

Supplementary Appendix 1

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Falini B, Mecucci C, Tiacci E, et al. Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. *N Engl J Med* 2005;352:254-66.

Appendix 1A

(GIMEMA LAM99P and EORTC AML12 Protocols)

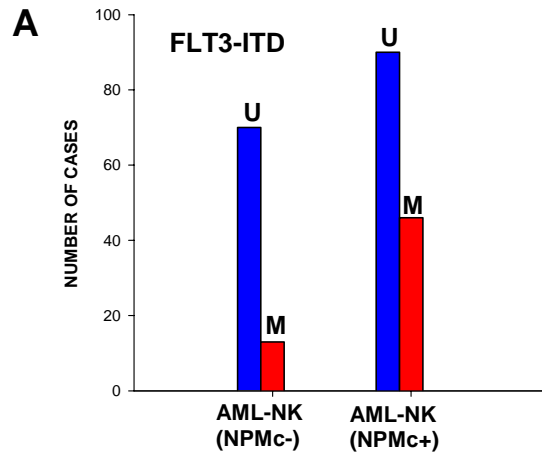
Between 1999 and 2002, the GIMEMA Cooperative Group treatment for adult AML (age:15-60) was based on the GIMEMA LAM 99P protocol which included:

- A 5-day pre-treatment with hydroxyurea (HU) at 2 g/sqm/day from days –4 to 0;
- Induction treatment with a 3-drug regimen: Daunorubicine (DNR) 50 mg/sqm/day on days 1, 3 and 5; Cytosine-Arabinoside (ARA-C) 100 mg/sqm/day on days 1 to 10; Etoposide 100 mg/sqm/day on days 1 to 5; to be repeated in case of partial remission (PR);
- Consolidation therapy with DNR (50 mg/sqm/day on days 4 to 6) and intermediate-doses ARA-C (500 mg/sqm/12 h on days 1 to 6) for patients achieving CR after either the first or the second induction cycle.

Post-consolidation treatment consisted of allogeneic stem cell transplantation (SCT) for patients with HLA-identical siblings, and peripheral blood stem cell autograft for patients without donors.

Since 2001, the GIMEMA Centers have started using the new EORTC AML12 protocol, which includes a double randomization. The first randomization is between two induction schedules, including either standard or high-dose ARA-C. After achieving CR, patients who are not eligible for allogeneic SCT undergo autologous SCT and then are randomized between either rIL-2 maintenance or no treatment. As the EORTC-GIMEMA AML12 study is still ongoing, no data about clinical outcome have been disclosed by the GIMEMA Data Center. Therefore, only clinical and biological characteristics at diagnosis are available for the patients who were studied for subcellular expression of NPM.

Appendix 1B: FLT3 mutations and NPM



Legend: FLT3-ITD mutations according to subcellular NPM expression (NPMc+ vs NPMc-) in AMLs with normal Karyotype (NK). U= Unmutated; M= Mutated.

B

Indep. Var.	Parameter est.	Stand. error	Wald	DF	Sig.	Odds Ratio (OR)	OR 95,0% CI
Age	0.030	0.12	6.560		0.010	1.030	1.007-1.054
Karyotype *	1.781	0.306	33.840	1	0.000	5.936	3.257-10.816
D835(M vs U)	0.417	0.485	0.739	1	0.390	1.518	0.586-3.929
ITD(M vs U)	1.201	0.332	13.057	1	0.000	3.322	1.732-6.371
Constant	- 2.953	0.580	25.949	1	0.000	0.052	

Ind. Var.: Independent variables; est.: estimate; Stand.: Standard; DF: Degree of freedom; Sig: Significance; *: Normal vs abnormal karyotype (excluding major genetic events); M: Mutated; U: Unmutated

Legend: To investigate the relationship between NPM localization and FLT3-ITD/D835 mutations, adjusted for age and cytogenetics, a logistic regression model was applied with these factors as independent variables and NPM as dependent variable. AML with major translocations were excluded because their absolute association with nucleus-restricted NPM expression did not provide a valid parameter estimate. The multivariate

logistic regression model establishes the independent association between cytoplasmic NPM (dependent variable) and FLT3-ITD.

Appendix 1C: NPM mutational analysis in 52 NPMc+

Code GIMEMA Trial	FAB	CD34	Karyotype	FLT3 ITD	FLT3 D835	Type of mutation	cDNA	gDNA	Predicted Protein	Remission cDNA/gDNA
1 181A/16	M2	Neg	NK	U	U	Dup TCTG (+960) Mutation A	Pt: gatctctgTCTGcagTgaggaagTcctctttaagaaaatag	-	Pt: -DLCIAVEEVSLEK.	
2 264A/30	M1	Neg	NK	U	U	Dup TCTG (+960) Mutation A	Pt: gatctctgTCTGcagTgaggaagTcctctttaagaaaatag	Dup TCTG (+960)	Pt: -DLCIAVEEVSLEK.	WT/WT
3 223A/28	M5b	Neg	NK	M	U	Dup TCTG (+960) Mutation A	Pt: gatctctgTCTGcagTgaggaagTcctctttaagaaaatag	-	Pt: -DLCIAVEEVSLEK.	WT/nd
4 367A/31	M1	Neg	NK	M	U	Dup TCTG (+960) Mutation A	Pt: gatctctgTCTGcagTgaggaagTcctctttaagaaaatag	Dup TCTG (+960)	Pt: -DLCIAVEEVSLEK.	WT/nd
5 497A/30	M5a	Neg	NK	M	U	Dup TCTG (+960) Mutation A	Pt: gatctctgTCTGcagTgaggaagTcctctttaagaaaatag	Dup TCTG (+960)	Pt: -DLCIAVEEVSLEK.	WT/nd
6 551A/22	M1	Pos	NK	U	n.a.	Dup TCTG (+960) Mutation A	Pt: gatctctgTCTGcagTgaggaagTcctctttaagaaaatag	-	Pt: -DLCIAVEEVSLEK.	
7 654A/22	M4	Neg	NK	U	U	Dup TCTG (+960) Mutation A	Pt: gatctctgTCTGcagTgaggaagTcctctttaagaaaatag	-	Pt: -DLCIAVEEVSLEK.	
8 66245	M2	Neg	NK	U	M	Dup TCTG (+960) Mutation A	Pt: gatctctgTCTGcagTgaggaagTcctctttaagaaaatag	-	Pt: -DLCIAVEEVSLEK.	WT/nd
9 145A/26	M2	Neg	NK	U	U	Dup TCTG (+960) Mutation A	Pt: gatctctgTCTGcagTgaggaagTcctctttaagaaaatag	-	Pt: -DLCIAVEEVSLEK.	
10 66219	M1	Neg	NK	M	U	Dup TCTG (+960) Mutation A	Pt: gatctctgTCTGcagTgaggaagTcctctttaagaaaatag	-	Pt: -DLCIAVEEVSLEK.	
11 19297	M1	Neg	NK	M	U	No mutations		-		
12 16402	M2	Neg	NK	M	U	Dup TCTG (+960) Mutation A	Pt: gatctctgTCTGcagTgaggaagTcctctttaagaaaatag	-	Pt: -DLCIAVEEVSLEK.	
13 406A/04	M2	Pos	NK	M	U	Dup TCTG (+960) Mutation A	Pt: gatctctgTCTGcagTgaggaagTcctctttaagaaaatag	-	Pt: -DLCIAVEEVSLEK.	
14 511A/08	M5a	Neg	NK	M	U	Dup TCTG (+960) Mutation A	Pt: gatctctgTCTGcagTgaggaagTcctctttaagaaaatag	-	Pt: -DLCIAVEEVSLEK.	
15 515A/19	M1	Neg	NK	U	U	Dup TCTG (+960) Mutation A	Pt: gatctctgTCTGcagTgaggaagTcctctttaagaaaatag	-	Pt: -DLCIAVEEVSLEK.	
16 537A/02	M4	Neg	NK	U	U	Dup TCTG (+960) Mutation A	Pt: gatctctgTCTGcagTgaggaagTcctctttaagaaaatag	-	Pt: -DLCIAVEEVSLEK.	
17 31683	M5b	Neg	del9	U	U	Dup TCTG (+960) Mutation A	Pt: gatctctgTCTGcagTgaggaagTcctctttaagaaaatag	-	Pt: -DLCIAVEEVSLEK.	
18 22887	M2	Neg	NK	U	U	Ins CDTG (+960) Mutation C	Pt: gatctctgCSTGcagTgaggaagTcctctttaagaaaatag	-	Pt: -DLCIAVEEVSLEK.	
19 19948	M2	Neg	failure	M	U	GGAGG->CTCTTGCCC Mutation E	Pt: gatctctgGcagTCTCTGcagTgaggaagTcctctttaagaaaatag	-	Pt: -DLCIAVEEVSLEK.	
20 19872	M1	Neg	n.a.	n.a.	U	Dup TCTG (+960) Mutation A	Pt: gatctctgTCTGcagTgaggaagTcctctttaagaaaatag	-	Pt: -DLCIAVEEVSLEK.	
21 111A/85	M4	Neg	NK	M	U	Dup TCTG (+960) Mutation A	Pt: gatctctgTCTGcagTgaggaagTcctctttaagaaaatag	-	Pt: -DLCIAVEEVSLEK.	
22 750A/28	M5a	Neg	NK	U	U	Ins CATG (+960) Mutation B	Pt: gatctctgCATGcagTgaggaagTcctctttaagaaaatag	-	Pt: -DLCIAVEEVSLEK.	
23 88059	M4	Neg	NK	U	U	Dup TCTG (+960) Mutation A	Pt: gatctctgTCTGcagTgaggaagTcctctttaagaaaatag	-	Pt: -DLCIAVEEVSLEK.	
24 814A/26	M5a	Neg	NK	M	U	Dup TCTG (+960) Mutation A	Pt: gatctctgTCTGcagTgaggaagTcctctttaagaaaatag	-	Pt: -DLCIAVEEVSLEK.	
25 210A/28	M4	Pos	Tris 8	U	U	Dup TCTG (+960) Mutation A	Pt: gatctctgTCTGcagTgaggaagTcctctttaagaaaatag	-	Pt: -DLCIAVEEVSLEK.	
26 114A/04	M4	Neg	NK	U	M	Dup TCTG (+960) Mutation A	Pt: gatctctgTCTGcagTgaggaagTcctctttaagaaaatag	-	Pt: -DLCIAVEEVSLEK.	
27 66298	M5b	Neg	NK	U	U	Ins CCTG (+960) Mutation D	Pt: gatctctgCCTGcagTgaggaagTcctctttaagaaaatag	Ins CCTG (+960)	Pt: -DLCIAVEEVSLEK.	
28 536A/28	M5a	Neg	NK	U	U	Dup TCTG (+960) Mutation A	Pt: gatctctgTCTGcagTgaggaagTcctctttaagaaaatag	Dup TCTG (+960)	Pt: -DLCIAVEEVSLEK.	
29 19108	M5b	Neg	Tris 8	M	M	Dup TCTG (+960) Mutation A	Pt: gatctctgTCTGcagTgaggaagTcctctttaagaaaatag	Dup TCTG (+960)	Pt: -DLCIAVEEVSLEK.	
30 27696	M4	Neg	NK	U	U	Dup TCTG (+960) Mutation A	Pt: gatctctgTCTGcagTgaggaagTcctctttaagaaaatag	-	Pt: -DLCIAVEEVSLEK.	
31 28698	M2	Neg	Failure	M	U	Dup TCTG (+960) Mutation A	Pt: gatctctgTCTGcagTgaggaagTcctctttaagaaaatag	-	Pt: -DLCIAVEEVSLEK.	
32 27900	M4	Neg	NK	U	U	Dup TCTG (+960) Mutation A	Pt: gatctctgTCTGcagTgaggaagTcctctttaagaaaatag	-	Pt: -DLCIAVEEVSLEK.	
33 27926	M5b	Neg	NK	M	U	Ins CATG (+960) Mutation B	Pt: gatctctgCATGcagTgaggaagTcctctttaagaaaatag	-	Pt: -DLCIAVEEVSLEK.	
34 28650	M1	Neg	NK	U	U	Ins CATG (+960) Mutation B	Pt: gatctctgCATGcagTgaggaagTcctctttaagaaaatag	-	Pt: -DLCIAVEEVSLEK.	

Appendix 1D

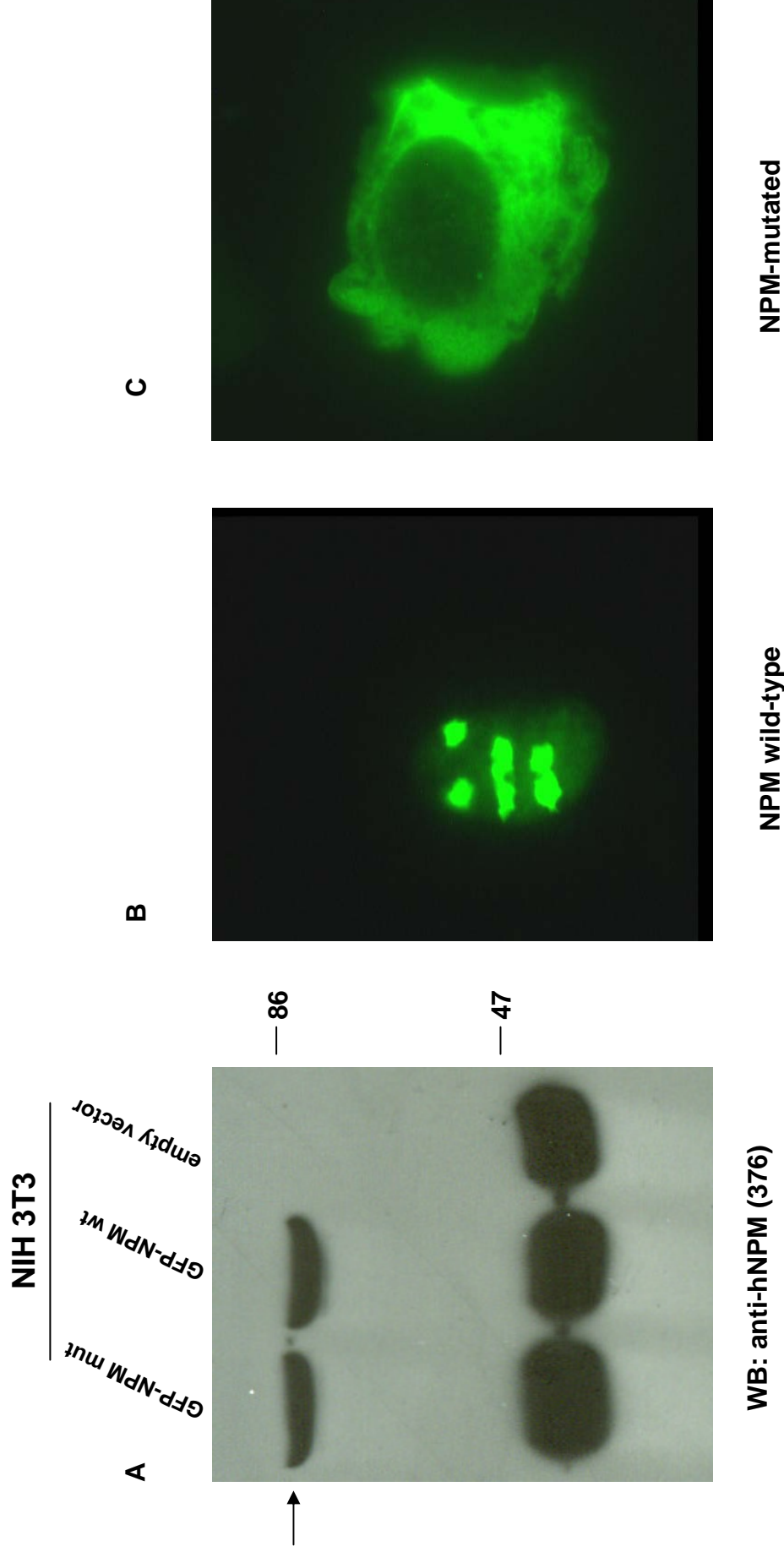


Figure Legends: NIH 3T3 cells were transfected with pEGFP-C1 empty vector and with the GFP fusion construct of normal and mutated human NPM protein. **A:** Cell lysates were used for Western blotting assay. Immunodetection was performed with anti-human NPM mAb (clone 376). Both GFP-NPMwt and GFP-NPMmut proteins were detected (arrow). The lower bands (40KDa) represent endogenous murine NPM protein (an epitope of which is recognized by the 376 clone). **B:** The NPMwt localizes in the nucleoli. **C:** The mutated NPM localizes aberrantly in the cytoplasm.