

# Incidence and Prognostic Value of NPM1 and FLT3 Gene Mutations in AML with Normal Karyotype

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**Abstract:** NPM1 belongs to a new category of genes that function both as oncogenes and tumor suppressor genes, depending on gene dosage, expression levels, interacting partners, and compartmentalization. Nucleophosmin mutations within exon 12 have been described as the most frequent acquired molecular abnormalities in adult and pediatric acute myeloid leukaemia (AML), mutation can be observed in nearly half of patients with a normal karyotype and is associated with a favorable outcome. NPM1 mutations are characterized by the aberrant cytoplasmic localization of NPM, the absence of CD34, involvement of several cell lineages myeloid, monocytic, erythroid and megakaryocytic but not lymphoid and a high frequency of FLT3-ITD mutation. We aimed to study the prevalence, association with Flt3 mutations, and prognostic impact of NPM1 exon-12 mutations in 71 AML patients with normal karyotype. We studied NPM1 and FLT3 by RT-PCR. NPM1 gene mutation was detected among 34 patients (47.9%) and was associated with a high white blood cell count, involvement of the monocytic lineage, CD34 negativity, and high frequency of FLT3ITD. DFS and OS did not differ between mutated and unmutated NPM patients. Prospective studies are needed to confirm the definitive place of NPM mutation among patients with normal karyotype.

**Keywords:** AML, normal karyotype, NPM1, FLT3.

## INTRODUCTION

Acute myeloid leukemia (AML) is clinically, cytogenetically and molecularly heterogeneous [1]. About 30% of cases carry recurrent chromosomal abnormalities that identify leukemia entities with clinical and prognostic features, [2] and 10% to 15% of AMLs have non random chromosomal abnormalities.

In cytogenetic analysis, 40% to 50% of AMLs show normal karyotype (AML-NK) and biologically and clinically, are the most poorly understood group [1, 3, 4].

Mutation of the nucleophosmin gene (NPM) has recently been described as the most frequent mutation in acute myeloid leukemia [1]. NPM mutation targets 50% to 60% of adult AML-NK [5]. No case of NPM mutation has been reported among AML with recurrent cytogenetic abnormality t(15;17), t(8;21), inv(16), t(16;16) and 11q23 abnormalities or with complex karyotype.

NPM is a protein that shuttles between the nucleus and cytoplasm, [6] is most prominent in nucleoli [7]. NPM is a molecular chaperone [8] that may prevent protein aggregation in the nucleolus and regulate the assembly and transport of peribosomal particles through the nuclear membrane [6]. It is also a target of CDK<sub>2</sub>-cyclin E complexes in centrosome duplication [9] and has been implicated in the regulation of

the alternate-reading-frame protein adenosine diphosphate ribosylation factor (ARF)-P53 tumor suppressor pathway [10-12].

In this study we evaluated the prevalence, association with Flt3 mutations, and prognostic impact of NPM1 exon-12 mutations in 71 AML patients with normal karyotype.

## PATIENTS AND SAMPLES

The study was focused on 71 AML patients (30 patients from hospital Saint Antoine Paris, and 41 from Faculty of Medicine haematology division, Alex. University and Armed Forces hospital in Alex) with normal karyotype (age 17-68 years, median 48.9) treated according to the acute leukemia French association-90 (ALFA 90) or standard cytarabine and daunorubicin protocol over one year. A normal karyotype was defined as more than 20 normal metaphases. Samples were obtained from bone marrow or peripheral blood (with at least 70% circulating blast cells) at diagnosis.

## Cytogenetic Study

Bone marrow samples were aspirated into heparinized syringes and chromosomal analyses by G-banding method was done on bone marrow cells after 1 to 3 days of unstimulated culture [13]. All cases with normal karyotype were confirmed by RT-PCR to exclude common translocations. t(8;21), t(9;11), t(15;17), t(16;16) and inv(16).

## Screening for Mutations of the FLT3

Total RNA was isolated from thawed samples using RNeasy columns (Qiagen, Valencia, CA) and reverse tran-

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scribed. Reverse transcription (RT) was performed followed by polymerase chain reaction (PCR) with primers R5 (5'-TGT CGA GCA GTA CTC TAA ACA-3') and R6 (5'-ATC CTA GTA CCT TCC C AACTC-3') [14]. Using the one-step RT-PCR kit (Invitrogen, Rockville, MD). Products were subjected to electrophoresis on 2.5% agarose gels to identify bands migrating above the expected 365 bp size of the wild-type FLT3 fragment. Dideoxy sequencing was performed on selected samples to confirm FLT3/ITD status. For identification of D835 point mutations. RT-PCR was performed on RNA samples using primers from exon 17 (5'-TTCACAGAGACCTG GCCG-3'), and (5'-TTG CCC CTGACAACATAG-3'). This was followed with EcoRV digestion and electrophoresis on 1.8% agarose gels [15].

**Screening for Mutations of the NPM1 Gene**

Analysis of NPM exon 12 mutation was done as described by Falini *et al.* [5].

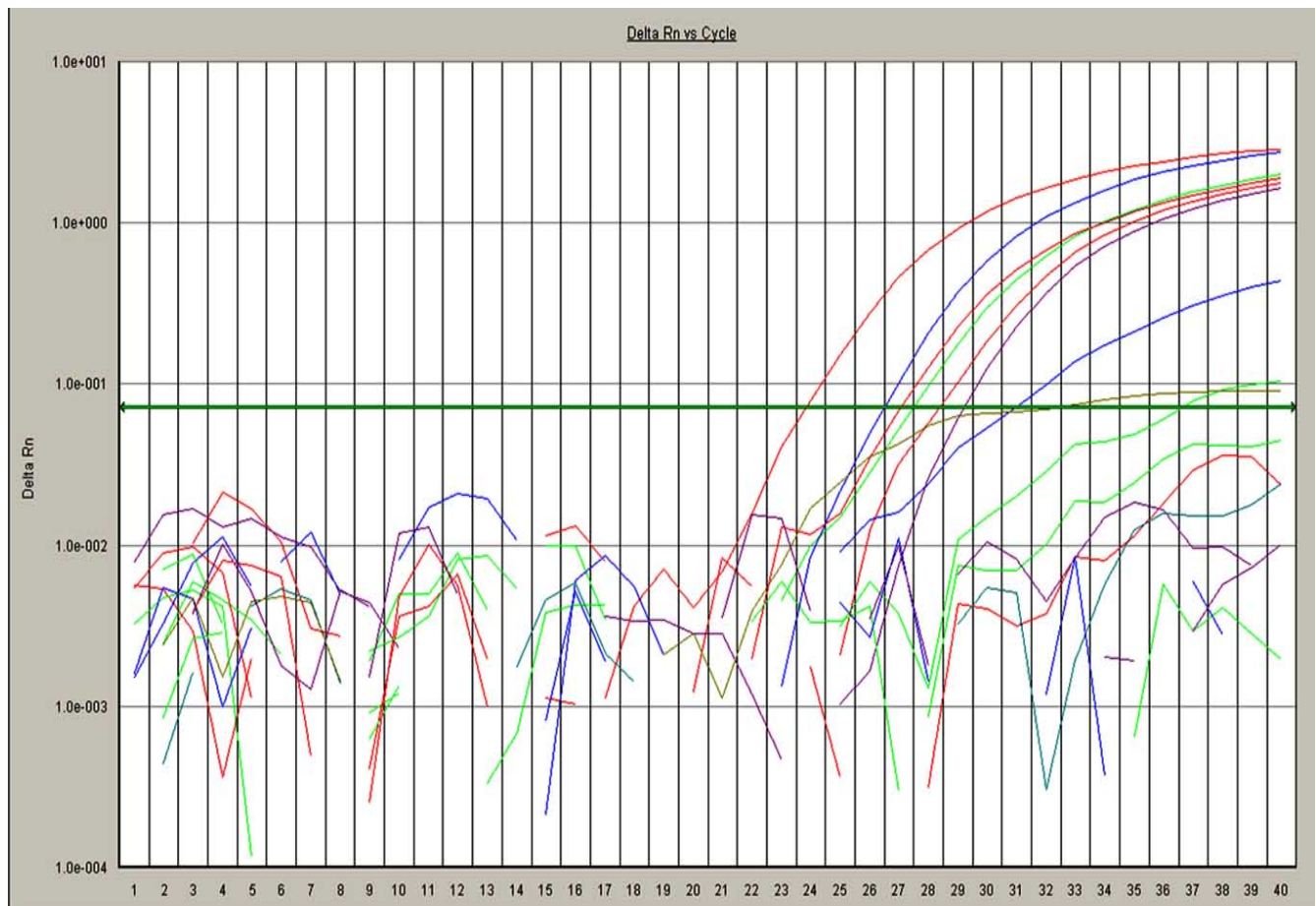
All samples taken at diagnosis were confirmed to contain more than 80% leukemia cells after enrichment by centrifugation and confirmed by microscopic review of Wright-stained, the isolated cells were frozen and stored at -80°C. RNA was extracted with the Qiagen total RNA extraction mini kit (Qiagen, Valencia, CA).

The isolated RNA was reverse transcribed into cDNA, (25°C for 10 minutes, followed 37°C for 69 minute, and

95°C for 5 minute).The final volume for PCR reaction was 35 µL containing 200 ng cDNA, 200 nmol/L deoxynucleotide triphosphate, 2 mmol/L MgSO<sub>4</sub>, 140 nmol/L of each primer, using the primers NPM1-F, (5'-TTAACTCTCTGG-TGGTAGAATGAA-3'), NPM1-R, (5'-CAAGACTATT TGCCATTCCTAAC-3'), and 2µl DNA Specific Master Hybridization Probes labeled with FAM and TAMRA dyes [5], and 1 unit of AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA). Total RNA concentration was quantified with using house keeping gene (GPDH) labeled with different dyes. Real-time polymerase chain reaction (PCR) assays using ABI PRISM<sup>®</sup> 7000 sequence Detection System (Applied Biosystems) was done by heating at 50°C for 2 minutes, 95°C for 10 minutes, followed by 35 cycles of 95°C for 40 seconds, 49°C for 1 minute, and 72°C for 1 minute, with a final step for 10 minutes at 72°C.

Real-time polymerase chain reaction (PCR) assays using ABI PRISM<sup>®</sup> 7000 sequence Detection System (Applied Biosystems) data wereanalyzed using Real-time polymerase chain reaction (PCR) assays using ABI PRISM<sup>®</sup> 7000 sequence Detection.

Amplification curves of RNA extracted from BM of AML patients by Real time PCR. The X-axis represents the PCR cycle number at which the cDNA started the amplification: the Y-axis represents the normalized reporter signal minus the base line signal.



## Statistical Analysis

Survival curves were calculated for overall survival (OS), disease-free survival (DFS) according to Kaplan-Meier and compared using the 2 sided log-rank test. OS and DFS were calculated from time of diagnosis to the end of follow-up. Fisher exact test compared NPM1 mutation status and student *T* test compared NPM1 mutation status and continuous variables. For all analysis, results were significant at a *p* level less than .05 at both sides. SPSS version 15.00 software (Chicago,IL) was used for statistical analysis.

## RESULTS

The study was focused on 71 patients with normal karyotype (Age range 17-68 years median 48.9). 45 patients achieved complete remission, among whom 42 underwent transplantation (26 allogeneic and 16 autologous). NPM1 gene mutation was detected among 34 patients (47.9%). Patients characteristics at presentation are mentioned in Table 1.

### NPM1 Morphology and Immunophenotypes

Frequency of NPM1 gene mutation among FAB subgroups was heterogenous, ranged from 11.5% among FABM1 to 52.9% among FABM5. The mutation was significantly higher among FABM5 compared with the total group. (*P* < 0.001). We didn't find the mutation among FAB M0. NPM1 mutated group had significantly lower expression of CD<sub>34</sub> (*P* < 0.001) in comparison with unmutated NPM1 group.

### NPM and Biologic Parameters

In the NPM1 mutated group, the white blood cell count was significantly higher (mean 40.58 *P* < 0.031) than the unmutated group, there was no significant difference in age and sex ratio between NPM<sup>+</sup> and NPM<sup>-</sup> groups. (*P* > 0.005).

### NPM and Flt3 Gene Mutations

- Among 34 patients who were positive for NPM1, 17(50%) were positive for dual NPM1 and Flt3ITD mutations.
- Flt3 mutation has been detected in 36.6% of our AML NK patients. Patients with FLT3ITD mutations who received BMT had significantly improved DFS in comparison with FLT3ITD positive who were treated with multiple consolidation courses. (*P* < 0.024).

### Prognosis of NPM Mutations in Adult AML with Normal Karyotype

Of the 71 patients, 45 (63.4%) achieved complete remission after induction chemotherapy, there was difference in CR rates between mutated NPM1 and unmutated NPM1 patients not reaching significant level. (67.6% versus 59.5%). CR rate was not influenced by Flt3 mutations.

The global 2 years OS of this study was 56%, there was no significant difference regarding outcome between mutated and unmutated NPM1 groups (82.4% versus 78.4%). No difference in DFS between mutated NPM1 and unmutated NPM1 AML NK patients (58.5% versus 59.5%).

The presence of Flt3 was not a prognostic factor for DFS (NPM<sup>+</sup> Flt3<sup>+</sup> DFS: 58.8%, versus 58.8% in NPM<sup>+</sup> Flt3<sup>-</sup>) (Fig. 1).

A difference in outcome (OS) was observed between NPM<sup>+</sup> Flt3<sup>-</sup> and NPM<sup>+</sup> Flt3<sup>+</sup> 82.4% versus 76.5% without reaching a statistical significance (Fig. 2).

## DISCUSSION

Our study was focused on 71 AML patients with normal karyotype among them 34 (47.9%) patients were positive for NPM mutation, this incidence is nearly similar to that reported by Falini *et al.* who discovered that NPM1 gene mutation target 50 to 60% of adult AML NK [5].

NPM mutations closely associate with de novo AML, as AML secondary to myeloproliferative disorders, myelodysplasia and therapy-related rarely show cytoplasmic NPM mutations [5].

In our study the frequency of NPM mutation among FAB subgroups was heterogenous being markedly higher among FAB M5, this finding coincide with several reports which found that NPM mutation was frequent among FAB groups with monocytic differentiation [16].

There was no sex or age prevalence for NPM mutation where other clinical studies found that NPM mutations are significantly more frequent in females. Cases of NPM<sup>+</sup> are associated with higher white blood cell counts and lower expression of CD<sup>34</sup> [17].

FLT3ITD mutation was seen near twice as often in cases of NPM<sup>+</sup> AML as it was in cases of NPM<sup>-</sup> AML, the close association between Flt3ITD and NPM mutations in clinical practice suggests that these genetic lesions may co-operate to promote leukemic transformation [16-19].

Similar to previous Italian study, there was difference in CR rates between NPM<sup>+</sup> and NPM<sup>-</sup> patients (76.6% versus 59.5%). The association of NPM1 mutation with better response to induction therapy can be explained by the ability of mutated NPM proteins to maintain the dimerization domain and are therefore able to form heterodimers with the wild type NPM1, this may lead to subcellular dislocation of the wild type protein by the mutated NPM1. This altered distribution plays a key role in the regulation of ARF-P53 Pathway and centrosome duplication [20-23].

As it was shown that daunorubicin induce nucleoplasmic dislocation of NPM1, which was associated with increased apoptosis, [24] the subcellular relocation of wild type NPM1 in NPM mutated AML may lead to increase sensitivity to chemotherapeutic agents.

Monitoring of minimal residual disease in AML NK is hampered by the lack of reliable molecular markers. Flt3 and NPM mutations can be used for this purpose, as FLT3 and NPM1 mutations have been detected among 36.6% and 47.9% AML NK patients respectively.

In contrary to Falini study, [25] there was no difference in DFS between NPM<sup>+</sup> and NPM<sup>-</sup> patients and no significant association between NPM<sup>+</sup> and patient outcome (OS). This can be explained by the high association of this mutation with numerous bad prognostic factors such as high WBC count, FAB M5 and the presence of Flt3 mutations.

Most importantly, this study demonstrated that DFS in NPM<sup>+</sup> patients was not influenced by concomitant Flt3

Table 1. Patients Characteristics

Patient N=	Age	Sex	FAB	WBcx10 <sup>9</sup> /L	NPM1	Flt3/ITD	Transplantation
1	67	M	M0	11,42	-	-	1
2	46	M	M2	7,3	+	-	2
3	66	M	M4	44	-	-	0
4	60	M	M5	26	-	-	0
5	50	M	M1	34	-	+	0
6	37	M	M1	42	+	+	2
7	38	F	M1	37,38	-	-	2
8	26	M	M4	2,5	-	-	2
9	35	F	M6	2,3	+	-	2
10	38	M	M5	87	+	-	1
11	54	M	M2	144	-	-	2
12	66	M	M1	126	+	+	1
13	44	M	M2	17,7	-	-	2
14	59	F	M5	64	+	+	1
15	44	M	M0	1,2	-	-	2
16	42	M	M5	49	+	-	0
17	58	M	M4	28	-	+	0
18	59	F	M2	13	-	-	0
19	55	M	M1	39	+	+	0
20	68	F	M1	27	+	+	0
21	20	M	M5	33	+	-	1
22	40	M	M6	2,15	-	-	2
23	45	F	M2	2,21	-	+	0
24	25	M	M1	2,5	+	-	2
25	36	M	M5	62	+	-	1
26	36	M	M1	18	-	-	2
27	44	M	M5	23	-	-	0
28	51	F	M4	43,1	+	-	2
29	39	M	M4	9,5	-	-	0
30	31	M	M4	209	+	+	2
31	37	M	M4	71	+	+	2
32	42	M	M0	13,8	-	-	1
33	46	M	M4	32	+	-	2
34	35	M	M4	1,9	-	-	0
35	25	F	M5	12,7	-	-	2
36	43	M	M1	27,9	-	+	0

Table 1. contd....

Patient N=	Age	Sex	FAB	WBcx10 <sup>9</sup> /L	NPM1	Flt3/ITD	Transplantation
37	38	M	M2	14,2	+	+	2
38	35	M	M1	17	-	-	2
39	29	M	M4	2,4	-	-	0
40	50	M	M6	11,5	+	-	0
41	49	M	M1	16	-	-	1
42	35		M5	56	+	-	2
43	60	M	M1	12,7	+	+	1
44	42	M	M2	13	-	-	0
45	39	F	M1	22	-	-	0
46	51	M	M5	44	+	+	2
47	40	M	M5	35	+	-	1
48	39	F	M4	22	-	+	0
49	63	M	M1	40	+	+	1
50	27	F	M2	1,8	-	-	0
51	55	F	M2	24,2	+	+	1
52	22	M	M2	72,8	+	-	0
53	52	M	M5	7,1	-	-	2
54	43	F	M6	4,3	-	+	0
55	40	F	M1	3,3	+	-	0
56	32	F	M5	53	+	-	1
57	35	M	M0	12,6	-	-	2
58	40	M	M5	49	-	+	2
59	39	F	M4	53	+	-	0
60	39	M	M4	3,4	-	-	0
61	42	F	M2	13,3	-	-	0
62	50	M	M4	17,8	+	+	2
63	57	M	M5	32,5	+	+	1
64	36	M	M4	47	+	+	2
65	42	M	M0	11	-	-	2
66	39	F	M5	22	+	-	1
67	45	M	M4	63	-	+	0
68	50	F	M2	3,6	-	-	0
69	38	M	M4	25	-	-	0
70	50	F	M1	16,4	+	+	1
71	17	F	M4	39	+	+	0

0=no transplantation, 1=autologous, 2=allogeneic.

mutation which is a recognized predictor of bad prognosis [26].

The improved DFS for FLT3/ITD patients who received BMT as consolidation is again consistent with the observation of others [27]. Thus the intensity of treatment could affect the clinical outcome of patients with AML expressing FLT3/ITD mutation.

Finally, NPM mutation is associated with responsiveness to induction therapy, although its role (alone or in combination with Flt3) in predicting the outcome of AML NK after remissions remains to be defined. Prospective studies are needed to confirm the definitive place of NPM mutation among patients with normal karyotype.

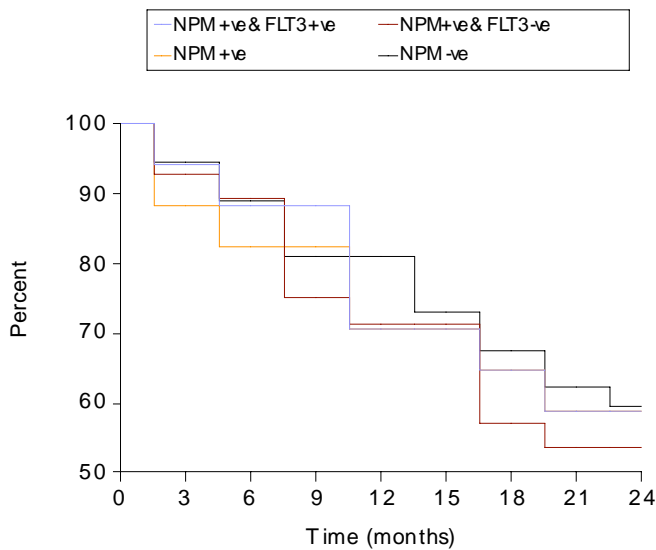


Fig. (1). Kaplan-Meier analysis of AML with normal karyotype bearing mutated or unmutated NPM1 and FLT3 status for DFS. Results were significant at a level of P <0.05.

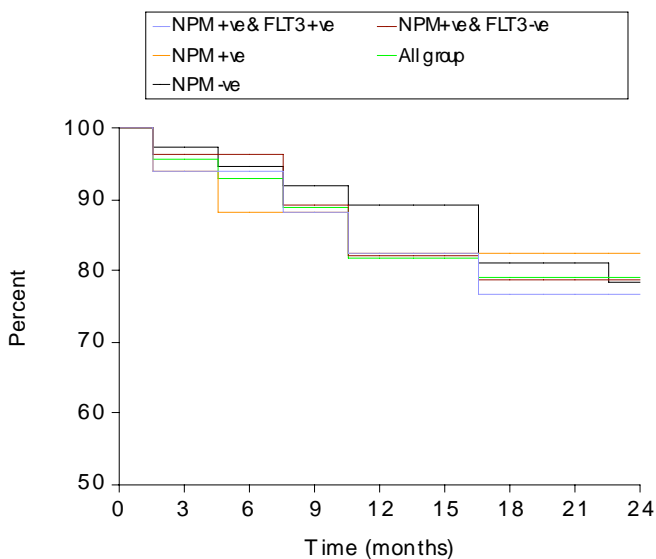


Fig. (2). Kaplan-Meier analysis of AML with normal karyotype bearing mutated or unmutated NPM1 and FLT3 status for OS. Results were significant at a level of P <0.05.

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