Prognostic implications of NPM1 mutations and FLT3 internal tandem duplications in Egyptian patients with cytogenetically normal acute myeloid leukemia

Sameh Shamaa[1], Nabil Laimon[2], Doaa A. Aladle[3], Emad Azmy[4], Doaa M. Elghannam[3], Dalia A. Salem[3], Mona M. Taalab[4]

[1]Department of Medical Oncology, Faculty of Medicine, Mansoura University, Egypt, [2]Department of Internal Medicine, Faculty of Medicine, Mansoura University, Egypt, [3]Department of Clinical Pathology, Faculty of Medicine, Mansoura University, Egypt, [4]Department of Clinical Hematology, Faculty of Medicine, Mansoura University, Egypt

Nucleophosmin (NPM1) and fms-like tyrosine kinase 3-internal tandem duplication (FLT3-ITD) gene mutations represent the most frequent molecular aberrations in patients with cytogenetically normal-acute myeloid leukemia (CN-AML). We analyzed the prognostic impact of these mutations and their interactions in adults with CN-AML. NPM1 mutation (NPM1mut) and FLT3-ITD mutation (FLT3-ITD+) were analyzed by polymerase chain reaction and GeneScan assays of bone marrow samples obtained from newly diagnosed 104 CN-AML patients. FLT3-ITD+ and NPM1mut were detected in 36 (34.6%) and 30 (28.8%) out of 104 subjects, respectively, 16 cases (15.4%) had double NPM1mut/FLT3-ITD+. The incidence of FLT3-ITD+ was significantly higher in the NPM1mut group than in the NPM1 wild (NPM1wt) group ($P = 0.018$). Statistical analysis revealed that isolated NPM1mut group had a better clinical outcomes in terms of higher complete response (CR) rate ($P = 0.01$) and a trend towards favorable overall survival (OS) and disease-free survival (DFS) ($P = 0.28$, 0.40, respectively). In contrast, the isolated FLT3-ITD+ group had an unfavorable outcome in terms of lower CR rate ($P = 0.12$), shorter OS, and DFS ($P < 0.0001$ for both). The NPM1mut/FLT3-ITD-group had the best OS and DFS, while the NPM1wt/FLT3-ITD+ group had the worst OS and DFS than other groups (NPM1mut/FLT3-ITD+ or NPM1wt/FLT3-ITD−) ($P < 0.0001$ for both). Multivariate Cox regression analysis showed that age and FLT3/ITD+ were independent poor prognostic factors for OS ($P = 0.006$, <0.0001, respectively), while FLT3/ITD+ was independent predictor for DFS ($P = 0.04$). However, NPM1mut did not have a significant impact on OS and DFS. In conclusion, adult patients with CN-AML carrying isolated NPM1mut and isolated FLT3-ITD+ exhibit different clinical outcomes than those with NPM1mut/FLT3-ITD+ or NPM1wt/FLT3-ITD−. Patients with NPM1mut/FLT3-ITD− had the best prognosis in terms of higher CR, OS, and DFS, while those with NPM1mut/FLT3-ITD+ had the worst CR rate, and NPM1wt/FLT3-ITD+ had the lowest OS and DFS.

Keywords: AML, NPM1, FLT3-ITD, Acute myeloid leukemia, Prognosis

Introduction

Acute myeloid leukemia (AML) is a phenotypically and genetically heterogeneous disease. Furthermore, cytogenetically normal (CN)-AML has been identified as a heterogeneous disease with various mutations mostly in the nucleophosmin (NPM1) and fms-like tyrosine kinase 3 (FLT3) genes.[1]

Correspondence to: Doaa M. Elghannam, Department of Clinical Pathology, Hematology Unit, Faculty of Medicine, Mansoura University, Egypt. Email: doaamahmoud1970@yahoo.com

NPM1, encoded by the NPM1 gene on chromosome 5q35, is a multifunctional nucleocytoplasmic shuttling protein localized primarily in the nucleolus but shuttles rapidly between the nucleus and the cytoplasm. It may assist in ribosomal protein assembly and maintain genomic stability through its participation in DNA repair. However, it is also thought to have a tumor suppressor function and to regulate the p53 pathway through its chaperoning activity.[2–4]

It is suggested that, a loss of nuclear NPM1 function is associated with mutations at exon-12 of its gene.

DOI 10.1179/1607845413Y.0000000085

NPM1 exon-12 mutations can encode mutant proteins with a novel nuclear export signal (NES) motif inserted at the C-terminus and disruption of the nucleolar localization signal due to mutations of tryptophan residues 288 and 290.[5–7] Such mutations are classified according to the type of NES motif inserted into the mutant protein, so far more than 40 different NPM1 mutations have been demonstrated.[8]

NPM1 mutations represent the most frequent mutation in AML and can be detected in about 35% of all patients and in nearly half of patients without cytogenetic aberrations. Of note is that NPM1 is associated with a wide spectrum of morphological subtypes of AML, a normal karyotype, and FLT3 mutations. Patients with CN-AML carrying isolated NPM1mut show better clinical outcomes in terms of the responsiveness to chemotherapy or disease-free survival (DFS).[6,9]

The FLT3 gene maps to chromosome band 13q12, encodes a member of class III receptor tyrosine kinase family, and is expressed in early hematopoietic stem cells and in a subset of dendritic cell progenitors.[10] FLT3 signaling activates intracellular pathways that promote proliferation and inhibition of apoptosis. The most common FLT3 mutation described in AML is the internal tandem duplication (ITD) mutation of the juxtamembrane segment, can be generated by the in frame insertion of 18 to more than 100 bp within the juxtamembrane region of FLT3. This mutation leads to loss of the autoinhibition exerted by the juxtamembrane domain over the tyrosine kinase domain, generating a constitutively active FLT3 molecule.[11,12]

FLT3-ITD mutations are found in 20–30% of patients with AML and are more common in CN-AML. Patients who have FLT3-ITD+ CN-AML have a higher leukocyte count, a complete response (CR) rate similar to that of FLT3-ITD− patients, but shorter DFS and overall survival (OS), mainly because of frequent relapses.[13–15]

The present study investigated the frequency, interactions, and the prognostic effect of both NPM1 and FLT3-ITD mutations in a cohort of 104 newly diagnosed CN-AML patients.

Subjects and methods

Patients

A total of 104 newly diagnosed AML patients who admitted to the Oncology Center, Mansoura University (OCMU) from June 2009 to December 2011 were enrolled in this study. The mean age of the 69 male and 35 female patients was 46.8 ± 17.88 years (range, 19–74 years). AML was diagnosed according to the standard diagnostic methods including clinical, cytomorphological, cytochemical, immunological, and cytogenetic evaluation.[16] Two millitres of ethylenediaminetetraacetic acid anticoagulated venous blood was withdrawn on presentation for molecular studies.

Patient characteristics are given in Table 1. Informed consent was obtained from all patients and approval for this study was obtained from institutional review board. Inclusion criteria for the study were newly diagnosed AML and normal karyotyping by conventional cytogenetics on bone marrow aspirate (BMA) at the time of diagnosis. To establish CN-AML, 20 or more metaphase cells from the samples had to be examined to assure normal karyotypes.[17] Patients with acute promyelocytic leukemia and therapy-related AML were excluded from the study.

Treatment protocol

AML patients <60 years (98 patients) and >60 years old with performance status 0–2 and minimal comorbidity (6 patients) received the standard '3 + 7′ induction chemotherapy protocol: doxorubicin (30 mg/m^2/day) for 3 days and cytarabine (100 mg/m^2/day as a continuous 24 h intravenous infusion) for 7 days.[18] BMA was done between 21 and 28 days after initiation of chemotherapy to demonstrate morphological remission. Consolidation is comprised of 3–4 courses of high-dose cytosine arabinoside (3 g/m^2 every 12 hours on days 1, 3, and 5; total, 18 g/m^2). Patients were followed up once every 3 months with clinical examination and complete blood counts. BMA was done if there was any doubt of a relapse on clinical examination or peripheral smear.

DNA isolation and polymerase chain reaction

Genomic DNA was extracted from the diagnostic BM samples using QIAamp DNA blood minikit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. For the screening of NPM1 exon-12 mutations, single-step fluorescent polymerase chain reaction (PCR) was done using primers NPM1 11f: 5′-CTGGTAGAATGAAAAATAGAT-3' (6 FAM labeled), and NPM1 12r: 5'-CTTGGCAATAGA ACCTGGAC-3' (Applied Biosystems, Foster City, CA, USA). The first primer was fluorescently labeled to enable post-PCR gene scan analysis. PCR reactions were performed in a total volume of 25 μl containing 20–50 ng of extracted DNA (1 μl), 10 pmol of each primer (Applied Biosystems), 12.5 μl of Maxima hot

Table 1 Clinical characteristics of the studied patients

Parameter	Range	Median	Mean ± SD
Age (years)	19–74	45.3 ± 19.23	46.8 ± 17.88
TLC (×10^9/l)	3.1–435	45.95	67.65 ± 83.19
Hemoglobin (g/dl)	5.1–11.1	7.55	7.67 ± 1.5
Platelet (×10^9/l)	5–150	35.7	50.38 ± 43.68
Peripheral blood blast (%)	9–98%	68%	61.88 ± 24.92
Bone marrow blast (%)	20–96%	77%	72.25 ± 20.39

start PCR master mix 2X (Thermo Scientific) (containing Maxima hot Taq DNA polymerase, hot start PCR buffer, 400 μM of each dNTP, and 4 mM Mg + 2), and the reaction was completed to 25 μl using molecular grade water. The amplification was carried out in a GeneAmp PCR System 9700 (Applied Biosystems) using the following steps: preactivation at 95°C for 4 minutes, 35 cycles at 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 60 seconds, and post-extension steps: 94°C for 30 seconds and 60°C for 45 seconds.

To detect FLT3/ITD, exon-11 and exon-12 were amplified by single-step fluorescent PCR using primers 11f (5'-GCAATTTAGGTATGAAAGCC AGC-3') and 12r (5'–CTTTCAGCATTTTGACG GCAACC-3') (Applied Biosystems). PCR reactions were performed in a total volume of 25 μl containing the same contents used as in the NPM1 PCR above apart from FLT3 primers. The amplification was done using the following steps: preactivation at 95°C for 4 minutes, 30 cycles at 95°C for 30 seconds, 62°C for 30 seconds, and 72°C for 30 seconds, and final extension at 72°C for 5 minutes.

Capillary gel electrophoresis and interpretation of results

To detect the precise PCR product size by Gene Mapper, 1 μl of fluorescently labeled PCR product was mixed with 10 ml of deionized HiDiformamide (Applied Biosystem) and 1 ml of genescan internal size standard (Applied Biosystem). The mixture was electrophoresed through an acrylamide containing polymer, POP4 (Applied Biosystems), which was then analyzed using an ABI 310 Genetic Analyzer via gene mapper software. The expected peak size for the wild-type (WT) FLT3 PCR product is 330 bp. FLT-3 ITD fragments could be 18–108 bp larger than this. Only ITD-positive cases are reported if the ITD represent at least 5% of the peak area of FLT3 WT fragment. As regard WT NPM1, its PCR product expected peak size is 287 bp, while NPM1 mutant fragment is usually 4 bp larger.

Criteria of response and survival definitions

CR was defined as a normocellular BM containing less than 5% blasts and showing evidence of normal maturation of other BM elements, with neutrophil count of $\geq 1 \times 10^9$/l and a platelet count of $\geq 100 \times 10^9$/l. Remission failures were classified as either partial remission (defined as 5–15% blasts or 5% blasts but a hypocellular BM), resistant disease (RD > 15% blasts in the BM), or induction death (ID, related to treatment or hypoplasia). OS was defined as the time from diagnosis to date of death. For patients achieving CR, DFS was the time from the date of first CR to an event (death in first CR or relapse).

Statistical analysis

Data entry and analyses were performed using Excel program and statistical package of social science (SPSS) version 10 (Chicago, IL, USA). The quantitative data were presented as a mean, standard deviation (SD), median, and range. Student's *t*-test was conducted to compare the mean of continuous variable for two different groups of individuals. One-way analysis of variance procedure was used to compare means and SD of more than two groups. The qualitative data were presented as number and percentage. The χ^2 was used to find the association between variables of qualitative data. Kaplan–Meier survival analysis was used to find out OS and DFS with log-rank test for comparisons of factor probably affecting survival. Hazard ratio (HR) and confidence intervals (CI) were calculated using Cox regression analysis for multivariate analysis. The *P* values of ≤0.05 and ≤0.001 indicate significant and highly significant results, respectively, at CI 95%.

Results

Frequencies of FLT3 and NPM1 mutations

Among the 104 studied patients, 30 patients (28.8%) were NPM1mut and 36 (34.6%) were FLT3-ITD+. Isolated NPM1mut was detected in 14 of 104 (13.5%) and isolated FLT3/ITD+ was detected in 20 of 104 patients (19.2%). On the other hand, 16 patients (15.4%) were positive for both mutations (NPM1mut/FLT3-ITD+), and 54 patients (51.9%) were negative for both mutations (NPM1wt/FLT3-ITD−) (Table 2). The incidence of FLT3-ITD+ was higher in the NPM1mut group than in the NPM1wt group (53.3 vs. 27%; $P = 0.018$). Examples of NPM1mut and FLT3-ITD+ results detected by gene mapper are shown in Figs. 1 and 2.

Gene mutations and clinical/laboratory characteristics

To follow the recently updated National Comprehensive Cancer Network (NCCN) guidelines, we categorized the patients into four groups: NPM1mut/FLT3-ITD − , NPM1mut/FLT3-ITD + , NPM1wt/FLT3-ITD + , NPM1wt/FLT3-ITD−.

Table 2 Frequency of nucleophosmin and FLT3 mutations

FLT3and NPM1 status	No. (total = 104)	Percentage
NPM1mut	30	28.8
FLT3-ITD+	36	34.6
NPM1mut/FLT3-ITD−	14	13.5
NPM1mut/FLT3-ITD+	16	15.4
NPM1wt/FLT3-ITD+	20	19.2
NPM1wt/FLT3-ITD−	54	51.9

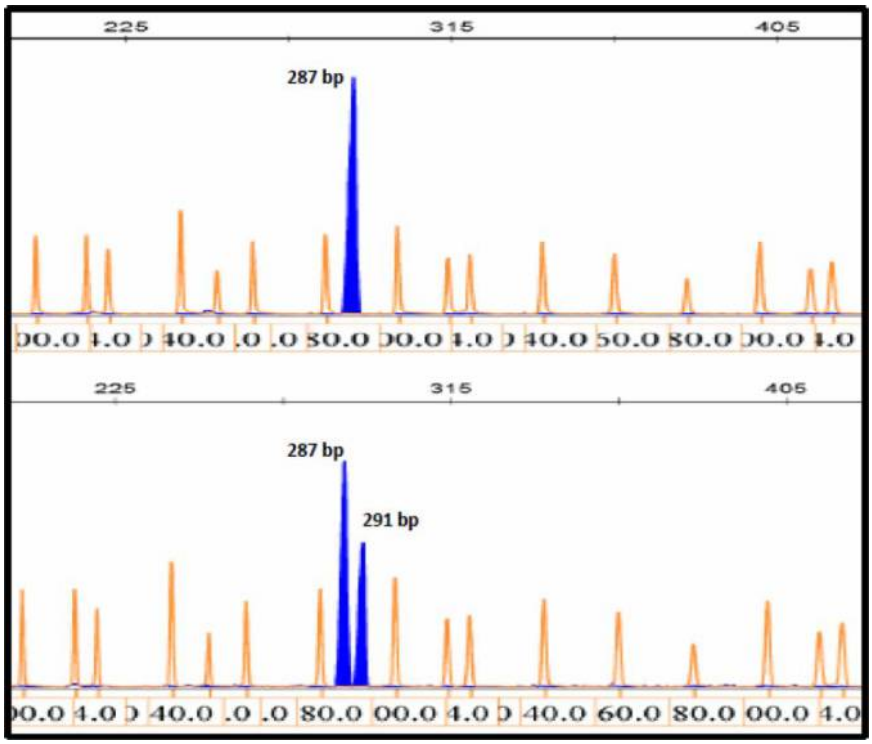

Figure 1 PCR product of AML patients with NPM1mutation. Upper electropherogram shows the WT NPM1 (287 bp) and the lower one shows the mutant NPM1 with the additional 4 bp.

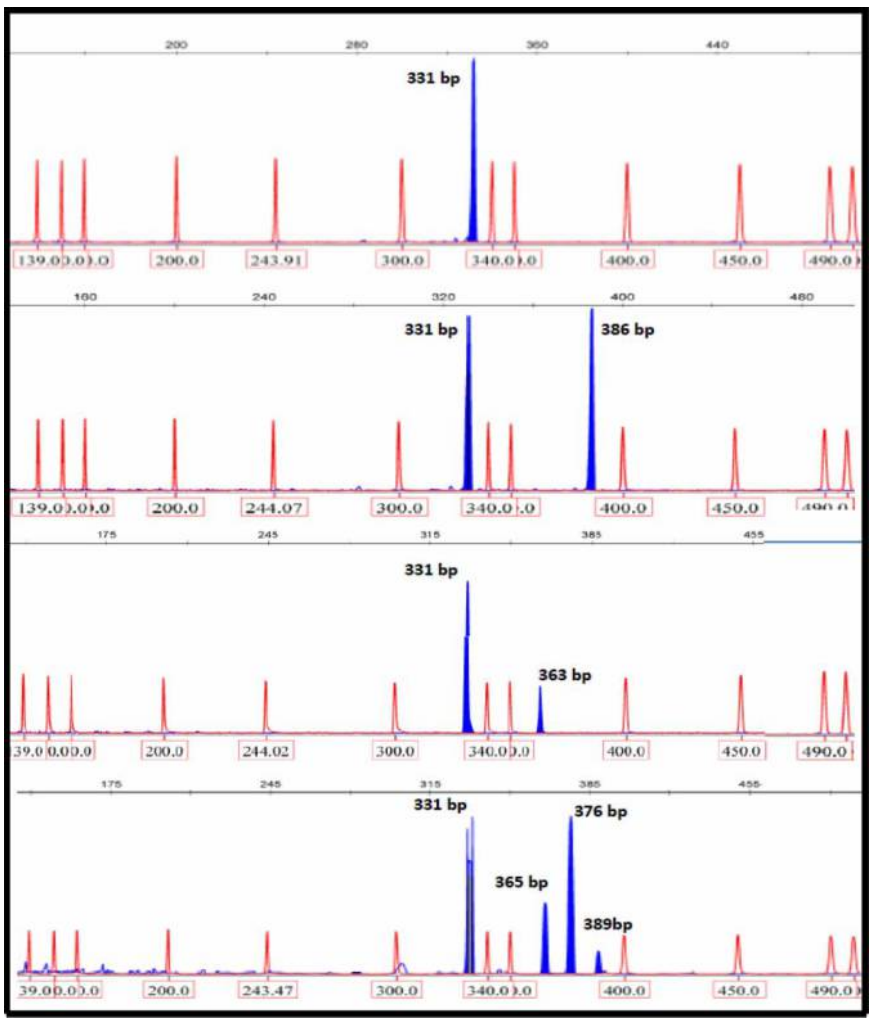

Figure 2 PCR product of AML patients with FLT3/ITD mutation having three mutants. Upper electropherogram shows the WT FLT3 (331) while the others show examples of different sizes FLT3-ITD.

Table 3 shows the baseline characteristics of these groups. There was no statistically significant difference among the groups with regard to age and gender. The NPM1mut/FLT3-ITD+ group showed the highest initial white blood cell (WBC) count and lowest hemoglobin (Hb) concentration with no statistical significance ($P = 0.59$, 0.42, respectively). Meanwhile, NPM1wt/FLT3-ITD+ group showed the highest mean platelet count with no statistical significance ($P = 0.50$) and highest BM blasts with a statistical significance ($P = 0.04$).

Response to induction therapy

Of the 104 patients, 62 (59.6%) achieved CR, 26 patients (25%) had RD and failed to achieve CR after the first round of induction chemotherapy, hence received re-induction chemotherapy. ID was reported in 16 of 104 patients (15.4%).

Looking at each of the two studied molecular markers separately, it is found that FLT3-ITD+ patients has significantly lower CR, higher RD, and higher ID (33.3, 22.2, and 44.4%, respectively) rates than FLT3-ITD− patients (73.5, 26.5, and 0%, respectively) ($P < 0.0001$). On the contrary, there was no significant change in the CR, RD, or ID rates between NPM1mut (60, 26.7, and 13.3%, respectively) and the NPM1wt (59.5, 24.3, and 16.2%, respectively) groups ($P = 0.923$). Within the NPM1wt group, the FLT3-ITD+ patients showed a lower CR rate compared with the FLT3-ITD− patients (40 vs. 66.7%, $P = 0.12$). On the other hand, within the FLT3-ITD− patients, the CR rate was significantly higher in patients with NPM1mut than without them (100 vs. 66.7%, $P = 0.01$) (Table 4). CR rate was also significantly negatively associated with age and WBCs count ($P = 0.03$ and 0.02, respectively) (data not shown).

When the two gene mutations were simultaneously analyzed, the highest CR rate was achieved in the NPM1mut/FLT3-ITD-group (14/14; 100%) while the lowest CR rate were found in NPM1mut/FLT3-ITD+ group (4/16; 25%). RD was higher in the NPM1mut/FLT3-ITD+ group (8/16; 50%) than in the NPM1wt/FLT3-ITD− (18/54; 33.3%). ID was higher in the NPM1wt/FLT3-ITD+ group (12/20;

Table 3 Patients' characteristics according to the NPM1 and FLT3 mutational status

Parameter	NPM1 mut/FLT3-ITD−	NPM1 mut/FLT3-ITD+	NPM1wt/FLT3-ITD+	NPM1wt/FLT3-ITD−	*P*
Patients' no. (%)	14 (13.4%)	16 (15.3%)	20 (19.2%)	54 (51.9%)	
Age (years)					
Mean	49.3 ± 14.2	44.6 ± 16.2	45.73 ± 18.33	44.16 ± 14.25	0.49
Range	23–73	19–74	19–74	21–73	
Sex					
Male	9 (64.3%)	10 (62.5%)	15 (75%)	35 (64.8%)	0.51
Female	5 (35.7%)	6 (37.5%)	5 (25%)	19 (35.2%)	
WBC (10^3/l)					
Mean	28.5 ± 21.5	80.7 ± 63.3	65.7 ± 33.1	72.7 ± 105.6	0.59
Range	3.1–64	25.3–244	37.9–133	3.7–435	
Hb (g/dl)					
Mean	7.28 ± 1.29	7.16 ± 1.18	7.78 ± 1.40	7.89 ± 1.37	0.42
Range	5.4–9.4	5.8–9.2	5.1–11.1	5.1–11.1	
Platelets (10^9/l)					
Mean	50.4 ± 25.74	45.48 ± 21.2	66.42 ± 29.7	42.78 ± 27.8	0.50
Range	11–97	19–120	12.4–192	7.7–140	
BM blasts (%)					
Mean	55.2 ± 26.4	78.7 ± 10.01	82.8 ± 10.3	71.75 ± 21.5	0.04
Range	20–90	60–96	70–95	20–95	

Table 4 Outcome data according to NPM and FLT/ITD mutational status

Groups	Fate			*P*
	CR	RD	ID	
NPM1mut	18/30 (60%)	8/30 (26.7%)	4/30 (13.3%)	0.923
NPM1wt	44/74 (59.5%)	18/74 (24.3%)	12/74 (16.2%)	
FLT3-ITD+	12/36 (33.3%)	8/36 (22.2%)	16/36 (44.4%)	<0.0001
FLT3-ITD−	50/68 (73.5%)	18/68 (26.5%)	0/68 (0%)	
NPM1mut/FLT-ITD − *	14/14 (100%)	–	–	<0.0001
NPM1mut/FLT3-ITD +	4/16 (25%)	8/16 (50%)	4/16 (25%)	
NPM1wt/FLT3-ITD + **	8/20 (40%)	–	12/20 (60%)	
NPM1wt/FLT-ITD − *,**	36/54 (66.7%)	18/54 (33.3%)	–	

CR, clinical remission; RD, resistant disease; ID, induction death.
**P* value as regards CR rate for these two groups = 0.01.
***P* value as regards CR rate for these two groups = 0.12.

Table 5 OS and DFS according to the FLT3 and NPM1 mutational status

Mutational status	Mean OS (months)	*P*	Mean DFS (months)	*P*
NPM1wt	10.8	0.281	12.6	0.409
NPM 1mut	15.5		16.3	
FLT3/ITD−	16.1	<0.0001	17.3	<0.0001
FLT3/ITD+	4.9		7.6	
NPM1mut/FLT3-ITD−	26.9	<0.0001	26.0	<0.0001
NPM1mut/FLT3-ITD+	6.2		11.9	
NPM1wt/FLT3-ITD+	2.9		1.5	
NPM1wt/FLT3-ITD−	13.3		14.7	

60%) than in the NPM1mut/FLT3-ITD+ group (4/16; 25%) with a statistical significance among the groups ($P < 0.0001$) (Table 4).

Multivariate logistic regression analysis showed that age, WBCs count, and FLT3-ITD were independent unfavorable factors for achieving CR while NPM1mut was an independent favorable predictor of CR (data not shown).

Survival analysis

The median follow-up time was 30 months. FLT3-ITD+ group showed a shorter mean OS (4.9 vs. 16.1 months) and DFS (7.6 vs. 17.3 months) than the FLT3/ITD group with a significant statistical difference ($P < 0.0001$ for both) (Table 5 and Fig. 3). On the other hand, NPM1-mutated patients showed a trend of higher OS (15.5 vs. 10.8 months) and DFS (16.3 vs. 12.6 months) than the NPM1wt group with no statistical difference ($P = 0.281$, 0.409, respectively) (Table 5 and Fig. 4). Survival analysis of the four genotypes revealed that the NPM1wt/FLT3-ITD+ group had the longest OS and DFS (26.9, 26.0 months, respectively), and the NPM1wt/FLT3-ITD+ group had the shortest OS and DFS (2.9, 1.5 months, respectively) with a statistical significance among the four groups ($P < 0.0001$) (Table 5).

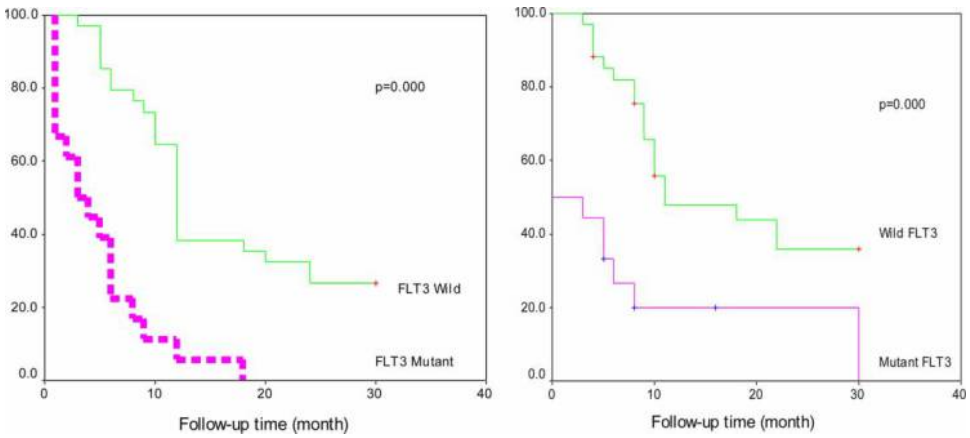

Figure 3 Kaplan–Meier curve of OS and DFS according to FLT3/ITD mutations.

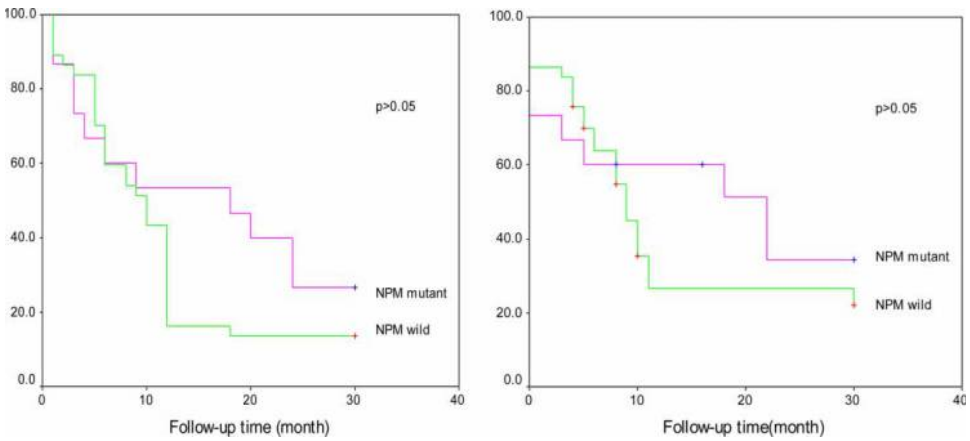

Figure 4 Kaplan–Meier curve of OS and DFS according to NPM1 gene mutations.

Table 6 Multivariate analysis for OS and DFS

Overall survival		
	HR (95% CI)	*P*
Age (years)	2.96 (1.35–6.46)	0.006
TLC		
$20–100 \times 10^9$/l	1.33 (0.55–3.20)	0.51
$>100 \times 10^9$/l	0.93 (0.26–3.26)	0.91
FLT3/ITD mutation	5.72 (2.55–19.83)	0.000
Disease-free survival		
	HR (95% CI)	*P*
Age (years)	1.69 (0.45–6.35)	0.43
TLC		
$20–100 \times 10^9$/l	1.33 (0.47–3.78)	0.51
$>100 \times 10^9$/l	1.49 (0.25–8.80)	0.56
FLT3/ITD mutation	5.72 (2.55–19.83)	0.04

Multivariate Cox regression analysis with stepwise selection showed that age ($P = 0.006$) and FLT3/ITD+ ($P < 0.0001$) were independent poor prognostic factors for OS, while only FLT3/ITD+ was independent predictor for DFS ($P = 0.04$) (Table 6). In contrast, NPM1 mutation failed to be an independent prognostic factor for either OS or DFS.

Discussion

We evaluated the prevalence and prognostic impact of NPM1mut, FLT3-ITD+, and their interactions in adult patients with CN-AML. The incidence of FLT3-ITD+ in our patients was 34.6%, which was obviously higher than that reported by some studies (19.7–28%),[19–21] but consistent with that reported by others (30%, 31%).[22,23] On the contrary, the frequency of NPM1mut in our study (28.8%) was lower as compared with most of the previously published studies that demonstrated 45–64% incidence of NPM1mut.[24,25] However, de Jonge *et al.*[26] reported closer frequency of NPM1 mutation to our results (25%). The different incidence may be due to smaller sample number in our study, ethnic variation, higher background of WT allele, or a lower percentage of FLT3/ITD or NPM1 mutation-positive cells in some cases.

The incidence of FLT3-ITD+ was significantly higher in the NPM1mut group (16/30) than in the NPM1wt group (20/74). Previous studies have reported a considerable correlation between these two mutations, suggesting that they are secondary events from a primary process that predisposes myeloid stem and progenitor cell errors in DNA replication.[22,27] Others have demonstrated that the NPM1mut/WT ratio is higher than the FLT3-ITD+/WT ratio, suggesting that NPM1mut occurs prior to FLT3-ITD+ in cases with both mutations.[6]

Certain associations between the two gene mutations and other laboratory findings have been reported. We could demonstrate a non-significantly increased WBC count in the NPM1mut/FLT3-ITD+ group ($P = 0.59$) as well as a significant increased percentage of BM blasts in the NPM1wt/FLT3-ITD+ group ($P = 0.04$). Other studies revealed that both NPM1 and FLT3-ITD mutations were associated with a significantly higher WBC count as well as an increased percentage of BM blasts.[6,13,14,20] Although the effect of FLT3/ITD on inducing leukemogenesis was not directly proved, the ligand-independent constitutive activation of FLT3 induced by ITD mutation could activate some downstream signal molecules which contribute to cell proliferation and survival advantages.[28–30]

In our study, CR rate was statistically lower in FLT3-ITD+ (33.3%) than that in FLT3-ITD− (73.5%) subjects. Many studies have shown that FLT3/ITD has an unfavorable prognostic impact in adult patients with AML. FLT3/ITD contributes to a short CR duration and lower CR rate.[6,20] On the other hand, our results revealed that, NPM1mut did not show a significant impact on CR rate. Data previously published on the prognostic impact of NPM1 mutations in CN-AML have been somewhat controversial, with some studies showing a significant effect on CR,[22,31] while others did not reveal this significant effect.[24] When the two gene mutations were analyzed together, the highest CR rate was observed in the NPM1mut/FLT3-ITD− group (100%), followed by the NPM1wt/FLT3-ITD− group (66.7%), then the NPM1wt/FLT3-ITD+ group (40%), and lowest CR rate was observed in the NPM1mut/FLT3-ITD+ group (25%). These results were comparable with those concluded by earlier reports.[9,20]

Survival analysis demonstrated a significant worse mean OS and DFS in FLT3-ITD+ subjects ($P < 0.0001$ for both). Scholl *et al.*[32] also studied the impact of FLT3-ITD on the achievement of OS and DFS and declared that FLT3-ITD+ was associated with inferior 2-year DFS and OS. Subjects with NPM1mut did not show better impact on OS or DFS in our study. This lacking effect is mainly due to a high relapse rate as these groups are studied depending on NPM1 mutation solely regardless of the presence of FLT3-ITD, and NPM1 mutation might be a favorable prognostic factor for OS and DFS in the presence of FLT3-ITD. These data correlated with a previous publication that could not demonstrate a difference of DFS and OS for patients with NPM1mut.[32] However, others have reported that OS and DFS are significantly better in the NPM1mut group.[33,34]

Survival analyses of the four genotypes revealed that the mean OS and DFS were significantly better in the NPM1mut/FLT3-ITD− group (26.9, 26.0 months) and worse in NPM1wt/FLT3-ITD+ (2.9, 1.5 months) than the other groups ($P < 0.0001$). Some

studies have also shown that the 5-year DFS and OS of CN-AML patients with NPM1mut/FLT3-ITD− are comparable to those with favorable cytogenetics,[35,36] suggesting that this group of patients could be considered a favorable group.

Multivariate analysis has been done to evaluate other factors influencing OS and DFS. It identified age and FLT3-ITD+ as independent prognostic factors for OS, meanwhile FLT3/ITD+ was the only independent predictor for DFS. Others identified FLT3-ITD+ and NPM1mut as independent prognostic factors for OS and DFS.[6,9] Therefore, the NCCN guidelines for AML consider patients with isolated NPM1mut as a favorable-risk group. Further, patients carrying isolated FLT3-ITD+ constitute a poor-risk cytogenetic group.[37] However, other studies have reported variable results about the clinical outcomes according to the presence of these mutations.[25,38]

In conclusion, adult patients with CN-AML carrying isolated NPM1mut or FLT3-ITD+ showed different clinical outcomes than those bearing both mutated or WT NPM1 and FLT3. Patients with NPM1mut/FLT3-ITD− had the best prognosis in terms of higher CR, OS, and DFS while those with NPM1mut/FLT3-ITD+ had the worst CR rate and NPM1wt/FLT3-ITD+ had the lowest OS and DFS.

References

1 Avivi I, Rowe JM. Prognostic factors in acute myeloid leukemia. Curr Opin Hematol. 2005;12:62–7.
2 Colombo E, Marine JC, Danovi D, Falini B, Pelicci PG. Nucleophosmin regulates the stability and transcriptional activity of p53. Nat Cell Biol. 2002;4:529–33.
3 Kurki S, Peltonen K, Latonen L, Kiviharju TM, Ojala PM, Meek D, *et al.* Nucleolar protein NPM interacts with HDM2 and protects tumor suppressor protein p53 from HDM2-mediated degradation. Cancer Cell. 2004;5:465–75.
4 Lee SY, Park JH, Kim S, Park EJ, Yun Y, Kwon J. A proteomics approach for the identification of nucleophosmin and heterogeneous nuclear ribonucleoprotein C1/C2 as chromatin-Binding proteins in response to DNA double-strand breaks. Biochem J. 2005;388:7–15.
5 Falini B, Bolli N, Shan J, Martelli MP, Liso A, Pucciarini A, *et al.* Both carboxy-terminus NES motif and mutated tryptophan(s) are crucial for aberrant nuclear export of nucleophosmin leukemic mutants in NPMc+ AML. Blood. 2006;107:4514–23.
6 Thiede C, Koch S, Creutzig E, Steudel C, Illmer T, Schaich M, *et al.* Prevalence and prognostic impact of NPM1 mutations in 1485 adult patients with acute myeloid leukemia (AML). Blood. 2006;107:4011–20.
7 Falini B, Nicoletti I, Martelli MF, Mecucci C. Acute myeloid leukemia carrying cytoplasmic/mutated nucleophosmin (NPMc+AML): biologic and clinical features. Blood. 2007; 109:874–85.
8 Nakagawa M, Kameoka Y, Suzuki R. Nucleophosmin in acute myelogenous leukemia (letter). N Engl J Med. 2005;352: 1819–20.
9 Gale RE, Green C, Allen C, Mead AJ, Burnett AK, Hills RK, *et al.* The impact of FLT3 internal tandem duplication mutant level, number, size, and interaction with NPM1 mutations in a large cohort of young adult patients with acute myeloid leukemia. Blood. 2008;111:2776–84.
10 Rosnet O, Buhring HJ, Marchetto S, Rappold I, Lavagna C, Sainty D, *et al.* Human FLT3/FLK2 receptor tyrosine kinase is expressed at the surface of normal and malignant hematopoietic cells. Leukemia. 1996;10:238–48.
11 Nakao M, Yokota S, Iwai T, Kaneko H, Horiike S, Kashima K, *et al.* Internal tandem duplication of the FLT3 gene found in acute myeloid leukemia. Leukemia. 1996;10:1911–18.
12 Gilliland DG, Griffin JD. The roles of FLT3 in hematopoiesis and leukemia. Blood. 2002;100:1532–42.
13 Kottaridis PD, Gale RE, Frew ME, Harrison G, Langabeer SE, Belton AA, *et al.* The presence of FLT3 internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials. Blood. 2001;98:1752–9.
14 Frohling S, Schlenk RF, Breitruck J, Benner A, Kreitmeier S, Tobis K, *et al.* Prognostic significance of activating FLT3 mutations in younger adults (16 to 60 years) with acute myeloid leukemia and normal cytogenetics: a study of the AML Study Group. Blood. 2002;100:4372–80.
15 Thiede C, Steudel C, Mohr B, Schaich M, Schäkel U, Platzbecker U, *et al.* Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis. Blood. 2002;99:4326–35.
16 Schoch C, Kern W, Schnittger S, Hiddemann W,, Haferlach T. Karyotype is an independent prognostic parameter in therapy-related acute myeloid leukemia (t-AML): an analysis of 93 patients with t-AML in comparison to 1091 patients with de novo AML. Leukemia. 2004;18:120–5.
17 Mrózek K, Carroll AJ, Maharry K, Rao KW, Patil SR, Pettenati MJ, *et al.* Central review of cytogenetics necessary for cooperative group correlative and clinical studies of adult acute leukemia: the cancer and leukemia group B experience. Int J Oncol. 2008;33:239–44.
18 Greer J, Baer M, Kinnery M. Acute myelogenous leukemia. In: Lee G, Foester J, Leukens J, Pareskerva F, Greer J, Rogers G, (eds.) Wintrobe's clinical hematology. 12th ed. Philadelphia: Lippincott Williams and Wilkins; 2009. p. 2045–62.
19 Suzuki T, Kiyoi H, Ozeki K, Tomita A, Yamaji S, Suzuki R, *et al.* Clinical characteristics and prognostic implications of NPM1 mutations in acute myeloid leukemia. Blood. 2005; 106(8):2854–61.
20 Wang L, Lai W, Meng H, Qian W, Mai W, Tong H, *et al.* FLT3 and NPM1 mutations in Chinese patients with acute myeloid leukemia and normal cytogenetics. Science 2010;11(10):762–70.
21 Haferlach T, Bacher U, Alpermann T, Haferlach C, Kern W, Schnittger S. Amount of bone marrow blasts is strongly correlated to NPM1 and FLT3-ITD mutation rate in AML with normal karyotype. Leuk Res. 2012;36(1):51–8.
22 Döhner K, Schlenk RF, Habdank M, Scholl C, Rücker FG, Corbacioglu A, *et al.* Mutant nucleophosmin (NPM1) predicts favorable prognosis in younger adults with acute myeloid leukemia and normal cytogenetics: interaction with other gene mutations. Blood. 2005;106(12):3740–6.
23 Ghanem H, Tank N, Tabbara IA. Prognostic implications of genetic aberrations in acute myelogenous leukemia with normal cytogenetics. Am J Hematol. 2012;87(1):69–77.
24 Kim Y, Kim H, Lee S, Ahn J, Yang D, Lee J, *et al.* Prognostic significance of nucleophosmin mutations and FLT3 internal tandem duplication in adult patients with cytogenetically normal acute myeloid leukemia. Korean J Hematol. 2010;45: 36–45.
25 Döhner K, Döhner H. Molecular characterization of acute myeloid leukemia. Hematologica. 2008;93(7):976–82.
26 de Jonge HJ, Huls G, de Bont ES. Gene expression profiling in acute myeloid leukaemia. Neth J Med. 2011;69(4):167–76.
27 Falini B, Mecucci C, Tiacci E, Alcalay M, Rosati R, Pasqualucci L, *et al.* Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. N Engl J Med. 2005;352(3): 254–66.
28 Tse KF, Allebach J, Levis M, Smith BD, Bohmer FD, Small D. Inhibition of the transforming activity of FLT3 internal tandem duplication mutants from AML patients by a tyrosine kinase inhibitor. Leukemia. 2002;16(10):2027–36.
29 Grundler R, Miething C, Thiede C, Peschel C, Duyster J. FLT3-ITD and tyrosine kinase domain mutants induce 2 distinct phenotypes in a murine bone marrow transplantation model. Blood. 2005;105(12):4792–9.
30 Rocnik JL, Okabe R, Yu JC, Lee BH, Giese N, Schenkein DP, *et al.* Roles of tyrosine 589 and 591 in STAT5 activation and transformation mediated by FLT3-ITD. Blood. 2006;108(4): 1339–45.

31 Cilloni D, Messa F, Rosso V, Arruga F, Defilippi I, Carturan S, *et al.* Increase sensitivity to chemotherapeutical agents and cytoplasmatic interaction between NPM leukemic mutant and NF-kappaB in AML carrying NPM1 mutations. Leukemia. 2008; 22:1234–40.
32 Scholl S, Fricke HJ, Sayer HG, Hoffken K. Clinical implications of molecular genetic aberrations in acute myeloid leukemia. J Cancer Res Clin Oncol. 2009;135:491–505.
33 Schnittger S, Schoch C, Kern W, Mecucci C, Tschulik C, Martelli MF, *et al.* Nucleophosmin gene mutations are predictors of favorable prognosis in acute myelogenous leukemia with a normal karyotype. Blood. 2005;106(12):3733–9.
34 Verhaak RG, Goudswaard CS, van Putten W, Bijl MA, Sanders MA, Hugens W, *et al.* Mutations in nucleophosmin (NPM1) in acute myeloid leukemia (AML): association with other gene abnormalities and previously established gene expression signatures and their favorable prognostic significance. Blood. 2005; 106(12):3747–54.
35 Brown P, McIntyre E, Rau R, Meshinchi S, Lacayo N, Dahl G, *et al.* The incidence and clinical significance of nucleophosmin mutations in childhood AML. Blood. 2007;110: 979–85.
36 Hollink IH, Zwaan CM, Zimmermann M, Arentsen-Peters TC, Pieters R, Cloos J, *et al.* Favorable prognostic impact of NPM1 gene mutations in childhood acute myeloid leukemia, with emphasis on cytogenetically normal AML. Leukemia. 2009;23: 262–70.
37 National Comprehensive Cancer Network. Version 2. 2010 (accessed 2010 Jan 8). Available at: http://www.nccn.org/professionals/physician_gls/PDF/aml.pdf
38 Cheson BD, Bennett JM, Kopecky KJ, Büchner T, Willman CL, Estey EH, *et al.* Revised recommendations of the international working group for diagnosis, standardization of response criteria, treatment outcomes, and reporting standards for therapeutic trials in acute myeloid leukemia. J Clin Oncol. 2003;21: 4642–9.