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

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Abstract

Following the discovery of \textit{NPM1}-mutated AML in 2005 and its subsequent inclusion as a provisional entity in the 2008 World Health Organization (WHO) classification of myeloid neoplasms, several controversial issues remained to be clarified. It was unclear whether the \textit{NPM1} mutation was a primary genetic lesion and whether additional chromosomal aberrations and multilineage dysplasia had any impact on the biological and prognostic features of \textit{NPM1}-mutated AML. Moreover, it was uncertain how to classify AML patients that were double-mutated for \textit{NPM1} and \textit{CEBPA}. Recent studies have shown that: i) the \textit{NPM1} mutant perturbs hemopoiesis in experimental models; ii) leukemic stem cells from \textit{NPM1}-mutated AML patients carry the mutation; and iii) the \textit{NPM1} mutation is usually mutually exclusive of biallelic \textit{CEBPA} mutations. Moreover, the biological and clinical features of \textit{NPM1}-mutated AML do not seem to be significantly influenced by concomitant chromosomal aberrations or multilineage dysplasia. All together, these pieces of evidence point to \textit{NPM1}-mutated AML as a founder genetic event that defines a distinct leukemia entity accounting for about one-third of all AML.
Introduction

The remarkable molecular heterogeneity of acute myeloid leukemia (AML) 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. The four molecularly distinct entities that had been described in the 2001 WHO classification were expanded to comprise AML with t(6;9), AML with inv(3) or t(3;3), AML (megakaryoblastic) with (1;22) and two provisional entities, i.e. AML with mutated CEBPA and AML with mutated nucleophosmin (NPM1) (Table 1). The latter accounts for about one-third of all AML and has distinct genetic, pathological, immunophenotypic and clinical characteristics. The WHO synonym for AML with mutated NPM1, that is NPMc+ AML (c+ stands for “cytoplasmic positive”), focuses on its most distinguishing functional feature, i.e. aberrant expression of nucleophosmin in the cytoplasm of leukemic cells. This unique immunohistochemical pattern, which led in 2005 to the discovery of NPM1 mutations in AML, is an excellent surrogate marker for molecular studies since it is fully predictive of NPM1 mutations.

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

As expected for a founder genetic lesion, the \textit{NPM1} mutation defines a subgroup of AML with a distinct gene expression profile (including down-regulation of \textit{CD34} and up-regulation of \textit{HOX} genes)\textsuperscript{20-22} and microRNA signature\textsuperscript{22-24} (including up-regulation of \textit{miR-10a} and \textit{miR-10b}). Sequencing of the whole genome from two cases of AML with normal karyotype (AML-NK) at 91\%\textsuperscript{25} and 98\% resolution\textsuperscript{26}, respectively, did not reveal any recurrent lesion, other than the \textit{NPM1} mutation, which showed features of a primary genetic hit. In fact, in one case\textsuperscript{25}, the \textit{NPM1} and \textit{FLT3} genes were involved, while the other patient\textsuperscript{26} harboured a mutated \textit{NPM1} gene and concomitant \textit{NRAS} and \textit{IDH1} gene mutations. Mutations of \textit{FLT3} and \textit{NRAS} in AML are widely recognized as secondary genetic events which are associated with tumour progression. The impact of \textit{IDH1} mutation\textsuperscript{26,27} on the molecular pathogenesis of AML remains to be elucidated. Interestingly, one \textit{NPM1}-mutated/\textit{IDH1}-mutated AML patient was recently reported to have lost \textit{IDH1} mutation at relapse while retaining the \textit{NPM1} mutation, suggesting that at least in this case \textit{IDH1} mutation was probably a secondary event\textsuperscript{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.

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) since the latter are probably secondary genetic events. As an example, FLT3-ITD and FLT3-TKD are less stable, being lost at relapse in about 9% and 50% of cases, respectively\(^\text{29,30}\). Instability has been also reported for NRAS\(^\text{31}\) and WT1\(^\text{32}\) 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 emerges as the most likely candidate as the primary, driving genetic lesion in about 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 (see below).

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

**How does mutated NPM1 promote leukemia?**

The NPM1 gene encodes for a protein which, although nucleolar at steady state\(^\text{6}\), shuttles between nucleus and cytoplasm\(^\text{34}\). Acting as a molecular chaperone to establish multiple protein-protein interactions, NPM1 is involved in critical cell functions\(^\text{35}\), such as...
control of ribosome formation and export, stabilization of the oncosuppressor p14Araf protein in the nucleolus, and regulation of centrosome duplication. Although the NPM1 gene was strongly implicated in cancer pathogenesis\textsuperscript{35}, how the NPM1 mutant protein promotes leukemia remains elusive. Since the NPM1 mutation always results in aberrant cytoplasmic dislocation of the mutant protein\textsuperscript{36,37}, this event appears critical for leukemogenesis\textsuperscript{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 hyper-shuttling NPM1 mutant work in a deregulated fashion). Moreover, the NPM1 mutant could have neomorphic features, e.g. capability to interact with new protein partners in cytoplasm\textsuperscript{4,6}.

NPM1 mutant-mediated cytoplasmic delocalization of nuclear proteins\textsuperscript{6} was implicated in knocking-down the oncosuppressor Arf\textsuperscript{39,40} and activating the c-MYC oncogene\textsuperscript{41}. In addition, the function of wild-type nucleophosmin in NPM1-mutated AML cells is profoundly affected by its reduction at the nucleolar physiological site. Reduction of wild-type NPM1 in the nucleolus is due to both heterozygosity and dislocation into cytoplasm through forming heterodimers with NPM1 mutant\textsuperscript{6}. In the Npm knock-out mouse, Npm inactivation led to genomic instability which, in turn, promoted \textit{in vitro} and \textit{in vivo} cancer susceptibility. Npm heterozygous cells were more susceptible to oncogenic transformation and Npm\textsuperscript{+/−} mice developed spontaneous tumours, especially myeloid malignancies\textsuperscript{42} indicating how NPM1 acts as haploinsufficient tumor suppressor \textit{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 signalling in leukemic cell cytoplasm\textsuperscript{43}. In the future, functional alterations of other NPM1 interactors are expected to be identified in NPM1-mutated AML.

\textit{In vitro} studies demonstrated the NPM1 mutant promoted oncogenic transformation of primary cells in cooperation with oncogenic E1A\textsuperscript{44}. \textit{In vivo}, the NPM1 mutant impacted
directly on myelopoiesis, favoring myeloid proliferation in transgenic mice\textsuperscript{45} and in a zebrafish embryonic model\textsuperscript{46}. In the transgenic mouse model, the most frequent human \textit{NPM1} mutation (type A) was driven by the myeloid-specific human \textit{MRP8} promoter. NPMc+ transgenic mice developed a non-reactive myeloproliferation with mature GR-1+;Mac-1+ cells accumulating in bone marrow and spleen\textsuperscript{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 (\textit{gata1+}/\textit{lmo2}bright) and hematopoietic stem cells (\textit{c-myb+}/\textit{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 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 \textit{NPM1}-mutated AML exactly. In the zebrafish embryo, follow-up for AML development was not possible due to 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\textsuperscript{6,38} and/or being accompanied by a secondary cooperating event\textsuperscript{44}. Knock-in mice models mimicking human \textit{NPM1}-mutated AML more closely are needed to address these issues.

**Origin of \textit{NPM1}-mutated AML**

Consistent CD34 negativity in the great majority of \textit{NPM1}-mutated AML cases\textsuperscript{3} raises the question of whether a minimal pool of CD34+/CD38- \textit{NPM1}-mutated progenitors exists. In \textit{NPM1}-mutated AML we and other investigators\textsuperscript{47,48} found that the small fraction of CD34+ hemopoietic progenitors, including CD34+/CD38- cells, carried the \textit{NPM1}
mutation. When transplanted into immunocompromised mice, CD34+ cells generated a leukemia which recapitulated the patient’s original disease, morphologically and immunohistochemically (aberrant cytoplasmic NPM1 and CD34 negativity)\(^4\).7\(^4\).

The engraftment potential of the CD34-negative fraction in \(NPM1\)-mutated AML appears more controversial. In one study\(^4\), no or limited engraftment was observed in NOG mice. In contrast, Taussig et al.\(^4\) reported a more consistent engraftment of the CD34-negative 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 upregulated in \(NPM1\)-mutated AML\(^2\)\(^0\)-\(^2\)\(^2\). 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, pathological, immunophenotypic and clinical features\(^4\),\(^5\) (Table 2) that differentiate it from other myeloid neoplasms in the 2008 WHO classification.

1) “Other AML with recurrent genetic abnormalities”

AML with mutated \(NPM1\) is mutually exclusive of 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\(^1\)\(^8\),\(^2\)\(^1\). These cases remain controversial because it is unclear whether the genetic alterations occurred in the same, or in different, leukemic cell
populations. The significance of the rare association of $NPM1$ and $CEBPA$ gene mutations in AML is discussed below.

ii) AML with MD-related changes

The 2008 WHO classification did not recognize a clear demarcation between $NPM1$-mutated AML and AML with MD-related changes. Recent findings suggest they may be two distinct entities (this issue is discussed in detail below).

iii) Therapy-related myeloid neoplasms

About 10% of therapy-related AML are $NPM1$-mutated. Although clinical and biological features overlap, it is still unclear whether therapy-related AML with mutated $NPM1$ is a treatment-induced secondary leukemia (such as occurs with other AML carrying recurrent cytogenetic abnormalities) or a de-novo $NPM1$-mutated AML in patients with a history of therapy.

iv) AML not otherwise specified (NOS)

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), morphological, immunophenotypic and clinical features.

v) 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, and relapse in extramedullary organs. Skin and lymph nodes are most frequently affected even though all anatomical sites can be involved. In a large retrospective study in paraffin-embedded samples, about 15% of myeloid sarcoma carried cytoplasmic mutated nucleophosmin at immunohistochemistry. 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\textsuperscript{52}.

\textit{vi) 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 (Falini B., unpublished results, December 2009).

\textit{vii) Blastic plasmacytoid dendritic cell neoplasm (BPDC)}

$NPM1$-mutated AML and blastic plasmacytoid dendritic cell neoplasm (BPDC) may sometimes present with similar clinical and pathological 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\textsuperscript{53}, as $NPM1$-mutated AML consistently shows nucleophosmin expression in cytoplasm while BPDC is characterized by nucleus-restricted nucleophosmin positivity (predictive of $NPM1$ gene in germline configuration)\textsuperscript{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 $NPM1$\textsuperscript{54} (Figure 3).

\textit{Molecular analysis}

Since AML with mutated $NPM1$ was first identified in 2005, highly specific and sensitive molecular assays have been developed for detecting $NPM1$ mutations\textsuperscript{55}. One of the most frequently used at diagnosis is fragment analysis (genescan analysis)\textsuperscript{18} which has the advantage of multiplexing with $FLT3$-specific or $CEBPA$-specific assays\textsuperscript{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 characterisation. 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 PCR (RQ-PCR) assays with mutation specific primers which can be applied on DNA\(^58\) as well as on RNA\(^57,58\). RNA based RQ-PCR is able to detect 1:100,000 cells. Another alternative is LNA-mediated PCR clamping which is rapid and has a sensitivity of 1:100-1:1,000\(^59\). 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 about one-third of adult AML (50-60% of all AML with normal cytogenetics)\(^3,4\) but only in 6.5%-8.4% of pediatric AML\(^63-65\); they were absent in children <3 years of age\(^64\). Type A \(NPM1\) mutation (four base TCTG insertion) is the most frequent in adults (75-80% of cases) whilst \(NPM1\) 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 every day clinical practice.

\(\text{Detection of cytoplasmic nucleophosmin: a surrogate for molecular analysis}\)
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 since NPM1-mutated AML encompasses various FAB categories and absence of CD34 is also observed in other AML genetic subtypes. Appearing to fill the gap for AML with mutated NPM1 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 in bone marrow and in extramedullary sites (myeloid sarcomas) (Figure 4). This approach successfully assessed multilineage involvement in bone marrow samples from patients and tracked engraftment of CD34+ NPM1-mutated AML cells in immunocompromised mice. 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 means of, respectively, anti-PML (PG-M3) and anti-ALK monoclonal antibodies.

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/EDTA decalcified bone marrow trephines. Less reliable results were reported in bone marrow biopsies fixed in formalin and decalcified in formic acid. Preliminary findings from our laboratory suggest discrepancies may be due to the decalcifying agent (formic acid) rather than to formalin fixation (Falini B, unpublished results February 2010). Expression of cytoplasmic NPM was difficult to assess by immunocytochemistry in smears, probably because of artefact diffusion among cell compartments and even outside leukemic cells. More recently, flow cytometry was successfully used to detect nucleophosmin accumulation in leukemic cell cytoplasm (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 visualise subcellular expression of nucleophosmin? Some anti-NPM antibodies recognize both wild-type and mutated NPM1 while others identify only the NPM1 mutant. Immunohistochemistry as first line screening for NPM1-mutated AML is best achieved using the former, since 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 A74 fail to identify some mutants and may be more suitable for flow cytometry monitoring of minimal residual disease.

**Prognostic features of NPM1-mutated AML**

AML with mutated NPM1 is highly responsive to induction chemotherapy. About 80% of patients achieve complete remission with clearance of leukemic cells as early as 16 days after starting treatment. 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 < 60 years old, the outcome is similar to the “good-risk” AML categories carrying t(8;21) or inv(16), unless a concomitant FLT3-ITD mutation is present. This is hardly surprising as FLT3-ITD impacts negatively on the prognosis of other AML genetic subtypes, including AML with mutated CEBPA. Similarly, the good prognosis of AML with t(8;21)/RUNX1/RUNX1T1-positive is worsened by the presence of concomitant Kit-D816 mutations. As a certain number of patients succumb to their disease even in the prognostically favourable 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\(^2^6\). Some investigators reported that, when concomitant, *IDH1* mutations may adversely impact the favourable prognosis associated with *NPM1*-mutated/*FLT3*-ITD negative genotype\(^2^7,\)\(^8^0\), leading to the suggestion that *IDH1* mutation analysis might serve to refine prognostic stratification in *NPM1*-mutated AML cases without *FLT3*-ITD\(^2^7,\)\(^8^0\). However, these findings were not confirmed in other studies\(^2^8,\)\(^8^1\) where the unfavourable effect on prognosis of *IDH1* mutation was mainly found in AML patients with the unmutated *NPM1* genotype.

Although the prognostic impact of *NPM1* mutations was largely demonstrated for AML patients under 60 years of age, several studies included elderly patients\(^5^7\) who were recently investigated in depth. In patients >60 years old, Büchner et al.\(^8^2\) found a 52.1% incidence of *NPM1* mutated AML-NK compared with 66.4% in patients <60 years (p=0.0189). The favourable 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 >60 years old, Röllig et al.\(^8^3\) revealed that karyotype, age, *NPM1* mutation status, WBC count, LDH and CD34 expression were independent prognostic markers for overall survival. The authors defined a novel prognostic model and found *NPM1* mutation status significantly influenced overall survival whereas *FLT3*-ITD status did not. Finally, in AML-NK patients ≥70 years old, Becker et al.\(^2^2\) found that at multivariate analysis, the *NPM1* mutation was the only factor influencing prognosis. Overall survival was about 40% if an *NPM1* mutation was present but only 5% in cases carrying an unmutated *NPM1* gene\(^2^2\). Taken together, the above 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\textsuperscript{1}. This policy was also proposed for AML with mutated \textit{NPM1} in the absence of concurrent \textit{FLT3}-ITD, since no apparent benefit seems to derive from allogeneic transplantation in these patients\textsuperscript{76} who account for about 16\% of all newly diagnosed de novo AML under 60 years of age\textsuperscript{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 \textit{NPM1}: new insights into controversial issues of the 2008 WHO classification**

In the 2008 WHO classification, \textit{NPM1}-mutated AML was listed as a provisional entity since uncertainties persisted about the biological significance and prognostic impact of additional chromosomal aberrations and multilineage dysplasia in AML with mutated \textit{NPM1} and how AML patients who were double-mutated for \textit{NPM1} and \textit{CEBPA} should be classified. Recent studies provided insights into these areas.

\textit{i) What is the biological and clinical significance of chromosomal aberrations in AML with mutated \textit{NPM1}?

About 15\% of AML with mutated \textit{NPM1} harbour chromosomal aberrations other than typical recurrent cytogenetic abnormalities\textsuperscript{3}. The significance of these chromosomal abnormalities was addressed in 631 AML patients with mutated/cytoplasmic \textit{NPM1}\textsuperscript{84}. Chromosomal aberrations were found in 14.7\%, with the most frequent anomalies being
+8, +4, -Y, del(9q) and +21\textsuperscript{84} (Table 4). Several findings suggested these chromosomal aberrations were secondary events\textsuperscript{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\textsuperscript{84}. They were often subclones within the leukemic population with normal karyotype\textsuperscript{3} (mosaicism). More importantly, 4/31 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), inv(12) (n=1)\textsuperscript{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\textsuperscript{84}. NPM1-mutated AML with normal or abnormal karyotype showed the same gene expression profile and immunophenotype\textsuperscript{84}. Finally, in two independent clinical trials the karyotype did not appear to impact on the favourable prognosis (overall and event-free survival) of NPM1-mutated/FLT3-ITD negative AML patients\textsuperscript{84}. However, another study observed that an abnormal karyotype had a negative impact on event-free survival of NPM1-mutated AML\textsuperscript{85}. The discrepancy may be due to the small number of patients analyzed by Micol et al.\textsuperscript{85} and/or differences in therapy or type of chromosomal aberrations\textsuperscript{85}.

The major problem with these studies is that, due to 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.

\textit{ii) What is the biological and clinical significance of myelodysplasia-related (MD) changes in AML with mutated NPM1?}
According to the new WHO classification\(^\text{86}\), 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 myelodysplastic syndrome (MDS) or MDS/myeloproliferative neoplasm (MPN); 2) myelodysplasia-related cytogenetic abnormalities; 3) multilineage dysplasia, i.e. 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 \textit{NPM1} mutation in the setting of morphologic dysplasia in a AML patient with NK was still unclear\(^\text{87}\). 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 \textit{NPM1} mutation\(^\text{86}\).

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

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

\textit{iii) What is the significance of rare AML cases carrying both NPM1 and CEBPA mutations?}

A minority (about 4%) of \textit{NPM1}-mutated AML also carry a \textit{CEBPA} mutation\(^\text{90}\). At the time of preparation of 2008 WHO classification, this fact was thought to be difficult to reconcile with the claim that \textit{NPM1} and \textit{CEBPA} mutations defined distinct AML entities. In
depth analysis of $NPM1/CEBPA$ double-mutated cases has clarified the issue, showing this rare association only occurs between $NPM1$ and monoallelic $CEBPA$ mutations. In contrast, $NPM1$ mutations are usually mutually exclusive of biallelic $CEBPA$ mutations\textsuperscript{91}. This observation is relevant for the genetic classification of these tumors, since only $CEBPA$ double mutations define a genetic entity, in accordance with their distinct gene expression profile (downregulation of $HOX$ genes) and favourable prognosis\textsuperscript{90,92-94}.

**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 help to assess whether they should be incorporated as such in the next revision of the WHO classification. Since $NPM1$-mutated/$FLT3$-ITD negative AML patients seem to have good prognosis, independently of normal or abnormal karyotype\textsuperscript{94}, 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\textsuperscript{18,21,57} has two major limitations: i) it excludes AML patients in whom cytogenetic analysis fails; and ii) it prevents AML patients from being assigned to the group with favourable genotype (e.g., $NPM1$ 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 comprises about 40\% of AML with normal karyotype, increasing application of whole genome sequencing is expected to 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 (MRD), by looking at the number of
NPM1 mutant copies at different intervals following therapy. Indeed NPM1 mutations appear particularly suited to this purpose as they are a more specific, sensitive and stable molecular marker than WT1 or FLT3-ITD. Recent findings suggested MRD assessment is predictive of early relapse and long term survival. Assessment of NPM1 mutant copies at two different checkpoints (after double induction therapy and completion of consolidation therapy) had a similar significant impact on prognosis.

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. 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. and further studies are required to clarify the issue. In the future, better understanding of the molecular mechanisms through which the NPM1 mutant induces leukemia will hopefully translate into development of new effective anti-leukemic drugs.

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Authors’ contributions

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 analysis; T.H. contributed to the clinical studies on the role of aberrant karyotype and myelodisplasia-related changes in NPM1-mutated AML. All the authors contributed to write the manuscript.

**Conflict of interest disclosure**

B.F. applied for a patent on clinical use of NPM1 mutants. T.F. is part owner of the Munich Leukemia Laboratory. All other authors have no conflicts of interest.
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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), (CBFβ/MYH11) | AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFβ/MYH11 |
| Acute promyelocytic leukemia AML with t(15;17)(q22;q12), (PML/RARα) and variants | Acute promyelocytic leukemia AML with t(15;17)(q22;q12); PML/RARα* |
| 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 RARα with partner genes other than PML are recognized separately since they may exhibit atypical APL features, including resistance to all-trans-retinoic acid (ATRA) therapy;

** As compared to 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.
Table 2. Distinctive features of AML with mutated *NPM1* (NPMc+ AML)

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

° Or its immunohistological surrogate (cytoplasmic NPM, NPMc+); GEP:gene expression profiling; * Lower incidence in Chinese children; ** In most but not all studies; ^Less than 10% CD34+ cells.
Table 3. Features of mutations most frequently associated with AML carrying a normal karyotype (AML-NK) compared to a primary genetic lesion [t(8;21)]

| Features                      | Primary genetic event in AML* e.g. 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   | Yes*     | No       | No    | n.a.  | Yes     | no    | No    |
| Distinct microRNA profile     | Yes                                         | Yes   | Yes   | Yes      | n.a.     | n.a.  | n.a.  | n.a.    | 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 | n.a. | Early | n.a. |
| % 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 | n.a. | All   | Allº |
| Loss at relapse               | No                                          | No    | No    | No       | Possible | Possible | Possible | Possible | No    | Rarelyºº |

*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 bi-allelic CEBPA-mutated cases; § Rarely occurs in ALL; # In NPM1/FLT3-ITD double mutated cases, NPM1 mutation appears to precede FLT3-ITD; ^^ Occasionally seen in AML carrying recurrent cytogenetic abnormalities and complex karyotype. n.a.: not available data. LSCs: Leukemic Stem Cells. º Ref. 26; ºº Ref. 28.
### Table 4. Clonal chromosome abnormalities detected in \textit{NPM1}-mutated AML and other AML with recurrent cytogenetic abnormalities\(^\text{a}\)

| Karyotype                                      | \textit{NPM1-mut}\(^\text{a}\) (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               | 2              |                 |
| -7                                             | 3                                        |                 | 2              |                 |
| +8                                             | 43                                       | 5               | 11             | 21              | 15              |
| +13                                            | 2                                        | 1               | 1              | 2               |
| +19                                            |                                          | 1               | 5              |
| +21                                            | 5                                        | 2               | 7              |
| +22                                            | 1                                        | 13              | 2              |
| del(7q)                                        | 1                                        | 2               | 3              | 4               |
| del(9q)                                        | 9                                        | 17              | 5              | 1               |
| del(11q)                                       | 2                                        |                 | 2              |
| ider(17)(q10)t(15;17)                          |                                          |                 | 10             |
| Other                                          | 67                                       | 15              | 9              | 48              | 40              |

\(^{a}\) The table is an update of the findings reported by the Authors in Ref. 84.

\(^{\text{a}}\) AML with mutated \textit{NPM1}.
Table 5. Differences between AML with myelodysplasia-related (MD) changes and AML with mutated NPM1

| Feature                       | AML with myelodysplasia-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                            | Favourable (if FLT3-ITD absent) |
FIGURE LEGENDS

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 depicted in the central column while its more differentiated CD34-negative progeny is depicted in the right and left columns. The primary, driving NPM1 mutation (red dot) in a 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 about 15% of NPM1-mutated AML can also acquire a chromosomal abnormality (X) whilst 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 a patient to another. For simplicity, occurrence of the second mutation and a chromosomal abnormality in the same cells is not depicted. According to the two-hit hypothesis only two mutations are indicated but additional mutations may be involved. Light grey circles indicate normal HSC and multipotent progenitors. Green circles indicate the normal hemopoietic progenitor compartment where primary NPM1 mutation (red dot) and secondary mutations (blue dot) and/or chromosomal aberrations (X) occur, giving raise to the leukemic bulk population.

Figure 2. NPM1 mutant in zebrafish model

In zebrafish, where mutant NPM1 was expressed ubiquitously, not only it caused expansion of primitive myeloid cells, but also resulted in increased numbers of both definitive erythro-myeloid progenitors (gata1+/lmo2bright) and hematopoietic stem cells (c-myb+/cd41+) in the ventral wall of the aorta.

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 upon detection of aberrant cytoplasmic expression of nucleophosmin (immunohistochemistry on tissue
sections or flow cytometry) or the mutant protein with specific antibodies (Western blotting). The two approaches are complementary (bi-directional arrows). Evaluation of the FLT3 status should be carried out in all NPM1-mutated AML patients since it is instrumental to identify the subgroup of cases with NPM1-mutated/FLT3-ITD negative genotype that has a more favourable prognosis. Primers can be designed that allow to monitor minimal residual disease (MDR). WB indicates Western blotting; NPMc+ indicates aberrant cytoplasmic expression of nucleophosmin.

Figure 4. Myeloid sarcoma expressing cytoplasmic NPM1 and flow cytometry 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 (double arrows). C) Leukemic cells express the histiocyte-restricted form of CD68 (monoclonal antibody PG-M1). D) Leukemic cells are CD34-negative; the arrow indicates a CD34-positive vessel that serves as internal control. B-D: alkaline phosphatase anti-alkaline phosphatase (APAAP) technique; hematoxylin counterstaining; images were collected using an Olympus B61 microscope and a UPlan FI 100x/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. AML M5b with mutant NPM1 gene 48% blasts phenotype: CD34-CD13+CD33+CD117-MPO-CD56+NPMc+. F) AML M1 with wild-type NPM1 gene 93% blasts phenotype: CD34+ CD13+ CD33+CD117+ MPO+CD56+NPMc- (Bottom left and right, by courtesy of Prof. Christian Thiede and Dr. U. Oelschlaegel).

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 two dysplastic megakaryocytes with multiple nuclei. Bone marrow, Pappenheim staining. A-C) All images were collected using a Zeiss Axio Imager.A1, 63x/1.4 oil objective Plan-Apochromat; 10x/23 eyepiece Sony camera 3CCD HD, Model MC-HD 1/3 Horn imaging DHS solution, Greifenberg, Germany. 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 CD3 with a part of the population lacking CD33 expression (F). (Slightly modified from Falini B et al, Blood;115:3776-3786).
Normal HSC

NPM1 mutation (○) ("Primary")

CD34(-) Leukemic bulk

2nd mutation (○) and/or chromosomal aberration (x) ("Cooperating")

CD34(-) Leukemic bulk

NPM1-mutated AML with NK

Other secondary mutations and/or chromosomal aberrations ("Cooperating")

CD34(-) Leukemic bulk

NPM1-mutated AML with AK

Figure 1
Figure 2

npm1 DOWNREGULATION

Anterior Lateral Plate Mesoderm

48 hpf: myeloid cells

NPMc+ EXPRESSION

Ventral Wall of the Aorta

32 hpf: definitive hematopoietic stem cells

Anterior Lateral Plate Mesoderm

19-24 hpf: primitive myeloid precursors

Posterior Blood Island

23 hpf: definitive erythro-myeloid precursors
LEUKEMIC SAMPLE

Mutation analysis of \textit{NPM1} gene

Alternative methods

Immunohistochemistry
Flow cytometry

Western Blotting

NPM1 gene mutation

NPMc+

NPM1 mutated protein

AML with mutated \textit{NPM1}

\textit{FLT3-ITD}^+

\textit{FLT3-ITD}^-

Bad prognosis

Good prognosis

MRD

[Diagram showing the flow of processes and outcomes related to \textit{NPM1} gene and \textit{FLT3-ITD} in AML.]

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Figure 5

A
Dys G

B
Dys E

C
Dys M

D
Melting Peaks

E

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

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