

## Brief report

# *Npm1* is a haploinsufficient suppressor of myeloid and lymphoid malignancies in the mouse

Paolo Sportoletti,<sup>1,2,4</sup> Silvia Grisendi,<sup>1,2</sup> Samia M. Majid,<sup>1,2,4</sup> Ke Cheng,<sup>1,2,4</sup> John G. Clohessy,<sup>1,2,4</sup> Agnes Viale,<sup>3</sup> Julie Teruya-Feldstein,<sup>2</sup> and Pier Paolo Pandolfi<sup>1,2,4</sup>

<sup>1</sup>Cancer Biology and Genetics Program, <sup>2</sup>Department of Pathology, <sup>3</sup>Genomics Core Laboratory, Memorial Sloan-Kettering Cancer Center (MSKCC), New York, NY; and <sup>4</sup>Cancer Genetics Program, Beth Israel Deaconess Cancer Center and Department of Medicine, Beth Israel Deaconess Medical Center (BIDMC), Harvard Medical School, Boston, MA

**Nucleophosmin (*NPM1*) gene has been heavily implicated in cancer pathogenesis both as a putative proto-oncogene and tumor suppressor gene. *NPM1* is the most frequently mutated gene in acute myeloid leukemia (AML), while deletion of 5q, where *NPM1* maps, is frequent in patients with myelodysplastic syndromes (MDS). We have previously shown that mice heterozygous for *Npm1* (*Npm1*<sup>+/-</sup>) develop a hematologic syndrome with**

**features of human MDS. Here we analyzed *Npm1*<sup>+/-</sup> mutants to determine their susceptibility to cancer. *Npm1*<sup>+/-</sup> mice displayed a greater propensity to develop malignancies compared with *Npm1*<sup>+/+</sup> mice. The *Npm1*<sup>+/-</sup> cohort frequently developed hematologic malignancies of both myeloid and lymphoid origin with myeloid malignancies displaying the highest incidence. Malignant cells retained the wild-type allele with normal localization**

**and expression of *Npm1* at the protein level, suggesting that complete *Npm1* loss is not a prerequisite for tumorigenesis. Our results conclusively demonstrate that *Npm1* acts as a haploinsufficient tumor suppressor in the hematopoietic compartment. (Blood. 2008;111:3859-3862)**

© 2008 by The American Society of Hematology

## Introduction

Nucleophosmin (NPM1) is a nucleolar phosphoprotein involved in critical cellular processes including ribosome biogenesis, maintenance of genomic stability, regulation of p53 and p19<sup>Arf</sup> tumor suppressor stability, modulation of DNA transcription, and response to stress stimuli.<sup>1</sup> The *NPM1* gene has been implicated in human cancer as it is frequently a target of genetic alterations primarily in hematopoietic tumors. It is found translocated with distinct partner genes in several diseases such as acute promyelocytic leukemia (APL), anaplastic large cell lymphoma (ALCL), acute myeloid leukemia (AML), and myelodysplasia.<sup>2-4</sup> Moreover, it is mapped to a region of chromosome 5 that is frequently deleted in therapy-related MDS and in nonhematopoietic tumors, such as non-small cell lung carcinoma.<sup>5,6</sup> More recently, *NPM1* has been found mutated and aberrantly localized in the cytoplasm of the leukemic blasts in a high proportion of AML patients (approximately 35%), rendering *NPM1* the most frequently mutated gene in AML.<sup>7</sup>

Characterization of primary cells from an *Npm1* hypomorphic series in vitro indicated that *Npm1* could exert tumor suppressive functions.<sup>8</sup> *Npm1* loss significantly affected genomic stability and *Npm1* was shown to be haploinsufficient for this function. *Npm1*<sup>+/-</sup> mouse embryonic fibroblasts (MEFs) displayed genomic instability, which resulted in increased susceptibility to oncogenic transformation in vitro.<sup>8</sup> Moreover, *Npm1*<sup>+/-</sup> mice displayed hematologic features similar to those observed in patients with MDS, a preleukemic condition in humans.<sup>8</sup> However, whether *Npm1* func-

tional loss may lead to cancer susceptibility per se remained unclear. In this report, we demonstrate that *Npm1* acts indeed as a haploinsufficient tumor suppressor gene in vivo.

## Methods

The Research Animal Resources Center at Memorial Sloan Kettering Cancer Center and Harvard Medical School Animal Facility approved and monitored all protocols that used animals in this study.

### Mouse line

The generation of *Npm1* heterozygous mice was previously described.<sup>8</sup>

### Histology and immunohistochemistry

Mouse tissues sections were stained with hematoxylin and eosin (H&E) for histopathological examination. Antibodies for immunohistochemical staining were CD45R/B220 for B-cell lineage, CD3 for T-cell lineage, polyclonal prediluted anti-lysozyme muramidase (Ventana, Tucson, AZ) for myeloid cells, and mouse antinucleophosmin/B23 (Zymed, South San Francisco, CA), used according to the manufacturer's instructions. Pictures of the stained tissue sections were obtained using an Olympus BX41 microscope and a DP20 camera (Olympus, Center Valley, PA). All image acquisition and processing was carried out with Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA).

Submitted June 27, 2007; accepted January 13, 2008. Prepublished online as *Blood* First Edition paper, January 22, 2008; DOI 10.1182/blood-2007-06-098251.

P.S. and S.G. contributed equally to this work.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

© 2008 by The American Society of Hematology

**Table 1. Tumor spectra and incidences in *Npm1*<sup>+/-</sup> and *Npm1*<sup>+/+</sup> mice**

Genotype/ID	Age, mo	Type of solid tumor	Hematologic malignancies
<b><i>Npm1</i><sup>+/-</sup>, n = 16/55 (29%)</b>			
70	20	—	Myeloid leukemia
36	24	—	Myeloid leukemia
80	23	—	Myeloproliferative disease–like myeloid leukemia
32D	20	—	Myeloid leukemia
90D	18	Lung carcinoma	—
75A	24	Salivary adenocarcinoma	—
72D	23	—	T cell lymphoma/leukemia
B24	10	—	B cell lymphoma
B3	23	—	Myeloproliferative disease–like myeloid leukemia
A73	24	—	Myeloid leukemia
A66	23	Hepatocellular carcinoma	—
B20	23	—	B cell lymphoma
B47	24	—	Myeloproliferative disease–like myeloid leukemia
B22	24	Lung adenocarcinoma	—
3B	24	—	Myeloproliferative disease–like myeloid leukemia
75	19	—	Myeloproliferative disease–like myeloid leukemia
<b><i>Npm1</i><sup>+/+</sup>, n = 3/47 (6.3%)</b>			
85	21	Cutaneous squamous cell carcinoma	—
A76	24	Hepatocellular adenoma	—
83	18	Hepatocellular carcinoma	—

*Npm1*<sup>+/-</sup> versus *Npm1*<sup>+/+</sup> *P* less than .01. The *P* value was calculated by chi-square test.

— indicates not applicable.

### Immunofluorescence

Immunofluorescence was performed as previously described<sup>8</sup> using anti-mouse  $\gamma$ -tubulin antibody (Sigma-Aldrich, St Louis, MO).

### CGH array analysis

Genomic DNA from tumors of 6 *Npm1* heterozygous mice was subjected to comparative genomic hybridization (CGH) array analysis at the MSKCC Genomic Core Lab Microarray Facility.

### Flow cytometry

Bone marrow cells were stained with phycoerythrin (PE)-conjugated anti-Mac1 and allophycocyanin (APC)-conjugated anti-Gr1 (BD Pharmingen, San Diego, CA) and analyzed using FACSCalibur (BD Biosciences).

### Southern blot analysis

Southern blot analysis was performed using the 5' probe used to identify the *Npm1*-null allele as previously described.<sup>8</sup>

## Results and discussion

To determine the susceptibility of *Npm1*<sup>+/-</sup> mice to tumorigenesis, we conducted a 2-year follow-up on a colony of 102 mice (47 wild-type and 55 heterozygotes mice), and have evaluated incidence, latency, and pathological features of neoplasias occurring in these mice compared with wild-type littermates.

Strikingly, *Npm1*<sup>+/-</sup> mice displayed higher susceptibility to develop malignancies than their wild-type counterparts. Of the 55 heterozygous mice under observation, 16 (29%) developed malignancies (Table 1). By comparison, only 3 cases (6.3%) were identified in the wild-type population (*P* < .01). Tumors were observed after a long latency that ranged from 10 to 24 months.

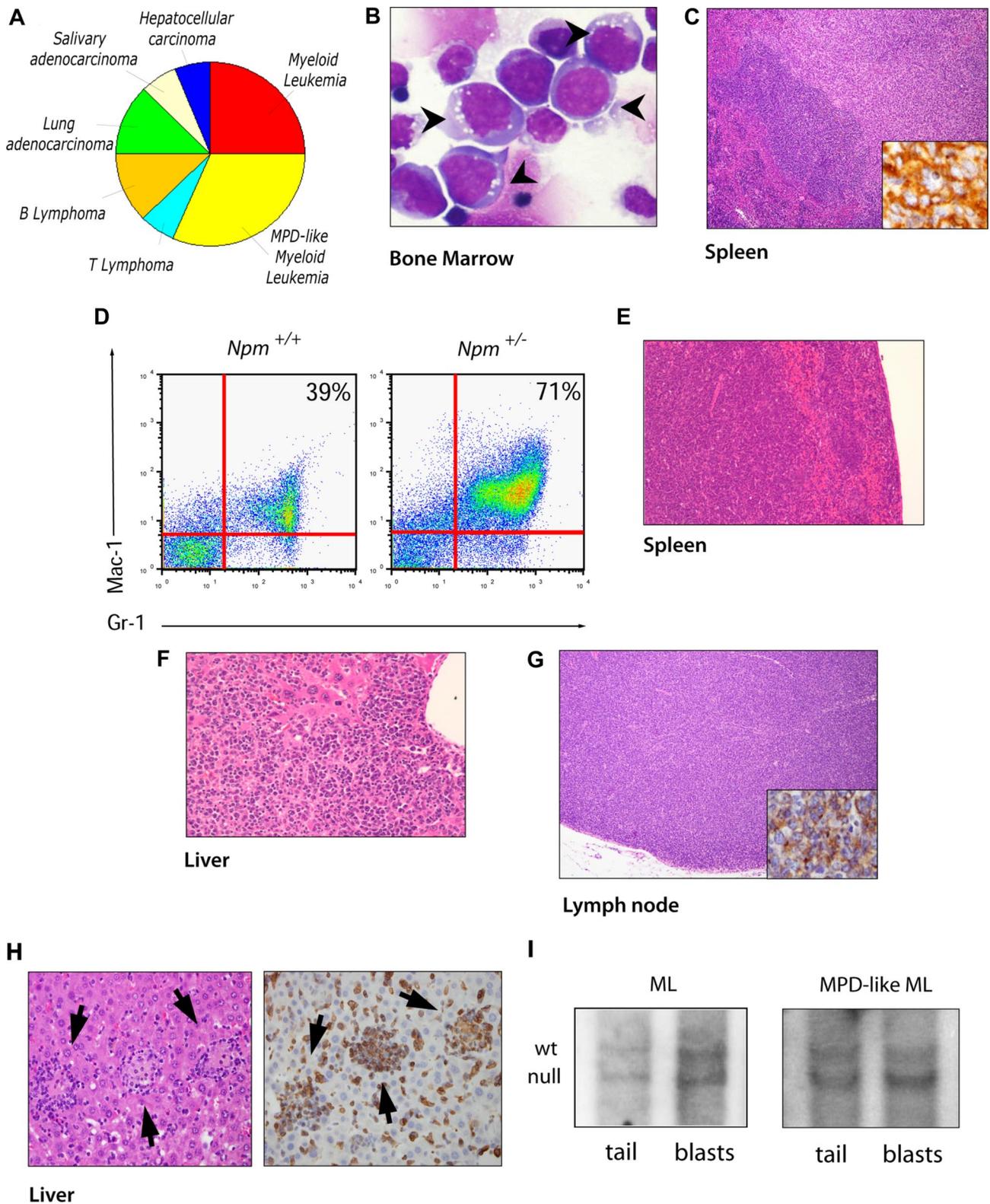
Pathological analysis revealed that 75% of the diseased *Npm1*<sup>+/-</sup> mice were affected by hematologic malignancies (Figure 1A), while no abnormalities were detected in the hematopoietic organs of wild-type animals analyzed. Interest-

ingly, among the solid tumors developed by the *Npm1*<sup>+/-</sup> mice, 2 were cancers of the lung.

Interestingly, three quarters of the hematologic neoplasms developed by *Npm1*<sup>+/-</sup> mice were myeloid malignancies, with a myeloproliferative disease–like myeloid leukemia (MPD-like ML) having the highest incidence (41.6% of all hematologic malignancies). MPD-like ML was characterized by leukocytosis (white blood cell [WBC] count =  $18.1 \pm 0.7 \times 10^9/L$ ) and extreme expansion of well-differentiated myeloid cells in bone marrow (Figure 1D), spleen (Figure 1E), and also in other nonhematopoietic tissues, such as liver (Figure 1F), and kidney and intestine (Figure S1C and S1D, respectively, available on the *Blood* website; see the Supplemental Materials link at the top of the online article).

The remaining mice affected by myeloid malignancies presented with myeloid leukemia (ML). ML was characterized by severe anemia (hemoglobin [HGB] level =  $69 \pm 8$  g/L), mild leukocytosis (WBC count =  $17.4 \pm 0.5 \times 10^9/L$ ), and thrombocytopenia (platelet [PLT] count =  $290 \pm 60 \times 10^9/L$ ). Both peripheral blood and bone marrow contained a significant percentage of leukemic blasts ( $20\% \pm 4\%$  and  $36\% \pm 2\%$ , respectively). Morphologically, blasts presented like immature myeloid cells with increased nucleus-cytoplasm ratio and highly vacuolated cytoplasm (Figure 1B arrowheads). Hepatomegaly and splenomegaly were invariably detected in these leukemic mice (Figure S1A). Pathologically, spleen and liver were filled with a large amount of leukemic blasts that compromised the architecture of the tissues (Figures 1C, S1B). Immunohistologic analysis of spleen and liver sections identified cells whose cytoplasm was positive for muramidase (lysozyme) staining, and positivity is usually associated with monocytic differentiation in human and mouse leukemias<sup>9</sup> (Figure 1C inset). These are all features distinctive of myeloid leukemia in the mouse.<sup>10</sup>

In addition to an increased rate of myeloid malignancies, *Npm1*<sup>+/-</sup> mice also developed B- and T-cell lymphomas (one quarter of all hematologic malignancies observed). These mice presented with enlarged lymph nodes with atypical lymphoid



**Figure 1. Features of *Npm1*<sup>+/-</sup> mice hematologic malignancies.** (A) Pie chart showing tumor spectrum in *Npm1*<sup>+/-</sup> mice. (B) Bone marrow cytopsin (60×/0.80 NA oil objective magnification) from an ML *Npm1*<sup>+/-</sup> mouse showing blasts with intracytoplasmic vacuoles, high nuclear/cytoplasmic ratio, basophilic cytoplasm, and multiple nucleoli (arrowheads). (C) Spleen section (10×/0.30 NA oil objective magnification) from the same mouse showing mass involving the red pulp composed of atypical mononuclear cells. The inset is a 40×/0.75 NA oil objective magnification of the immunohistochemical staining for muramidase indicating blasts originating from the myeloid lineage. (D) Flow cytometry analysis of bone marrow from an MPD-like ML *Npm1*<sup>+/-</sup> mouse demonstrates an expansion of Gr-1/Mac-1 mature cells. Numbers on graphs are percentages of cells in total bone marrow. (E) Spleen section (10×/0.30 NA oil objective magnification) from the same mouse in panel D showing both red and white pulp replaced by atypical mononuclear infiltrate. (F) Liver section (40×/0.75 NA oil objective magnification) from the same mouse in panel D showing infiltration of periportal area extended into the parenchyma as well. (G) Lymph node (10×/0.30 NA oil objective magnification) replacement by atypical lymphoid infiltrate of CD45R<sup>+</sup> cells (inset 40×/0.75 NA oil objective) supporting the involvement of a B-cell lymphoma. (H) Hematoxylin and eosin staining of liver lobular infiltrates (40×/0.75 NA oil objective magnification, arrows in left panel). Immunohistochemistry shows lobular lymphoid infiltrates in liver positive for CD3 (arrows in right panel). (I) Southern blot analysis on tail and blast DNA from representative ML (left panel) and MPD-like ML (right panel) affected *Npm1*<sup>+/-</sup> mice.

infiltrates affecting the normal architecture (Figure 1G). Lymphadenopathy was accompanied by hepatosplenomegaly with both spleen pulps and liver parenchyma replaced by nodules of atypical lymphoid cells (Figure 1H left panel; Figure S1F,G). In two thirds of lymphomas, the atypical lymphoid infiltrates were positive for CD45R staining (Figure 1G inset), supporting the diagnosis of B-cell lymphoma. In the other cases, neoplastic lymphoid infiltrates displayed strong positivity for CD3 staining (Figure 1H right panel), suggestive of T-cell lymphomagenesis.

We next analyzed the status of the *Npm1* gene in genomic DNA extracted from blasts of focal homogeneous infiltrates localized in the spleen of ML and MPD-like ML affected mice (6 mice in total), using the corresponding tail DNA as reference. Loss of the wild-type *Npm1* allele was never observed (Figure 1I). The nuclear expression of Npm1 in these tumors was confirmed by immunohistochemistry (Figure S2A).

As *Npm1* function is required for maintaining genomic stability, we have analyzed centrosome number and the presence of chromosomal abnormalities in *Npm1*<sup>+/-</sup> mice tumors. Bone marrow cytopins from MPD-like ML affected mice were immunostained for the centrosome marker  $\gamma$ -tubulin. Two independent cytopins clearly showed an increased number of cells displaying multiple centrosomes (Figure S2B). On average, 70% of cells displayed amplified centrosomes in these MPD-like ML, compared with 6% of bone marrow cells from nonleukemic *Npm1*<sup>+/-</sup> mice. These data demonstrate that centrosome amplification is associated with *Npm1* loss and may result in leukemia pathogenesis in *Npm1*<sup>+/-</sup> mice.

Genomic DNA analysis of 6 hematologic malignancies performed by comparative genomic hybridization invariably revealed the presence of numeric and structural chromosomal abnormalities (Figure S2C).

Although *Npm1*<sup>+/-</sup> mice display features resembling some of those identified in human MDS, it was not clear whether this represented a genuine preleukemic situation as is the case for the human disease. Here, we addressed this question by following a large cohort of *Npm1*<sup>+/-</sup> mice to establish whether *Npm1* heterozygosity results in an increased susceptibility to leukemogenesis. Indeed, a number of these mice were found to develop a variety of hematologic neoplasms, with myeloid malignancies having the

highest incidence. These data confirm our hypothesis that the *Npm1* phenotype originally characterized has the potential to progress to overt leukemia.

The absence of loss of heterozygosity (LOH) indicates that *Npm1* haploinsufficiency, rather than biallelic inactivation, is promoting the development of tumors in these mice. This suggests that while *Npm1* heterozygosity may render the cells more susceptible to oncogenic transformation, the complete somatic loss of the *Npm1* gene is neither required for the transformation nor selected for by the transformed cell itself.

In conclusion, we demonstrate that *Npm1* is a haploinsufficient suppressor of myeloid and lymphoid malignancies in the mouse. *Npm1*<sup>+/-</sup> mice are prone to spontaneous tumor formation and in particular to the development of disease of myeloid origin.

## Acknowledgments

We thank Linda Disantis and Owen Milbury for editing the paper, Manoj Kumar of BIDMC Genomics Center for help with CGH analysis, and members of the Pandolfi laboratory for helpful discussions.

This work was supported by National Institutes of Health grant (CA-71692) to P.P.P. P.S. was supported in part by the Associazione Umbra Leucemie e Linfomi (AULL).

## Authorship

Contribution: P.S. and S.G. designed and performed experiments, analyzed data, and wrote the paper; S.M.M. performed experiments; K.C. and J.G.C. performed experiments and edited the paper; A.V. performed the CGH analysis; J.T.-F. performed the pathological analysis of tumor samples; P.P.P. designed research, analyzed data, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Pier Paolo Pandolfi, Beth Israel Deaconess Medical Center, 330 Brookline Avenue, NRB 1038A, Boston, MA 02215; e-mail: ppandolf@bidmc.harvard.edu.

## References

- Grisendi S, Mecucci C, Falini B, Pandolfi PP. Nucleophosmin and cancer. *Nat Rev Cancer*. 2006; 6:493-505.
- Redner RL, Rush EA, Faas S, Rudert WA, Corey SJ. The t(5;17) variant of acute promyelocytic leukemia expresses a nucleophosmin-retinoic acid receptor fusion. *Blood*. 1996;87:882-886.
- Morris SW, Kirstein MN, Valentine MB, et al. Fusion of a kinase gene, ALK, to a nucleolar protein gene, NPM, in non-Hodgkin's lymphoma. *Science*. 1994;263:1281-1284.
- Yoneda-Kato N, Look AT, Kirstein MN, et al. The t(3;5)(q25.1;q34) of myelodysplastic syndrome and acute myeloid leukemia produces a novel fusion gene, NPM-MLF1. *Oncogene*. 1996;12: 265-275.
- Olney HJ, Le Beau MM. Evaluation of recurring cytogenetic abnormalities in the treatment of myelodysplastic syndromes. *Leuk Res*. 2007;31: 427-434.
- Mendes-da-Silva P, Moreira A, Duro-da-Costa J, Matias D, Monteiro C. Frequent loss of heterozygosity on chromosome 5 in non-small cell lung carcinoma. *Mol Pathol*. 2000;53:184-187.
- Falini B, Mecucci C, Tiacci E, et al. Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. *N Engl J Med*. 2005; 352:254-266.
- Grisendi S, Bernardi R, Rossi M, et al. Role of nucleophosmin in embryonic development and tumorigenesis. *Nature*. 2005;437:147-153.
- Kunder S, Calzada-Wack J, Holzwimmer G, et al. A comprehensive antibody panel for immunohistochemical analysis of formalin-fixed, paraffin-embedded hematopoietic neoplasms of mice: analysis of mouse specific and human antibodies cross-reactive with murine tissue. *Toxicol Pathol*. 2007;35:366-375.
- Kogan SC, Ward JM, Anver MR, et al. Bethesda proposals for classification of nonlymphoid hematopoietic neoplasms in mice. *Blood*. 2002;100: 238-245.