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Cytoplasmic Localization of Nucleophosmin in Bone Marrow Blasts of Acute Myeloid Leukemia Patients is not Completely Concordant with NPM1 Mutation and is not Predictive of Prognosis

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Abstract

Background—*NPM1* mutations are reported to predict a favorable prognosis in acute myeloid leukemia (AML) patients. Aberrant cytoplasmic localization of NPM protein is reported to be a surrogate for *NPM1* gene mutation.

Methods—Using immunohistochemical analysis (IHC), we assessed for NPM (clone 376) expression in formalin-fixed, formic acid-decalcified bone marrow biopsy specimens. DNA sequencing of the exon 12 of *NPM1* gene was performed in 104 patients.

Results—The study included 252 AML patients: 192 *de novo* AML, 33 AML following a myelodysplastic syndrome or chronic myelomonocytic leukemia, and 27 therapy-related AML. The median age was 62 years and 115 patients were \geq 60 years old. All patients received intensive chemotherapy. Cytoplasmic NPM was detected in 59 of 252 (23%) patients, including 48 of 192 (25%) *de novo* AML and 33 of 94 (35%) with a normal karyotype. DNA sequencing identified *NPM1* mutations in 30 of 38 cases with cytoplasmic NPM and 10 of 66 cases with nuclear NPM. Cytoplasmic NPM was associated with young patient age ($p=0.024$), *FLT3/ITD* ($p=0.005$), CD34-negative blasts ($p<0.001$), high peripheral blood blast counts ($p=0.041$), and high serum albumin level ($p=0.028$). No statistical differences in overall or event-free survival were found on the basis of NPM localization. Similar results were obtained in patients \geq 60 years old with normal karyotype and wild-type *FLT3* ($p=0.768$).

Conclusions—Immunohistochemical assessment for NPM localization did not predict prognosis in this patient cohort. The discordance between immunohistochemistry and DNA sequencing results indicates that DNA sequencing cannot be replaced by IHC assessment.

Keywords

AML; *NPM1* mutation; immunohistochemical analysis; prognosis; *FLT3/ITD*

Introduction

Nucleophosmin, encoded by the nucleophosmin (*NPM1*) gene on chromosome 5q35, is ubiquitously expressed in human tissues^{1, 2}. The *NPM1* gene has been shown to be commonly mutated in acute myeloid leukemia (AML)^{3, 4}. *NPM1* mutations are found in 25%–35% of all AML cases and 45%–64% of AML cases with a normal karyotype^{3, 4}. *NPM1* mutations have been associated with a favorable prognosis⁵ in the absence of a *fms-related tyrosine kinase-3 (FLT3)* gene mutation of internal tandem duplication type (*ITD*), a known adverse prognostic factor. In two retrospective studies, *NPM1*-mutated/*FLT3-ITD*-negative patients did not benefit from allogeneic stem cell transplantation unlike *NPM1*-wild-type/*FLT3-ITD*-negative patients^{5, 6}. These data led to the proposal that AML patients with a normal karyotype should be tested for *FLT3-ITD* and *NPM1* mutations to allow for risk-adapted therapy with *NPM1*-mutated/*FLT3-ITD*-negative patients being treated with chemotherapy without allogeneic stem cell transplantation, and *NPM1*-wild-type/*FLT3-ITD*-negative patients undergoing allogeneic stem cell transplantation⁷.

Wild type NPM is predominantly located in nuclei and nucleoli. *NPM1* gene mutations typically occur in exon 12, affecting the carboxy terminus and both disrupting the localizing domain and creating a nuclear export signal. As a result, mutated NPM protein is located in the nucleus and cytoplasm of neoplastic cells⁸. Others have suggested that immunohistochemical analysis for NPM localization can be used in lieu of molecular analysis for *NPM1* mutations⁹. In one study using immunohistochemical analysis of bone marrow biopsy specimens and mouse anti-NPM monoclonal antibody, the pattern of NPM expression was completely concordant with mutation status⁸. These data resulted in a proposed work flow to assess for *NPM1* mutations using immunohistochemical analysis as an initial screening step⁹. Cases with cytoplasmic NPM staining subsequently need molecular confirmation of *NPM1* mutations, and cases with nuclear NPM staining do not need further NPM work-up, thereby reducing unnecessary work and conserving resources⁹.

In this study, we investigated NPM localization using an immunohistochemical method in a large number of AML patients at our institution. The results were compared with DNA sequencing results in a subset of patients and were also correlated with clinical parameters, including overall and event-free survival.

Patients and Methods

After approval from the Institutional Review Board, we analyzed 252 bone marrow biopsy specimens from patients with AML: 70 archival and 182 that were obtained prospectively by the Department of Hematopathology at The University of Texas M. D. Anderson Cancer

Center (Houston, TX) between October 1, 2006, and September 1, 2008. For all patients, we obtained clinical and demographic data as shown in Table 1.

Formalin-fixed, paraffin-embedded tissue sections of bone marrow biopsy specimens (5 μ m thick) were deparaffinized in xylene (3 times for 5 min) and then rehydrated through graded concentrations of alcohol (3 times for 3 min in 100% ethanol, then for 3 min in 80% ethanol). For antigen retrieval, tissue sections were heated in EDTA (Biocare Medical, Concord, CA) for 30 min in a Decloaking Chamber (Biocare Medical, Concord, CA). Tissue sections were incubated with Biocare Medical peroxidase blocking reagent for 5 min. Primary antibodies used were a mouse monoclonal antibody against NPM protein (clone 376) (kindly provided by Dr. Brunangelo Falini) and a mouse monoclonal antibody against nucleolin/C23 (MS-3, Santa Cruz Biotech, Santa Cruz, CA). After washing with buffer, tissue sections were incubated with the primary antibodies for 60 min at room temperature (20°C). Sections were again washed, and incubated with secondary antibodies using Mach 4 AP Universal Polymer kit (Biocare Medical) according to manufacturer's instructions. For visualization, Vulcan Fast Red chromogen (Biocare Medical) was used for 10 min. All sections were counterstained with hematoxylin (Dako, Carpinteria, CA) for 3 min. Slides were then air dried and cover-slipped.

In order to assure proper assessment of nuclear localization of NPM, all cases were stained in parallel for nucleolin (C23, Santa Cruz Biotech, Santa Cruz, CA), a protein known to be restricted to the nucleus which served as a control⁸. Only cases that demonstrated appropriate nuclear distribution of C23 were analyzed for the cellular distribution of NPM. The NPM staining pattern was evaluated in blasts/immature cells, which were medium-sized cells with round-to-oval nuclei, fine nuclear chromatin, and moderate-to-scant cytoplasm in immunostained sections. Cytoplasmic NPM staining in megakaryocytes and dividing cells, that always represented less than 5% of all cells, was not scored.

Conventional cytogenetic analysis using standard GTG banding was performed on bone marrow aspiration specimens according to methods described previously¹⁰. The *FLT3* gene was assessed for *ITD* mutations using polymerase chain reaction (PCR) methods, as described previously¹¹.

In a subset of cases we extracted genomic DNA from bone marrow aspirate specimens and used it for a PCR to amplify a 168 bp fragment of exon 12 of the NPM1 gene using Tli polymerase (Promega, Madison, WI). The forward primer (5'-GATGTCTATGAAGTGTGTTGGTTCC-3') was labeled with 5-carboxyfluorescein (FAM) whereas the reverse primer (5'-GGACAGCCAGATATCAACTG-3') was unlabeled. PCR fragments were resolved according to size by gel electrophoresis (3100 Genetic Analyzer; Applied Biosystems, Foster City, CA). Wildtype fragments migrated at the predicted size of 168; shifts to a larger size indicated insertion of additional nucleotides.

The Kaplan-Meier method was used to estimate overall survival (OS) and event-free survival (EFS). All patients were included in the EFS assessment. For patients who did not experience a complete remission, EFS was designated as 0. We fitted univariate and multivariate Cox proportional hazards models to assess the effects of the following patient

and tumor characteristics on OS and EFS: age (> 60 vs ≤ 60 years), sex, ethnicity (black, Asian, Hispanic, or white), antecedent hematologic disorders as defined previously¹², peripheral blood blast count, bone marrow blast count, hemoglobin level, platelet count, and serum bilirubin and creatinine levels. For the multivariate analysis, we fitted a full Cox proportional hazards model on OS or EFS, including all patient characteristics listed above. A reduced model was built by stepwise variable selection such that all variables remaining in the model were significant at a *p* value of < 0.05. All analyses were performed using SAS software (SAS Institute, Cary, NC) and S-plus software (Insightful, Seattle, WA).

Results

There were 128 women and 124 men, with a median age of 62 years (range, 17–88 years); 115 patients were ≤ 60 year old (Table 1). The median duration of clinical follow-up was 10 months (range, < 1–96 months), with the most recent follow-up in January 2009. One hundred ninety-two patients had *de novo* AML, 33 had AML preceded by either a myelodysplastic syndrome (n=27) or chronic myelomonocytic leukemia (n=6) (designated here as secondary AML), and 27 developed AML after chemotherapy for other malignancies (i.e. therapy-related AML). Numerous blasts were found in all BM biopsy specimens, constituting a median of 62% all cells (range, 21%–97%). All patients underwent intensive therapy according to institutional protocols. The therapeutic combinations included idarubicin and cytarabine (n=85), fludarabine and cytarabine (n=49), idarubicin, cytarabine, and sorafenib (n=14), daunorubicin and cytarabine (n=13), decitabine (n=13), clofarabine (n=11), vidaza and decitabine (n=11), daunorubicin, cytarabine, etoposide, dexamethasone and thioguanine (n=7), decitabine, cytarabine, and clofarabine (n=6), high dose cytarabine (n=5), cytarabine, cytoxan, and topotecan (n=4), decitabine cytarabine (n=4), decitabine and mylotarg (n=4), fludarabine, clofarabine (n=3), idarubicin and cytarabine decitabine (n=3), idarubicin, cytarabine, and mylotarg (n=3), idarubicin, clofarabine (n=2), clofarabine and cytarabine (n=2), decitabine and valproic acid (n=2), decitabine plus valproic acid (n=2), idarubicin, cytarabine, and SAHA (n=2), mitoxantrone, cytarabine, and gemtuzumab (n=2), idarubicin, etoposide, and daunorubicin (n=1). Four patients with t(15;17) received all trans retinoic acid (ATRA) including 3 patients who received ATRA in combination with arsenic trioxide and one patient who received a liposomal form of ATRA as a monotherapy. Fifty-one patients underwent hematopoietic stem cell transplantation (allogeneic related, n=30; matched unrelated, n=18; cord blood, n=3). One hundred thirty-six patients (54%) patients experienced a complete remission of which 51 had recurrence; 116 (56%) did not experience complete remission.

After a median follow-up of 10 months (range, < 1–97 months), 151 patients died, including 19 with no evidence of disease. The 3-year OS rate was 30% (95% CI, 26%–34%), and the 5-year OS rate was 22% (95% CI, 18%–26%). There was no difference in age, sex, and survival between patients in the prospective and retrospective groups, which allowed us to combine patients into a single group for analysis.

The conventional cytogenetic analysis showed that 21 patients had favorable-risk, 152 intermediate-risk, and 77 unfavorable-risk cytogenetic results. The 21 patients with favorable results included 9 patients with inv(16)(p13.1q22), 8 patients with t(8;21)

(q22;q22), and 4 patients with t(15;17)(q22;q12). Ninety-four of 152 patients with intermediate cytogenetics had a normal karyotype. *FLT3/ITD* was detected in 53 of 231 (23%) patients tested.

Immunohistochemical analysis showed cytoplasmic NPM in 59 of 252 (23%) AML specimens (Fig. 1), including 48 of 192 (25%) *de novo*, 6 of 33 (18%) secondary, and 5 of 27 (19%) therapy-related AML. No statistically significant difference was detected in the prevalence of cytoplasmic NPM in *de novo* versus other AML cases ($p=0.382$). Cytoplasmic NPM was observed in 33 of 94 (35%) patients with a normal karyotype. No patients with inv(16), t(8;21), or t(15;17) had cytoplasmic NPM. Nuclear NPM was found in 193 cases, 172 of whom had no cytoplasmic NPM expression and 21 that had rare (<5%) immature cells with cytoplasmic NPM.

Cytoplasmic NPM was associated with young age ($p=0.024$), *FLT3/ITD* ($p=0.005$), lack of CD34 expression ($p<0.001$), high blast counts in bone marrow ($p=0.043$) and peripheral blood ($p=0.041$), and high albumin level ($p=0.028$). There was no association between cytoplasmic NPM and race, sex, leukocytosis, hemoglobin level, platelet count, or serum LDH, bilirubin, and creatinine levels.

In the univariate Cox model, prolonged OS was associated with young age ($p<0.001$), *de novo* AML ($p<0.001$), presence of antecedent hematologic disorders ($p<0.001$), favorable cytogenetics ($p<0.001$), hematopoietic stem cell transplantation ($p<0.001$), lower bone marrow blast count ($p=0.014$), lower peripheral blood blast count ($p=0.003$), lower white blood count ($p=0.021$), high platelet count ($p=0.045$), and high serum albumin level ($p<0.001$). No significant difference in OS was detected between patients with AML with cytoplasmic versus nuclear NPM expression, $p=0.822$ (Fig. 2). No significant difference in OS for cytoplasmic versus nuclear NPM was shown for the subset of patients with a normal karyotype and wild-type *FLT3*, $p=0.987$ (Fig. 3). Similar results were obtained when only patients ≥ 60 years old with a normal karyotype and wild-type *FLT3* were analyzed ($p=0.768$).

In the univariate Cox model, long EFS duration was associated with young age ($p=0.0002$), *de novo* AML ($p=0.008$), presence of antecedent hematologic disorders ($p=0.0008$), favorable cytogenetics ($p=0.002$), hematopoietic stem cell transplantation ($p=0.0009$), and high serum albumin level ($p=0.006$). No significant difference in EFS was detected between patients with AML with cytoplasmic versus nuclear NPM expression ($p=0.534$). No significant difference in EFS for cytoplasmic versus nuclear NPM was shown for the subset of patients with a normal karyotype and wild-type *FLT3* ($p=0.562$). Similar results were obtained when only younger patients (< 60 years old) with a normal karyotype and wild-type *FLT3* were analyzed ($p=0.635$).

In the multivariate Cox model, prolonged OS was associated with young age ($p=0.047$), a favorable cytogenetic profile ($p<0.001$), and hematopoietic stem cell transplantation ($p<0.001$). Prolonged EFS was associated with young age ($p=0.014$), a favorable cytogenetic profile ($p<0.001$), and hematopoietic stem cell transplantation ($p=0.002$).

We analyzed the *NPM1* gene for mutations in 104 patients with AML. There were 53 women and 51 men, with a median age of 61 years (range, 17–86 years). In this subset of patients, 78 patients had *de novo* AML, 17 had secondary AML, and 9 had therapy-related AML. Nine patients had favorable-risk, 68 intermediate-risk, and 27 unfavorable-risk cytogenetic results. Forty of the 68 patients with intermediate cytogenetics had a normal karyotype. *FLT3/ITD* was detected in 28 of 90 (31%) patients tested. There was no statistical difference in sex, age, type of AML, cytogenetic findings, or frequency of *FLT3/ITD* between the whole group of patients and this subset of 104 patients.

NPM1 mutations were detected in 40 (38%) cases. Immunohistochemical analysis showed cytoplasmic NPM in 30 of these 40 (75%). Cytoplasmic NPM was also identified in 8 of 64 (13%) AML cases with wild type *NPM1*. The positive predictive value of cytoplasmic NPM was 79%, and the negative predictive value was 85%. The sensitivity of immunohistochemical analysis was 75%, with a specificity of 88%. For the subset of 104 patients with molecular data, *NPM1* mutations as detected by sequencing did not impact OS ($p=0.867$) or EFS ($p=0.561$).

Discussion

NPM1 gene mutation status in AML is a subject of intense interest because several studies have shown that the presence of *NPM1* mutations has a prognostic effect in *FLT3-ITD*-negative AML patients^{5, 6}. The proposal to assign patients with a normal karyotype and wild-type *FLT3* to different therapeutic modalities (chemotherapy with or without allogeneic stem cell transplantation)⁷ has resonated among clinicians leading to the need for all new AML patients to undergo assessment for *NPM1* gene mutations. However, it is unknown whether the association between *NPM1* mutation and clinical variables shown in European studies will be reproduced in a patient population from the United States. Furthermore, *NPM1* mutational analysis which often involves relatively time-consuming sequencing methods may not be available in smaller diagnostic laboratories.

NPM1 mutations alter the carboxy terminus of NPM, leading to its cytoplasmic localization. Falini and colleagues proposed that immunohistochemical staining for NPM can be used as a screening tool for NPM mutational status⁸. In their study, cytoplasmic NPM had a positive predictive value of 100% for *NPM1* mutations. Conversely, nuclear localization of NPM has a negative predictive value of 100% for *NPM1* mutations⁸. These results support the notion that immunohistochemical analysis for NPM expression can be used as a screening tool, or even as a replacement for molecular assessment of *NPM1* gene mutations. For these reasons, we assessed the cellular localization of NPM in a large number of AML patients at our institution. We believe that our patient population is representative because standard prognostic indicators such as age, cytogenetic profile, and *de novo* AML vs. therapy-related or secondary AML were predictive of OS and EFS.

NPM1 gene mutations have been reported in 25%–35% of all adult AML patients and 45%–64% of adult AML patients with a normal karyotype. In this study, cytoplasmic NPM was detected in 23% of all patients, which is a lower frequency than that reported. Surprisingly, cytoplasmic NPM was detected in only 35% of patients with a normal karyotype, which is

substantially lower than what has been previously reported. As the prevalence of *NPM1* gene mutations in pediatric AML cases was reported to be substantially lower in Taiwan¹³ than in European countries,¹⁴ it is possible that geographic differences affect the incidence of *NPM1* gene mutations in adults in a similar manner.

In several European studies, *NPM1* mutations in AML were associated with female sex, *de novo* AML, lack of CD34 expression, high blast count, high serum LDH level, and presence of *FLT3-ITD*^{5, 15}. In this study, we found an association between cytoplasmic NPM and young age ($p=0.024$), *FLT3-ITD* ($p=0.005$), lack of CD34 expression ($p<0.001$), high bone marrow blast count ($p=0.043$), high peripheral blood blast count ($p=0.041$), and high serum albumin level ($p=0.028$). We did not show a correlation between NPM expression and sex. Of interest, no association was found between *NPM1* mutation and gender in another study from the United States reported in abstract form¹⁶. These findings support the hypothesis that geographic differences affect the prevalence of *NPM1* mutations as well as clinical associations.

Previously, *NPM1* mutations were reported to be closely associated with *de novo* AML, as AML arising after a myelodysplastic syndrome, myelodysplastic/myeloproliferative neoplasm or therapy-related AML rarely show cytoplasmic NPM⁸. However, in our study, the prevalence of cytoplasmic NPM was similar in *de novo*, secondary, and therapy-related AML cases. Our findings have been recently confirmed indirectly in a large study focusing on multilineage dysplasia in AML patients¹⁷. This study found a similar frequency of multilineage dysplasia (approximately 30% of cases) among patients with *NPM1* mutations and patients with wild type *NPM1*¹⁷. Taking into consideration the reported frequency of multilineage dysplasia in secondary AML compared with *de novo* AML (45.4% vs. 25.2%, $p<0.001$)¹⁷, we suspect that this study also found a high frequency of *NPM1* mutations among patients with secondary AML, although this was not specifically reported in the published data.

NPM status had no effect on the OS or EFS duration in our study, either in the total patient population or in the subset of patients with a normal karyotype and wild type *FLT3*. The inability of NPM to predict prognosis cannot be attributed solely to the use of immunohistochemical assessment as a surrogate for *NPM1* mutations because we obtained similar results when we analyzed DNA sequencing results, although in a smaller subset of 104 patients. Although our data contradict initial reports stressing the prognostic importance of *NPM1* mutations^{5, 6}, more recent studies have failed to show a prognostic impact of *NPM1* mutations on OS and EFS in AML patient populations including *FLT3-ITD* negative patients^{4, 17, 18}. Although some of these studies have had relatively small numbers of patients,^{4, 18} a study reported by Wandt et al¹⁷ analyzed 1766 patients. The explanation for the discordance of recent studies is unknown, but possible explanations include geographic variation and differences in study design.

We compared DNA sequencing results with those of immunohistochemical analysis and found that immunohistochemical analysis had good sensitivity and excellent specificity. However, both the positive and negative predictive values of cytoplasmic and nuclear NPM, respectively, were substantially lower than that reported by others⁸. NPM immunostaining is

nuclear in approximately 10% of molecularly proven *NPM1*-mutated cases (i.e. “missed” mutations). In addition, NPM cytoplasmic expression occurs in approximately 13% of AMLs without *NPM1* mutation (i.e. “overcalled” mutations). The explanation for the discordance between our results and those of Falini et al are unknown. One possible explanation is that *NPM1* mutations other than those occurring at exon 12 may disrupt nuclear cytoplasmic shuttling of NPM. Albiero and colleagues have reported an AML patient carrying an exon 11 mutation that encoded a truncated form of nucleophosmin with aberrant cytoplasmic localization.^{19,20} Another possibility, however, is that cytoplasmic localization of NPM is not entirely specific for the presence of *NPM1* mutations. Technical issues also complicate our understanding of the problem as bone marrow biopsy specimens can be processed in a myriad of ways. Falini and colleagues used BM biopsy specimens that were fixed in B5 fixative and decalcified by EDTA⁸. We used BM biopsy specimens that were routinely processed at our institution, fixed in formalin and decalcified by formic acid. It is unclear whether formalin fixation or our decalcification process compromises the sensitivity of immunohistochemical assessment of NPM cellular localization. Nevertheless, formalin is the most popular fixative to assess tissue samples in the United States. Being conservative, we conclude that immunohistochemical analysis for NPM cannot be used as a substitute for molecular assessment using our methods.

In summary, we found no correlation between cellular NPM localization detected by immunohistochemistry and patient prognosis in this group of AML patients. The differences between our study and European studies may be related to geographical variation or other differences. In addition, our results suggest illustrate a lack of complete concordance between cytoplasmic NPM detected by immunohistochemistry and direct sequencing of the *NPM1* gene. Therefore immunohistochemistry for cytoplasmic NPM appears not to be a completely reliable screening tool for detecting *NPM1* mutations. The differences between our results and those of others remain to be explained. *NPM1* mutations at sites other than exon 12 or technical differences related to methods of fixation, decalcification and staining are possible explanations.

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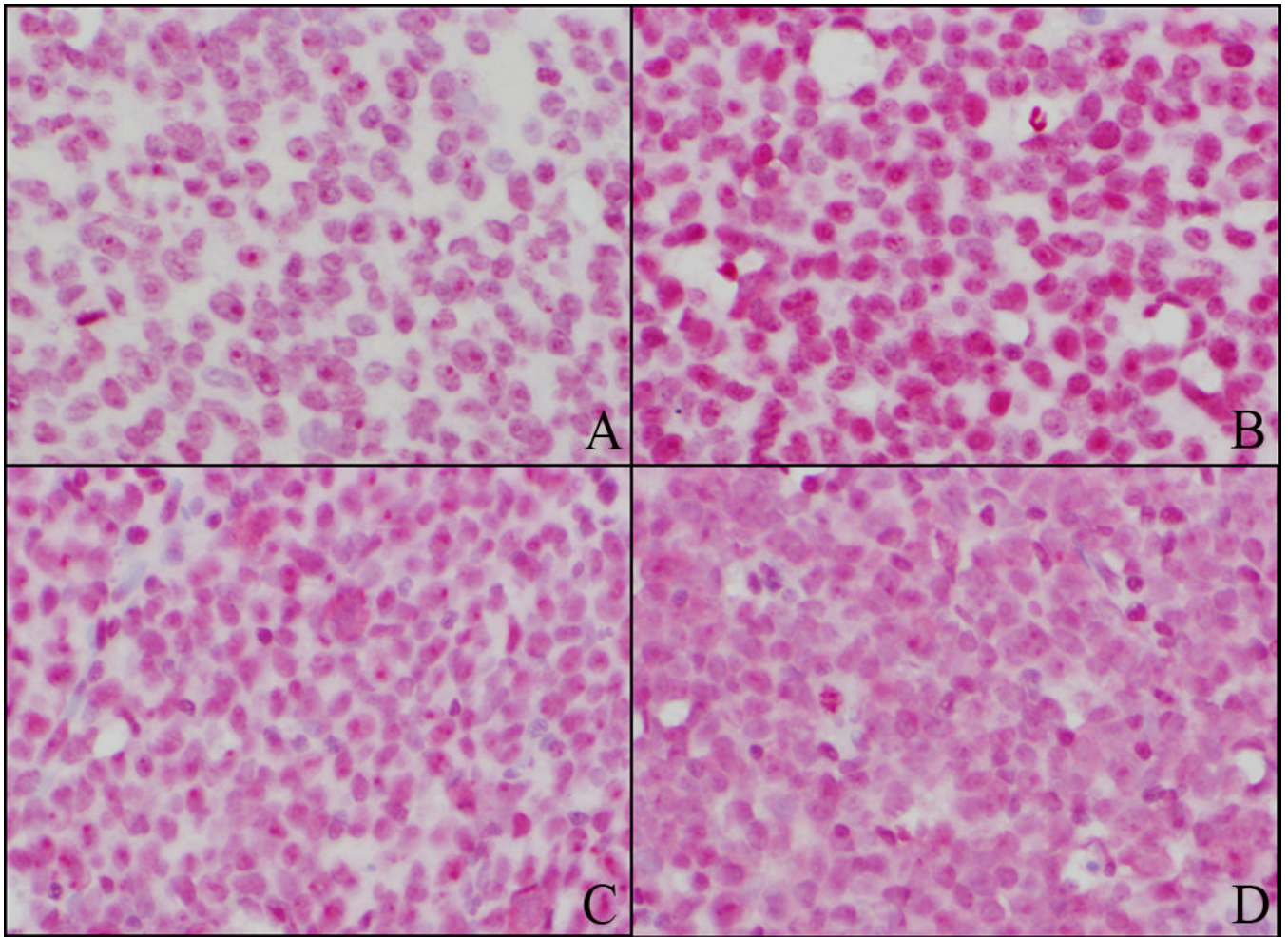


Figure 1.

Examples of acute myeloid leukemia (AML) cases with nuclear (A&B) and cytoplasmic (C&D) NPM expression. Anti-C23 antibody is used as a control and demonstrates nuclear staining in both cases (A and C). In a case of AML with nuclear NPM, anti-NPM antibody is similar to anti-C23 staining pattern (B). In a case of AML with cytoplasmic NPM, anti-NPM antibody demonstrates predominantly cytoplasmic staining (D). (A–D, immunohistochemistry, $\times 400$)

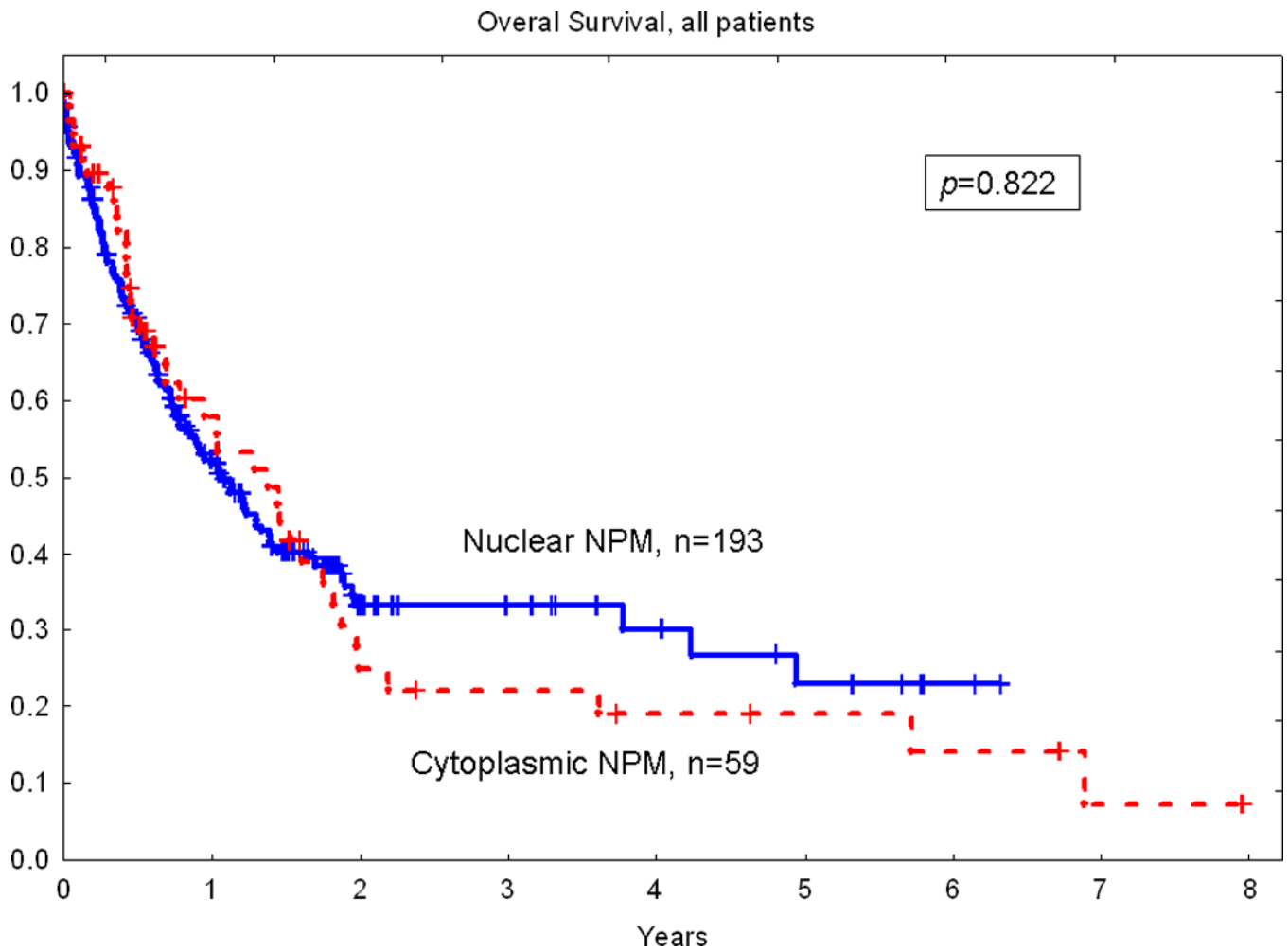


Figure 2.
No significant difference in overall survival between patients with cytoplasmic and nuclear NPM in total study group

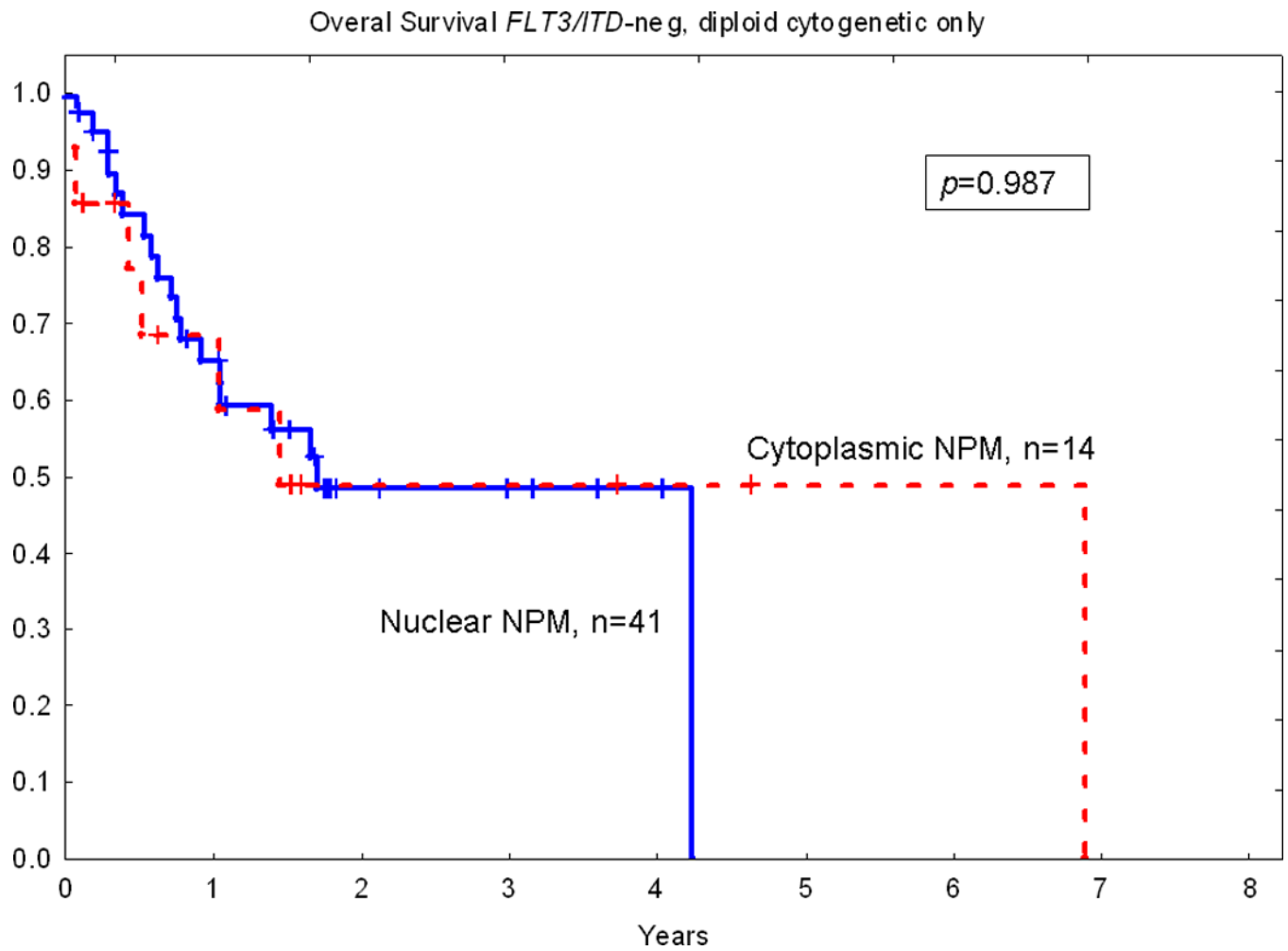


Figure 3. No significant difference in overall survival between patients with cytoplasmic and nuclear NPM in a subset of patients with diploid cytogenetic and wild type *FLT3*.

Table 1

Summary of clinicopathologic features

Variables	Study groups
Age (years)	Median=62 (range: 17–88)
60 year old and younger	115
Sex	
women	128
men	124
Race	
Asian	7
Black	14
Hispanic	33
White	198
Cytogenetics	
Favorable	21
Intermediate	152
Normal karyotype	94
Unfavorable	79
AML	
<i>De novo</i>	192
Preceding MDS or MDS/MPD	33
Therapy-related	27
<i>FLT3</i>	
<i>ITD</i>	53
Wild type	178
Not tested	21
Response	
Complete remission	136
Relapsed	51
Failure	116
Death	
alive	151
dead	101
Follow-up (months)	Median=10 (range, <1–97 months)

Table 2

Correlation of NPM immunohistochemistry staining and DNA Sequencing

DNA Sequencing	NPM IHC	
	Cytoplasmic	Nuclear
Mutant	30	10
Wild type	8	56