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Nucleophosmin1 (*NPM1*) abnormality in hematologic malignancies, and therapeutic targeting of mutant *NPM1* in acute myeloid leukemia

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***Abstract*:** Nucleophosmin (*NPM1*) is an abundant nucleolar protein that is implicated in a variety of biological processes and in the pathogenesis of several human malignancies. For hematologic malignancies, approximately one-third of anaplastic large-cell non-Hodgkin's lymphomas were found to express a fusion between *NPM1* and the catalytic domain of anaplastic lymphoma receptor tyrosine kinase. About 50–60% of acute myeloid leukemia patients with normal karyotype carry *NPM1* mutations, which are characterized by cytoplasmic dislocation of the *NPM1* protein. Nevertheless, *NPM1* is overexpressed in various hematologic and solid tumor malignancies. *NPM1* overexpression is considered a prognostic marker of recurrence and progression of cancer. Thus, *NPM1* abnormalities play a critical role in several types of hematologic malignancies. This has led to intense interest in the development of an *NPM1* targeting strategy for cancer therapy. The aim of this review is to summarize present knowledge on *NPM1* origin, pathogenesis, and therapeutic interventions in hematologic malignancies.

Keywords: hematologic malignancy, mutation, Nucleophosmin1, overexpression, therapy

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Introduction

Nucleophosmin (*NPM1*), also known as B23 protein, resides primarily in the nucleus, but shuttles continuously between the nucleus and cytoplasm.[1,2] The *NPM1* protein contains a numbers of motifs that act to mediate interactions with binding partners and affect its cellular localization.[1,3] Intracellular *NPM1* is predominantly oligomeric and binds to other proteins, including tumor suppressor proteins.[3] *NPM1* is also a multifunctional phosphoprotein that plays multiple roles in ribosome biogenesis, mRNA processing, chromatin remodeling, and embryogenesis.[4] For human hematologic malignancies, *NPM1* mutations are significantly implicated in newly diagnosed *de novo* acute myeloid leukemia (AML) cases,[1,5–7] which account for approximately one-third of all AML patients and have distinct genetic, pathologic, immunophenotypic, and clinical features.[1,8,9] Notably, mutated *NPM1* is a reliable marker of AML status in the majority of patients.[10] *NPM1* mutations can be detected in AML at relapse, even many years after the initial diagnosis.[11–14] Because of its distinct biological and clinical characteristics, NPM1-mutated AML has been defined as a distinct molecular leukemia entity in the recently updated World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia.[15–18] Nevertheless, *NPM1* abnormality has an enormous impact on the biological diagnosis, prognostic stratification, and monitoring of minimal residual disease (MRD) in hematologic malignancies. The discovery of *NPM1* gene alterations represents a rational basis for the development of molecular targeted drugs for leukemia and lymphoma.[19–22] The aim of this present review is to update our knowledge of the discoveries of *NPM1*

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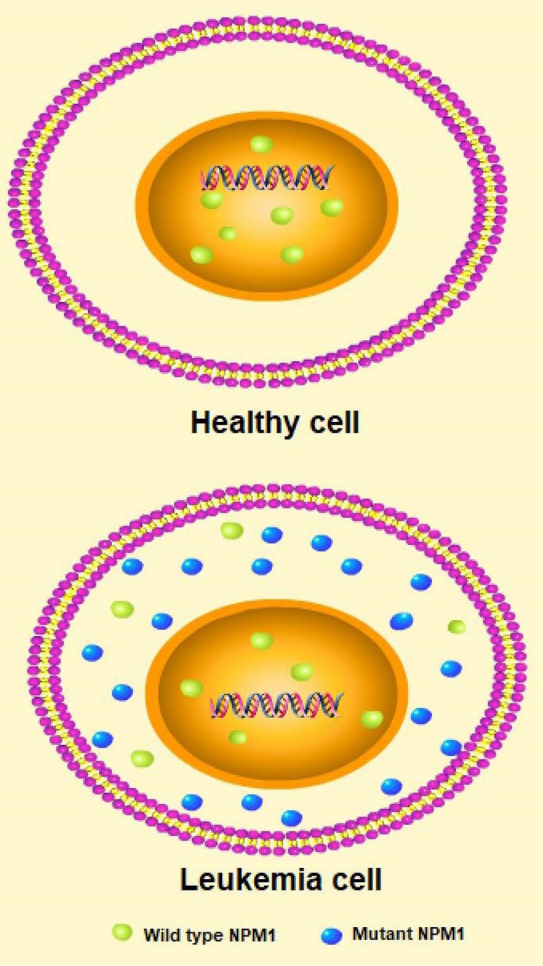

Figure 1. The cellular distribution of wild type and mutant NPM1 in a healthy cell and a leukemia cell. NPM1, Nucleophosmin.

and its alternations in different hematologic malignancies, as well as to deepen our understanding of recent findings concerning *NPM1* therapeutic targeting.

NPM1 structure and biological function

The human *NPM1* gene, located on chromosome 5q35, contains 12 exons ranging in size from 58 to 358 bp.[23–25] *NPM1*, a multifunctional phosphoprotein, is found localized primarily to the granular regions of the nucleolus. The protein can shuttle between the nucleus, the nucleoplasm, and the cytoplasm during the cell cycle,[2] and is involved in several biological processes, such as ribosome biogenesis, tumor suppression, nucleolar stress response, and cell apoptosis.[3] In the majority of patient cases, *NPM1* mutations are heterozygous, and localize to exon 12 of the gene. Around 50 different types of mutations have been found, all creating the cytoplasm-dislocated mutant *NPM1* (NPM1c+) protein.[8,9] The NPM1c+ protein in AML is critical to its oncogenicity. All *NPM1* mutations act to maximize the export of the mutant to the cytoplasm, including rare mutations found outside of exon 12.[26] The loss of NPM1c from the cytoplasm, either through nuclear relocalization or targeted degradation, results in immediate downregulation of homeobox (HOX) genes, and is followed by AML differentiation.[27] The cellular distribution of wild type (wt) and mutant *NPM1* is shown in Figure 1. Normally, *NPM1* molecules contain distinct domains that account for multiple biological functions. The N-terminal hydrophobic regions of *NPM1* are responsible for the self-oligomerization and chaperone activities of the molecule.[28] The C-terminus of *NPM1* accounts for the ribonuclease activity of the protein. The C-terminus also contains a short aromatic stretch with two tryptophan residues, which are crucial for *NPM1* binding to the nucleolus.[29]

The expression of *NPM1* is higher in proliferating cells than in quiescent cells,[30] which may modulate cell cycle progression and centrosome duplication.[31,32] Multifunctional characteristics of *NPM1* also appear to regulate the various post-translational modifications of *NPM1*, such as acetylation, phosphorylation, polyubiquitination, and sumoylation.[33–35] When *NPM1* expression aberrantly increases, the protein acts as an oncogene *via* promoting abnormal cell survival.[36] Conversely, *NPM1* may play a critical role in modulating the growth-suppressive pathway due to its decreased expression, inhibition of *NPM1* shuttling, or colocalization with other oncosuppressors, such as the ADP-ribosylation factor (ARF).[24,37] In general, *NPM1* involvement in cell proliferation is probably the result of several activities, which include modulation of ribosome biogenesis as well as interactions with histone oncosuppressor proteins.

Anti-*NPM1* antibodies for the diagnosis of hematologic malignancies

In recent years, several studies have explored the utility of anti-NPM1 antibodies for monitoring therapeutic outcomes, or as indicators of cancer

prognosis after treatment. Of those, the serum anti-NPM1 autoantibody has been shown to potentially function as a biomarker for the immunodiagnosis and prognosis of prostate cancer.[38] For diagnostic purposes, three different types of antibodies directed against fixative-resistant epitopes of *NPM1* have potential utility for immunohistochemistry in hematologic malignancies: those recognizing wt and mutant *NPM1* proteins, and those specifically directed against either the mutant or the wt *NPM1* protein. Monoclonal antibodies that recognize both wt and mutant *NPM1* are the most reliable reagents for immunohistochemical diagnosis of AML with mutated *NPM1*[1,39,40]; they label leukemic cells in cytoplasm (which contains mutant and wt *NPM1*) and the nucleus (which contains only wt *NPM1*).[24,41] Polyclonal antibodies that recognize mutant but not wt *NPM1* label only the cytoplasm of leukemic cells, providing more evidence that mutant *NPM1* is completely dislocated in the cytoplasm.[24,42,43] If a monoclonal antibody recognizing only wt *NPM1* stains leukemia cells in the nucleus and cytoplasm, then this is an indication of AML with mutated *NPM1*, since the mutant recruits wt *NPM1* into the cytoplasm. In this case, the best control for specificity of aberrant cytoplasmic expression of *NPM1* is immunostaining with an antibody against nucleolin (NCL), which is another abundant shuttling nucleolar protein; in AML with mutated *NPM1*, the protein will be located only in the nucleus.[40]

It has been reported that a monoclonal antibody (T26) that recognizes 10 of the 21 known *NPM1* mutants in AML cells did not cross react with wt *NPM1* or unrelated cellular proteins when assessed by immunofluorescence and flow cytometry analysis. These data indicate that T26 may become a helpful tool for rapid molecular diagnosis of AML.[44] The value of anti-NPM1 antibody-based immunohistochemistry in bone marrow biopsies and molecular analysis for the detection of *NPM1* mutations was further evaluated by Woolthuis and colleagues from the University of Groningen.[45] They observed a high percentage of concordance between the two methods of mutation detection. A small subgroup of patients showed discordant results from using the two methods, which could be caused by fixation and histotechnical factors as found in previously published studies.[1,41,45–48] Moreover, cases with mutated *NPM1* do not always show overt cytoplasmic staining of *NPM1* on bone marrow biopsies with formalin fixation. Cytoplasmic *NPM1* localization is not always caused by a conventional *NPM1* mutation, and the authors suggest that, for the screening of *NPM1* abnormalities, more information will be obtained *via* combining immunohistochemistry with molecular analysis.[45]

NPM1 mutations in human hematologic malignancies

NPM1 mutations in AML

Mutations in the *NPM1* gene are the most frequent genetic abnormalities of AML, and are highly specific to *de novo* AML.[1,5,7,44] *NPM1* mutations usually cause a frameshift in the region encoding the C-terminus of the protein. The altered reading frame results in the disruption of a nucleolar localization signal and the introduction of an additional nuclear export signal; this generally results in aberrant expression of the mutated NPM1c+ protein.[26,27,40,49,50] Mutations within *NPM1* are a founder genetic alteration in AML, and the presence of NPM1c+ is critical for disease maintenance.

Clinically, *NPM1* mutations have an important prognostic significance. Mutations in the *NPM1* gene have been associated with a favorable prognosis in the absence of concomitant internal tandem duplications (ITD) of the fms-related tyrosine kinase 3 (*FLT3*) gene in cytogenetically normal acute myeloid leukemia (CN-AML).[1,51–53] CN-AML is the largest and most heterogeneous cytogenetic AML subgroup. *NPM1* is the most frequently encountered mutation in CN-AML.[1] In the European Leukemia Net and other prognostic classifications, NPM1-mutated CN-AML in the absence of FLT3-ITD ($NPM1^{mut}$/FLT3-ITD-negative) is considered part of the favorable genetic group.[54,55] The comparative value of post-remission treatment in patients with CN-AML subclassified by *NPM1* and FLT3-ITD allelic ratio has been debated in a recent study. Among 521 patients with CN-AML in first complete remission (CR1), favorable overall survival (OS) was found for patients with mutated *NPM1* without FLT3-ITD ($71 \pm 4\%$). The outcome in patients with a high FLT3-ITD allelic ratio appeared to be very poor, with OS and relapse-free survival (RFS) of $23 \pm 8\%$ and $12 \pm 6\%$, respectively.[56] A favorable outcome is further

identified in older AML patients with NPM1^{mut}/FLT3-ITD-negative who underwent allogeneic hematopoietic cell transplantation in CR1. These data offer encouraging possibilities compared with results from historical nontransplant approaches.[57]

Several studies have provided definitive evidence demonstrating that *de novo* AML with mutated *NPM1* is frequently associated with a normal karyotype and frequent comutations in DNMT3A, IDH1/IDH2, and TET2, as well as, notably, in FLT3-ITD.[5,51,52,57–59] It has been reported that IDH1 mutations may adversely impact the favorable prognosis associated with the NPM1-mutated/FLT3-ITD-negative (NPM1^{mut}/FLT3-ITD^{neg}) genotype, indicating that IDH1 mutation analysis might serve to refine prognostic stratification in NPM1-mutated AML cases without FLT3-ITD.[60,61] Recently, a large study evaluated the potential prognostic impact of karyotype in 2426 intensively treated patients with NPM1^{mut}/FLT3-ITD$^{neg/low}$ AML in a pooled analysis of individual patient data from nine international cohorts. Of these, 2000 patients (82.4%) had a normal karyotype and 426 (17.6%) had an abnormal karyotype. A total of 1845 patients with NPM1^{wt}/FLT3-ITD$^{neg/low}$ and adverse-risk cytogenetics were identified in this international collaborative study. Of note, AML patients with *NPM1* mutations harboring the FLT3-ITD$^{neg/low}$ genotype and adverse-risk cytogenetics were found to share the same unfavorable prognosis as their counterparts with wt *NPM1*.[62] Interestingly, the investigators from the Wellcome Trust Sanger Institute in Cambridge found that the combination of NPM1c+ and Flt3-ITD had an early profound effect on gene expression and hematopoiesis. Also, both types of comutations drove AML in the majority of mice, and the leukemias in NPM1c Flt3-ITD mice were more aggressive and undifferentiated. Their data demonstrate that molecular synergy between NPM1c+ and Flt3-ITD underpins the co-occurrence patterns, phenotype, and prognosis of NPM1-mutant AML.[63] More studies further revealed that the co-occurrence of DNMT3A, *NPM1*, and FLT3-ITD mutations represents a distinct entity with a very poor AML outcome.[64–66] A recent study identified Hepatic Leukemia Factor (HLF) as a novel leukemic stem cell regulator in DNMT3A, *NPM1*, and FLT3-ITD triple-mutated AML, which is also genetically defined as a high-risk subgroup in AML.[64] Furthermore, there are still some mutations that rarely co-occur with *NPM1* mutations, such as partial tandem duplication in the mixed lineage leukemia (MLL) gene, and mutations in RUNX1, CEBPA, and TP53 genes.[67]

To characterize the genetic composition of NPM1-mutated AML, a study showed recently that NPM1c+ leukemia cells displayed increased transcription of stem-cell-associated genes, including HOXA, HOXB, and MEIS1.[27] Relocalization of NPM1c+ to the nucleus resulted in a downregulation of the HOX/MEIS1 gene signature and subsequent differentiation of AML cells. These results demonstrate the potential therapeutic benefit of inducing nuclear relocalization of *NPM1*. Interestingly, when the mutation status of five recurrently mutated oncogenes in 129 paired *NPM1* mutated samples obtained at diagnosis and relapse was assessed,[21] the authors found a mild shift in the genetic pattern from diagnosis to relapse including the loss of *NPM1* mutations. At the time of relapse, *NPM1* mutation loss patients feature distinct mutational patterns that share almost no somatic mutation with the corresponding diagnosis sample, and affect different signaling pathways. By contrast, profiles of patients with the persistence of the *NPM1* mutation are demonstrated to have a high overlap of mutations between diagnosis and relapse. A recent study further showed that upregulation of the HOXA5, HOXB5, HOXA10, PBX3, and MEIS1 genes was associated with AML cells harboring *NPM1* gene mutations, which was also correlated with a worse prognosis in AML. The *in vitro* data in this latter study suggests that a complex involving the HOX genes with PBX3 and MEIS1 cofactors may behave as an advanced therapeutic target in NPM1-mutated AML patients.[68] These results were consistent with previous findings showing that the gene expression profile of NPM1c+ AML is characterized by upregulation of genes involved in stem cell maintenance.[53,69]

Moreover, Brodska and colleagues described several fusion products with other genes resulting from chromosomal translocations in hematological malignancies in particular.[70] The fusion proteins contain the *NPM1* N-terminal domain, which serves mostly as an oligomerization interface promoting the oncogenic potential of the fusion partner. Of note, NPM-RAR, NPM-MLF1, or NPM-ALK fusions can be found

specifically in acute promyelocytic leukemia (APL, AML-M3), myelodysplastic syndrome (MDS), or non-Hodgkin's lymphoma (NHL), respectively. Recently, a unique NPM1–RARG–*NPM1* chimeric fusion was discovered in an older male subject, who presented with morphological and immunophenotypical features of hypergranular APL, but lacked response to all-trans retinoic acid (ATRA) and arsenic trioxide [ATO (As_2O_3)] therapies. Further study demonstrated that the NPM1–RARG–NPM1 fusion leads to both impairment of the *NPM1* protein and abnormal RARG, which contributes to impaired differentiation and leukogenesis.[71]

To investigate the correlation of *NPM1* mutation with clinical features and biological characteristics, the *NPM1* mutation was analyzed in bone marrow cells from 173 consecutive patients with *de novo* AML.[12] The results revealed a remarkable difference in the incidence of *NPM1* mutation between adult and pediatric patients. Children had a significantly lower incidence of *NPM1* mutations than adults. Of note, *NPM1* mutation presented at diagnosis and disappeared at complete remission (CR), and the same mutation reappeared at relapse, suggesting that the *NPM1* mutation is probably an early event in the development of AML but may play little role in the progression of the disease. Another group found that cytoplasmic localization of the promyelocytic leukemia gene (PML) could be mediated by interacting with mutant *NPM1*, which could stabilize PML through inhibiting proteasome-mediated degradation, and further enhance autophagic activity and cell survival in AML. Their results indicate conclusively that pharmacological inhibitors of PML or autophagy are potential therapeutics for NPM1-mutated AML therapy.[72]

NPM1 mutations in chronic myelomonocytic leukemia

Chronic myelomonocytic leukemia (CMML) is a clonal hematopoietic stem cell disorder, characterized by overlapping features of both a myeloproliferative neoplasm and a myelodysplastic syndrome.[18] It is a rare hematological malignancy that occurs in 0.3–0.52/100,000 patients.[73] *NPM1* mutations in the context of CMML are extremely infrequent. Zuo and colleagues reported that, of 152 patients with CMML who were tested, 8 (5%) were positive for the *NPM1* mutation.[74] Investigators from the Mayo Clinic identified 8 (2%) patients with *NPM1* mutation in a total of 373 WHO-defined CMML patients.[75] Similar results were analyzed in primary marrow samples from 150 patients with various chronic myeloid disorders. Of those, *NPM1* mutations were detected in three (2%) patients, all of whom had CMML and less than 1-year survival.[76] Notably, the previous findings show that patients with CMML and *NPM1* mutations have a more aggressive clinical course and a higher probability of AML progression.

In comparison with those harboring the wt counterpart, NPM1-mutant CMML patients were more likely to be anemic, have a 'dysplastic CMML subtype', have an increased frequency of DNMT3A and FLT3-ITD, and a lower incidence of TET2 and ASXL1 mutations.[75] To better understand the molecular events following acquisition of the *NPM1* mutation in CMML patient, Bolli and colleagues performed exome sequencing of bone marrow DNA at CMML diagnosis, AML diagnosis, and first CR. They found that DNMT3A and TET2 mutations were acquired first, followed by *NPM1* and CEBPA mutations. All four mutations had high variant allele frequencies (VAF) in CMML, which is consistent with a clonal sweep after acquisition of the last mutation (CEBPA or *NPM1*) and prior to AML transformation. Mutations affecting NRAS and *FLT3* were almost undetectable in CMML, but present at high VAF in AML samples.[77]

NPM1 mutations in MDS

MDS is a heterogeneous group of chronic myeloid neoplasms in which progression to secondary AML (sAML) is common.[78–81] To elucidate differential roles of mutations in MDS, the investigators analyzed clonal dynamics using whole-exome or targeted sequencing based on 699 patient samples. Of those, 122 samples were analyzed longitudinally. In comparison with high-risk MDS, FLT3, PTPN11, WT1, IDH1, *NPM1*, IDH2, and NRAS mutations tended to be newly acquired, and were associated with faster sAML progression and a shorter OS time.[78] Similar to the incidence in CMML, *NPM1* mutations are very rare in MDS or MDS/myeloproliferative neoplasm (MPN). Only 31 (1.6%) of 1900 patients with newly diagnosed MDS or MDS/MPN had *NPM1* mutations. The authors found

those NPM1-mutated patients had distinct clinical features, were younger, and likely to be female; they also had lower hemoglobin, higher median bone marrow blast percentage at diagnosis, and a higher frequency of normal karyotype compared with wt *NPM1* patients. In this regard, these data were in agreement with recent findings, which suggest that patients with NPM1-mutant MDS or MDS/MPN who are candidates for intensive therapeutic strategies and allogeneic stem cell transplantation may have improved outcomes and may benefit the most from chemotherapy, rather than MDS-based treatment approaches.[82]

NPM1 translocations in lymphomas

The *NPM1* gene is translocated in CD30$^+$ anaplastic large-cell lymphoma (ALCL) and in rare variants of AML.[83] Due to the loss of one functional allele of the *NPM1* gene, cells from these tumors contain an oncogenic fusion protein (*NPM1–ALK*, NPM–RARα or NPM1–MLF1) and a reduced level of wt *NPM1*. Because of ALK gene translocations, about 60% of ALCL express the ALK protein. ALK$^+$ T cell lymphoma is an aggressive neoplasm, and around 85% of ALK$^+$ ALCL carry the t(2;5)(p23;q35) chromosome translocation,[83,84] in which the ALK gene on chromosome 2 is fused with the *NPM1* gene on chromosome 5.[85] The chimeric gene encodes a fusion protein comprising the amino-terminal portion of *NPM1* and the entire cytoplasmic region of ALK. Lymphomas with this condition characteristically express the ALK protein both in the cytoplasm and, ectopically, in the nucleus. The *NPM1* oligomerization domain promotes NPM1–ALK heterodimer formation with wt *NPM1*, which, in turn, *via* shuttling, imports NPM1-ALK into the nucleoli. Meanwhile, due to the presence of the NPM1–ALK fusion protein, ALCL cells also show aberrant *NPM1* cytoplasmic expression.[86]

More than 80% of ALK$^+$ T cell lymphoma cases harbor the NPM1–ALK oncogene; NPM1–ALK functions as a constitutively activated cytoplasmic tyrosine kinase that is capable of translocating to the nucleus.[87] The activation of NPM1-ALK induces activation of several downstream signaling pathways, such as Janus kinases/signal transducers and activators of transcription (JAK/STAT) and Ras/mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK), all of which play crucial roles in cell survival and proliferation in ALK$^+$ NHLs.[88–92] A previous study indicated that NPM acts through its heterodimerization domain to bind sequences located in the transactivation domain (TAD) of the Forkhead Box M1(FOXM1), an oncogenic transcription factor.[93] A recent study showed that FOXM1 is highly expressed in ALK$^+$ lymphoma and contributes to its oncogenesis. The authors found that the *NPM1* portion in the *NPM1-ALK* fusion protein was crucial for binding to FOXM1, as the ALK portion alone cannot efficiently interact with FOXM1. This study provided evidence of the important pathogenetic role of *NPM1* in ALK$^+$ lymphoma. Disruption of the binding between FOXM1 and *NPM1* in heterodimers may serve as a highly specific anticancer therapeutic approach for NPM1-ALK$^+$ lymphoma.[94]

NPM1 overexpression in solid tumors and hematologic malignancies

NPM1 overexpression has been found in numerous human solid tumors, and has been extensively implicated as a biomarker of poor prognosis.[36,95–98] When *NPM1* expression is significantly increased, it may function as an oncogene by promoting aberrant cell growth through enhancement of ribosome machinery.[36] A previous study showed the upregulation of *NPM1* transcripts in colorectal cancer (CRC) cell lines, and increasing *NPM1* protein expression with progression from normal colon to adenoma to CRC in a tissue microarray. Modulation of *NPM1* expression occurred within CRC cells, affecting cellular viability, p53-dependent senescence, and cell-cycle progression, indicating that *NPM1* may play a fundamental role in colorectal carcinogenesis.[97] The high expression of *NPM1* has also been reported to link to gradient drug resistance in bladder cancer,[99,100] lung cancer,[15] hepatoma carcinoma,[101] and breast carcinoma.[102] The downregulation of *NPM1* expression markedly reversed the effects of multidrug resistance (MDR) in human hepatoma cells *via* inhibition of P-glycoprotein expression.[101]

To date, relatively little is known about the role of *NPM1* overexpression in hematologic malignancies. A recent study identified mutated chronic lymphocytic leukemia cells that were characterized by a MYC-related overexpression of *NPM1* and ribosome-associated components.[103] In our

previously reported work, we first validated that overexpression of *NPM1* and NCL may be involved in drug resistance, and might be an important indicator for prognosis evaluation in AML and in acute lymphoblastic leukemia (ALL).[104] We also reported that knockdown of *NPM1* by RNA interference may reverse MDR in resistant leukemic cells.[105] Further investigation by our group found that knockdown of *NPM1* reversed drug resistance by downregulating P-gp and the Akt/mTOR signal pathway, indicating that *NPM1* may serve as a potential modulator for drug resistance.[106] Our findings were in agreement with a previous report in human hepatoma cells.[101] Moreover, we recently found that high expression of NCL, another important nucleocytoplasmic multifunctional protein, promotes drug resistance in Burkitt's lymphoma, which may be related to the stabilization of *Bcl-2* mRNA and the decreased induction of apoptosis.[107] Currently, ongoing studies in our group are aimed at better understanding the interacting systems of *NPM1* and NCL in hematologic malignancies. Meanwhile, we are screening and assessing new therapeutic strategies and agents that we expect to facilitate the targeting *NPM1* and NCL and to improve the outcome of patients with hematologic malignancies.

Mutated *NPM1* as a biomarker for assessment of residual disease in AML

Mutated *NPM1* is a reliable biomarker for assessment of disease status in AML. A previous study in 173 patients of *de novo* AML demonstrated that *NPM1* mutations occur in an age-dependent fashion. The *NPM1* mutation disappeared with CR, but the same mutation reappeared at relapse.[12] A study involving a large cohort of intensively treated AML patients further showed that the *NPM1* mutation is an excellent marker for prediction of residual disease. The persistence of NPM1-mutated transcripts in blood after the second chemotherapy cycle was associated with a greater risk of relapse after 3 years of follow-up. *NPM1* mutations were detected in 69 of 70 AML patients at the time of relapse. In this study, the authors addressed that the presence of MRD, as determined by quantification of NPM1-mutated transcripts, and found that MRD might provide valuable prognostic information independent of other risk factors.[10] Moreover, another study recently found that mutated *NPM1* is of particular importance for monitoring disease dynamics in AML patients. A good initial response is essential to reach lower *NPM1* levels after treatment. A good initial mutated *NPM1* clearing cannot prevent relapse, but postpones it. However, this postponement positively correlates with OS. This study emphasized that the most informative time point for the determination of the minimal *NPM1* measurement as a predictor for survival and relapse risk is 9 months after therapy start.[108] These findings might impact the design of further studies and guide novel MRD-guided therapeutic strategies in AML.

Targeting *NPM1* mutation therapy in AML

NPM1 acts as a hub protein and is involved in trafficking other proteins to the nucleolus. Consequently, it is essential for the formation and maintenance of a functional nucleolus. *NPM1* mutations act deterministically to promote nuclear export of the mutants, and aberrant cytoplasmic delocalization of *NPM1* is thought to be critical for leukaemogenesis.[6,26,50] However, the manner in which the *NPM1* mutation disrupts cell control and induces leukemia remains unclear. Thus, the consequences of targeting *NPM1*, either wt or mutant, remain worth exploring. To date, the potential approaches for targeting NPM1-mutated AML include the following[22]: interfering with the aberrant transport of the *NPM1* mutant protein; interfering with wt *NPM1* functions; demethylating agents; immunotherapy with monoclonal antibodies; ATRA and arsenic trioxide. Promising molecules for the therapeutic targeting of mutant *NPM1* in AML are described in the following and summarized in Table 1.

NPM1-mutated leukemia cells display increased transcription of stem cell-associated genes such as the clustered *HOX* genes. Aberrant *HOX* expression is found in almost all AML cells that harbor a mutated *NPM1* gene, and *FLT3* is concomitantly mutated in about 60% of these cases.[52,68,113] Little is known about how mutant *NPM1* cells maintain aberrant gene expression. A recent study demonstrated that the chromatin regulators *MLL1* and *DOT1L* control *HOX* and *FLT3* expression in mutated *NPM1 AML*. These genes are therapeutically targetable *via* the pharmaceutical inhibition of menin-MLL1 and DOT1L. Small-molecule inhibition of these two histone modifiers results in the differentiation of mutated

Table 1. Promising molecules for therapeutic targeting of mutant NPM1 in AML.

Molecules	Target cells	Consequences in cellular processes	Study
ATRA/ATO	OCI-AML3 and IMS-M2 (*NPM1* mutated cell lines)	Proteasome-dependent degradation of NPM1c protein	Martelli[109]
	AML patient primary cells with *NPM1* mutation	Oxidative stress induction/cell apoptosis	
EAPB0503	OCI-AML3	Proteasome-mediated degradation of NPM1c protein	Nabbouh[110]
	OCI-AML3 xenograft mice	Restore wt-NPM1 nucleolar localization/growth arrest/apoptosis	
NSC348884	OCI-AML3	Interference with the oligomerization of NPM1	Balusu[111]
	AML patient primary cells with *NPM1* mutation	Cell apoptosis/sensitize NPM1c+ AML cells to ATRA	
MI-2-2	OCI-AML3	Inhibition of menin-MLL1 and DOT1L	Kuhn[112]
EPZ4777	AML patient primary cells with *NPM1* mutation	Suppression of *HOX* and *FLT3* expression	
MI-503	OCI-AML3 xenograft mice	Cell differentiation/inhibition $NPM1^{mut}$ leukemia initiation	

AML, acute myeloid leukemia; ATO, arsenic trioxide; ATRA, all-trans retinoic acid; EAPB0503, 1-(3-methoxyphenyl)-N-methylimidazoquinoxalin-4-amine; FLT3, fms-related tyrosine kinase 3; HOX, homeobox; NPM1, nucleophosmin; wt, wild type.

NPM1 AML cells *in vitro* and *in vivo*. This study indicated that both menin-MLL1 and DOT1L inhibitors, as single agents or in combination, represent novel therapeutic opportunities for NPM1-mutated AML.[112]

Imiquimod is a toll-like receptor 7 immunomodulator. It has been reported that the imiquimod analog 1-(3-methoxyphenyl)-N-methylimidazoquinoxalin-4-amine (EAPB0503) has promising antitumor activity, which could selectively induce NPM1c+ proteasomal degradation in NPM1c+ AML cells and lead to their apoptosis. Nevertheless, EAPB0503 treatment restores wt *NPM1* nucleolar localization *in vitro* and in *ex vivo* treated blasts, and it selectively reduces the leukemia burden in NPM1c+ AML xenograft mice.[110] These findings reinforce the idea of targeting the NPM1c+ oncoprotein to eradicate leukemic cells using this promising drug.

NPM1 is translocated or mutated in various lymphomas and leukemias, forming fusion proteins or NPM1-mutant products.[86,87] Several studies have shown that small molecule inhibitors may function as anticancer and antileukemic agents *via* disrupting *NPM1* dimer/oligomer formation, which can result in growth inhibition and apoptosis in cancer cells.[111,114,115] Among those inhibitors, NSC348884 has been shown to be lethal for various cancer cell-types with mutant *NPM1*.[111,116,117] For leukemia, NSC348884 induced apoptosis and sensitized cultured and primary AML cells with *NPM1* mutation to ATRA, but did not impact those AML cells coexpressing FLT3-ITD or normal $CD34^+$ progenitor cells expressing wt *NPM1*.[111] Currently, these molecules have not been approved for AML patients with *NPM1* mutations. However, there is growing interest in developing novel molecules aimed at targeting *NPM1* mutations, which might provide a more theoretical basis and scientific evidence for the anti-*NPM1* agents in future preclinical *in vivo* studies.

ATRA or ATO are highly effective molecular targeted therapies in APL with promyelocytic *PMLRARα* gene rearrangement.[118] The *NPM1* mutation is frequent and represents a founder genetic lesion in AML. Interestingly, data from a previous study showed that elderly patients with NPM1-mutant AML appeared to benefit from ATRA treatment.[119] Moreover, it was reported that AML cells carrying *NPM1* mutations are

more sensitive to ATO. The expression of NPM1-mutant protein with an acquired C-terminal cysteine-288 may enhance the sensitivity of AML cells to oxidative stress induced by ATO.[120] The mechanisms underlying this action were independently reported by two studies. The NPM1-mutant oncoprotein can be a target of ATO/ATRA, which induces proteasome-dependent degradation of *NPM1* leukemic protein and apoptosis in NPM1-mutated AML cell lines and the cells of primary patients. PML intracellular distribution is altered in NPM1-mutated AML cells, and reverted by ATO *via* oxidative stress induction.[109] The combination of ATO and ATRA significantly reduced leukemic blasts in the bone marrow of 3 NPM1-mutant AML patients, and restored nucleolar localization of *NPM1* and PML both *in vitro* and *in vivo*.[121] Collectively, these findings provide evidence that the ATO/ATRA strategy may represent a viable option in NPM1-mutant AML.

Other therapeutic strategies associated with *NPM1* mutation in AML

The precise mechanism of action of the *NPM1* mutant in AML is not well known, and other contributing events and therapeutic strategies still need to be investigated. Several studies have evidenced that NPM1-mutated AML cells strongly express CD33.[122–124] However, in the same study, the authors also found no correlation between *FLT3* gene mutations and CD33 expression in NPM1-mutated AML cells.[123] These results establish a rational basis for the therapeutic use of the anti-CD33 antibody in NPM1-mutated AML cells. A previous study reported that Dactinomycin may induce nucleolar stress by influencing ribosome biogenesis through the inhibition of RNA polymerase I,[125] which is active in Wilms' tumor and other cancers. Similar therapeutic effects of targeting *NPM1* mediated by Dactinomycin in AML cells were also presented by Falini and colleages.[20] They hypothesized that the nucleolus of NPM1-mutated AML cells might be vulnerable to Dactinomycin, and trigger a nucleolar stress response. They observed that one patient with NPM1-mutated AML without *FLT3* internal tandem duplication mutations achieved morphologic and immunohistochemical CR after two cycles of Dactinomycin therapy.[20] Moreover, Actinomycin D (actD), another chemotherapy drug used widely in various cancer types, has been found to induce cell death in all studied leukemic cell lines *via* an increase in nucleolar stress leading to a redistribution of mutated *NPM1* in the nucleoplasm.[126]

More recent work from the University of Nottingham showed that DNA damage caused by drugs may induce a switch in the aberrant cytoplasmic localization of NPM1c+ to a predominantly nucleolar localization in NPM1-mutated AML cells. Their results showed that the exploitation of nucleolar NPM1-replete cells to treat nucleolar stress would be effective only in the absence of DNA damage.[19] Going forward, immune responses may contribute to clinical outcomes *via* lysis of residual leukemic cells through specific T cells after chemotherapy. *NPM1* mutations are one of the most frequent molecular alterations in AML. Algorithm-based $CD4^+$ and $CD8^+$ T-cell epitopes derived from mutated *NPM1* were reported to feasibly elicit a coordinative immune response against NPM1-mutated AML cells, suggesting that *NPM1* mutations might constitute an ideal target structure for individualized immunotherapeutic approaches.[127] Recently, an unusual case of CMML harboring an *NPM1* mutation associated with extensive myeloid sarcomas was reported.[128] The patient had rapid resolution of lymphadenopathy, and attained remission after being administered idarubicin and cytarabine induction chemotherapy, in addition to a matched unrelated donor allogeneic bone marrow transplant. This study highlights that the *NPM1* chemosensitivity noted in AML might be applicable to other hematological conditions. Thus, other potential multiple therapeutic interventions are certainly worthy of further investigation.

Closing remarks

The answers to several questions regarding the role of *NPM1* abnormality in the development of hematopoietic malignancies remain unclear. More studies are required for a better understanding of the underlying mechanisms associated with the development of drug resistance caused by *NPM1* overexpression. Moreover, *NPM1* gene alternation status is not a single parameter in predicting clinical significance in hematopoietic malignancies. Many studies are currently ongoing with the aim of elucidating how *NPM1* abnormalities contribute to oncogenesis,

and to develop strategies to take advantage of specific characteristics for the improvement of therapy. In this review, we provide a summary of current knowledge in this field. Importantly, present investigations may eventually lead to the development of more specific antihematopoietic malignancy strategies in the future.

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