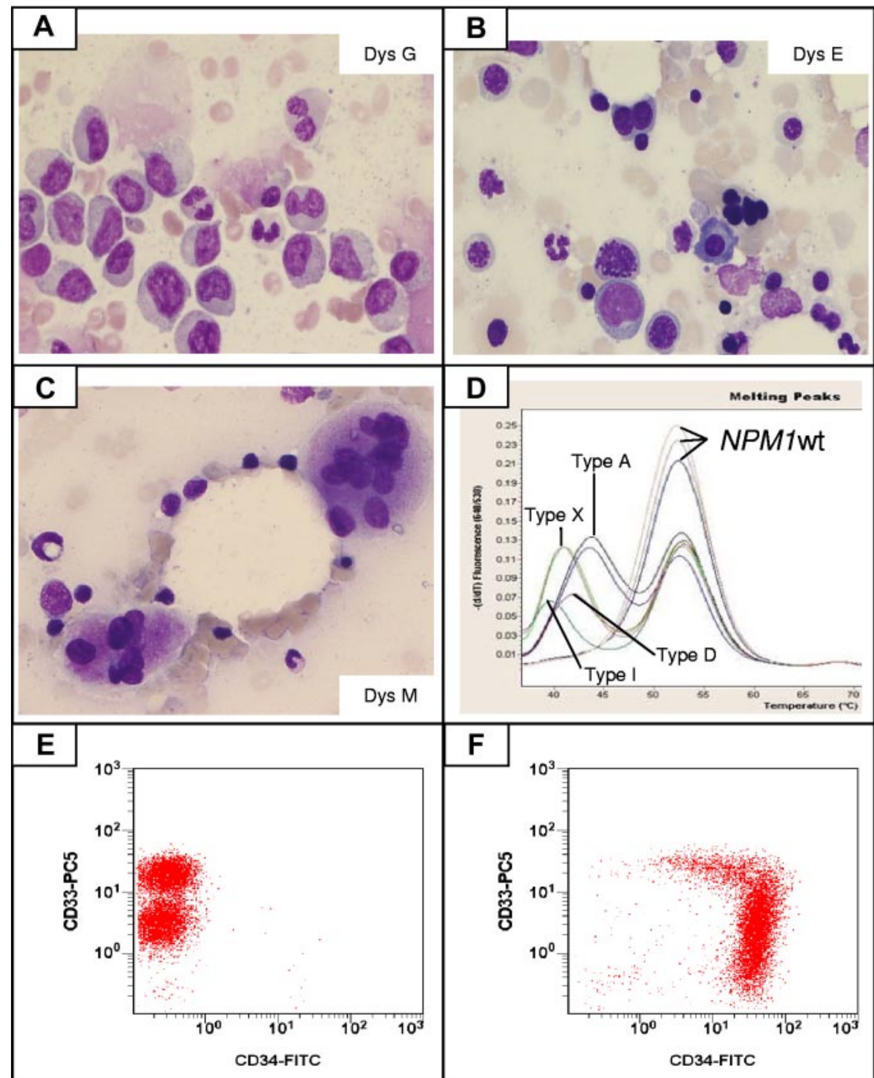

Figure 5. Multilineage dysplasia in *NPM1*-mutated AML. (A) Dysgranulopoiesis (Dys G) in a case of *NPM1*-mutated AML showing myeloid cells with hypogranulated cytoplasm and pseudo-Pelger cells. Bone marrow, Pappenheim staining. (B) Dyserythropoiesis (Dys E) in a case of *NPM1*-mutated AML showing nuclear irregularity with fragmentation, a mitosis, and multinucleation of red precursors. Bone marrow, Pappenheim staining. (C) Dysmegakaryopoiesis (Dys M) in a case of *NPM1*-mutated AML showing 2 dysplastic megakaryocytes with multiple nuclei. Bone marrow, Pappenheim staining. (A-C) All images were collected using a Zeiss Axio Imager.A1, 63×/1.4 oil objective Plan-Apochromat; 10×/23 eyepiece Sony camera 3CCD HD, Model MC-HD 1/3 Horn imaging DHS solution. (D) Lightcycler-based melting curve analyses showing different *NPM1* mutation types in AML with MLD changes: A (nt959insTCTG), D (nt959insCCTG), I (nt959insCTTG), X (nt959insTTCC), and wild-type patients. (E-F) Expression of CD34 by multiparameter flow cytometry. A case with *NPM1* mutation and MLD changes demonstrating a lack of expression of CD34 (E, note the different levels of CD33 expression between myeloblasts and monoblasts). A different AML MLD$^+$ case without *NPM1* mutation showing a strong expression of CD34 with a part of the population lacking CD33 expression (F). Slightly modified from Falini et al[88] with permission.

In-depth analysis of *NPM1*/*CEBPA* double-mutated cases has clarified the issue, showing that this rare association occurs only between *NPM1* and monoallelic *CEBPA* mutations. In contrast, *NPM1* mutations are usually mutually exclusive of biallelic *CEBPA* mutations.[91] This observation is relevant for the genetic classification of these tumors because only *CEBPA* double mutations appear to define a genetic entity, in accordance with their distinct gene expression profile (down-regulation of *HOX* genes) and favorable prognosis.[90,92-94]

Table 5. Differences between AML with MD-related changes and AML with mutated *NPM1*

Feature	AML with MD-related changes	AML with mutated *NPM1*
Nucleophosmin	Nuclear (unmutated)	Cytoplasmic (mutated)
WBC count	Often severe pancytopenia	Usually high WBC count
Previous history of MDS or MDS/MPN	Frequent	Usually absent
Karyotype	Usually abnormal	Usually normal (85%)
CD34	Usually positive	Usually negative
Prognosis	Usually poor	Favorable (if *FLT3*-ITD absent)

WBC indicates white blood cell.

Future perspectives

Recent findings point to “AML with mutated *NPM1*” and “AML with biallelic *CEBPA* mutations” as distinct leukemia entities. Additional information is expected to accumulate over the next few years that will further help to assess whether they should be incorporated as such in the next revision of the WHO classification. Because *NPM1*-mutated/*FLT3*-ITD-negative AML patients seem to have good prognosis, independently of normal or abnormal karyotype,[84] one critical issue requiring clarification will be how to best risk-stratify AML patients according to molecular criteria. The current assessment of the prognostic values of *NPM1, CEBPA*, and *FLT3*-ITD mutations in the framework of normal karyotype.[18,21,57] has 2 major limitations: (1) it excludes AML patients in whom cytogenetic analysis fails; and (2) it prevents AML patients from being assigned to the group with favorable genotype (eg, *NPM1* mutated/*FLT3*-ITD-negative), if a chromosomal aberration is present. Use of “normal karyotype” as initial framework for risk stratification may be more appropriate for AML patients without *NPM1* or biallelic *CEBPA* mutations. In this subgroup, which includes approximately 40% of AML with normal karyotype, increasing application of whole genome sequencing is expected to

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unravel novel causal mutations that may serve as new diagnostic and prognostic markers.

An important area of investigation in *NPM1*-mutated AML is the use of quantitative PCR techniques to monitor minimal residual disease, by looking at the number of *NPM1* mutant copies[58] at different intervals after therapy.[95] Indeed, *NPM1* mutations appear particularly suited to this purpose as they are a more specific, sensitive, and stable molecular marker than *WT1*[96] or *FLT3*-ITD.[12] Recent findings suggested minimal residual disease assessment is predictive of early relapse and long-term survival.[17,97] Assessment of *NPM1* mutant copies at 2 different checkpoints (after double induction therapy and completion of consolidation therapy) had a similar significant impact on prognosis.[98]

Recent findings on *NPM1*-mutated AML may also strengthen efforts to design therapeutic interventions focused on the underlying genetic lesion. The observation from Schlenk et al[99] that patients with *NPM1*-mutated/*FLT3*-ITD-negative AML may benefit from adding ATRA to chemotherapy goes in this direction. However, these results were not confirmed in the MRC trial conducted by Burnett et al,[100] and further studies are required to clarify the issue. In the future, a better understanding of the molecular mechanisms through which the NPM1 mutant induces leukemia will hopefully translate into development of new effective antileukemic drugs.

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Authorship

Contribution: B.F. had the original idea and wrote the manuscript; M.P.M. was responsible for biochemical studies and characterization of leukemic stem cell in *NPM1*-mutated AML; N.B. studied the mechanisms of transport of NPM1 mutant protein and the zebrafish model; P.S. described the transgenic mouse model; A.L. produced the specific antibody for NPM1 mutant protein and analyzed multilineage involvement in *NPM1*-mutated AML; E.T. performed gene expression profiling studies and immunohistochemical analyses; T.H. was involved in the clinical studies on the role of aberrant karyotype and myelodysplasia-related changes in *NPM1*-mutated AML; and all authors contributed to write the manuscript.

Conflict-of-interest disclosure: B.F. applied for a patent on clinical use of NPM1 mutants. T.H. is part owner of the Munich Leukemia Laboratory. The other authors declare no competing financial interests.

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