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FLT3 and *NPM1* mutations in Chinese patients with acute myeloid leukemia and normal cytogenetics

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Abstract: Mutations of fms-like tyrosine kinase 3 (*FLT3*) and nucleophosmin (*NPM1*) exon 12 genes are the most common abnormalities in adult acute myeloid leukemia (AML) with normal cytogenetics. To assess the prognostic impact of the two gene mutations in Chinese AML patients, we used multiplex polymerase chain reaction (PCR) and capillary electrophoresis to screen 76 AML patients with normal cytogenetics for mutations in *FLT3* internal tandem duplication (*FLT3*/*ITD*) and exon 12 of the *NPM1* gene. *FLT3*/*ITD* mutation was detected in 15 (19.7%) of 76 subjects, and *NPM1* mutation in 20 (26.3%) subjects. Seven (9.2%) cases were positive for both *FLT3*/*ITD* and *NPM1* mutations. Significantly more *FLT3*/*ITD* aberration was detected in subjects with French-American-British (FAB) M1 (42.8%). *NPM1* mutation was frequently detected in subjects with M5 (47.1%) and infrequently in subjects with M2 (11.1%). *FLT3* and *NPM1* mutations were significantly associated with a higher white blood cell count in peripheral blood and a lower CD34 antigen expression, but not age, sex, or platelet count. Statistical analysis revealed that the *FLT3*/*ITD*-positive group had a lower complete remission (CR) rate (53.3% vs. 83.6%). Survival analysis showed that the *FLT3*/*ITD*-positive/*NPM1* mutation-negative group had worse overall survival (OS) and relapse-free survival (RFS). The *FLT3*/*ITD*-positive/*NPM1* mutation-positive group showed a trend towards favorable survival compared with the *FLT3*/*ITD*-positive/*NPM1* mutation-negative group (P=0.069). Our results indicate that the *FLT3*/*ITD* mutation might be a prognostic factor for an unfavorable outcome in Chinese AML subjects with normal cytogenetics, while *NPM1* mutation may be a favorable prognostic factor for OS and RFS in the presence of *FLT3*/*ITD*.

Key words: Acute myeloid leukemia (AML), Normal cytogenetics, Prognosis, fms-like tyrosine kinase 3 internal tandem duplication (*FLT3*/*ITD*), Nucleophosmin (*NPM1*), Mutation

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1 Introduction

Acute myeloid leukemia (AML) is a phenotypically and genetically heterogeneous disease. Cytogenetics is regarded as an important prognostic factor for AML patients, which are classified into three risk groups: favorable, intermediate, and unfavorable. Normal cytogenetics is relegated to the intermediate risk group. In recent years, some somatic alterations have been identified in patients with AML (Schichman *et al*., 1994; Nakao *et al*., 1996; Pabst *et al*., 2001; Falini *et al*., 2005). Most of these abnormalities occur in cytogenetically normal AML (CN AML) and have been confirmed as important prognostic factors. They contribute to dividing CN AML into distinct prognostic subgroups, and represent potential targets for gene therapies. The fms-like

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tyrosine kinase 3 (*FLT3*) gene encodes a member of class III receptor tyrosine kinase family, which affects the proliferation, differentiation, and survival of hematopoietic stem cells. Many studies have found that *FLT3* internal tandem duplication (*FLT3*/*ITD*) mutation is associated with an adverse prognosis in CN AML patients (Yanada *et al*., 2005; Baldus *et al*., 2006; Thiede *et al*., 2006; Colovic *et al*., 2007). Nucleophosmin (NPM1), as a nucleolar phosphoprotein, usually impacts ribosomal protein assembly and transport, and prevents protein aggregation in the nucleolus. In multivariable analysis, the status of *NPM1* mutation without *FLT3*/*ITD* is an independent favorable prognostic factor on overall survival (OS) and relapse-free survival (RFS) in patients with CN AML (Döhner *et al*., 2005).

The objective of this study was to assess the prevalence and prognostic impact of *FLT3* and *NPM1* gene mutations in adult CN AML patients in China. We also evaluated the association between the two gene mutations and their clinical characteristics, such as age, white blood cell (WBC) count in peripheral blood, and French-American-British (FAB) subtype.

2 Materials and methods

2.1 Subjects

A total of 76 newly diagnosed patients with CN AML (except for FAB M3), who entered the First Affiliated Hospital of Zhejiang University from 2003 to 2005, were investigated in this study. AML was diagnosed according to the FAB classification (Bennett *et al*., 1985). Cytogenetic G-banding analysis was preformed with standard methods. Mononuclear cells were isolated by Ficoll density gradient centrifugation, and cryopreserved in 10% (v/v) dimethylsulphoxide (DMSO) at −80 °C. The subject characteristics are given in Table 1.

Table 1 Characteristics of patients included in this study

Parameter	Value
Number of patients	
Total	76
Male/female	45/31
WBC count at diagnosis $\geq 100\times10^9$ L^{-1}	17
Age (year)*	50 (13–78)
FAB subtype#	
M0	5 (6.6%)
M1	14 (18.4%)
M2	27 (35.5%)
M4	8 (10.5%)
M5	18 (23.7%)
M6	3 (3.9%)
AML following MDS	1 (1.3%)

* Value is expressed as median (range); # Values are expressed as number (percentage); FAB: French-American-British; AML: acute myeloid leukemia; MDS: myelodysplastic syndrome; WBC: white blood cell

2.2 Therapy protocol

Induction therapy consisted of one or two courses of DA (daunorubicin 45 mg/m^2 on Days 1 through 3, cytarabine 100 mg/m^2 every 12 h on Days 1 through 7) or HAA (homoharringtonine 2 mg/m^2 twice daily for 3 d, cytarabine 75 mg/m^2 every 12 h on Days 1 through 7, and aclarubicin 12 mg/m^2 on Days 1 through 7). Five patients were treated with HA regimen (homoharringtonine 2 mg/m^2 twice daily for 3 d, cytarabine 100 mg/m^2 every 12 h on Days 1 through 7) because of inferior general conditions. Consolidation therapy was applied every 1 month and consisted of 100 mg/m^2 cytarabine every 12 h on Days 1 to 7 in combination with a second drug. The second drug included 45 mg/m^2 daunorubicin by intravenous infusion on Days 1 to 3 (Courses 1, 2, 9, etc.), 10 mg/m^2 mitoxantrone by intravenous infusion on Days 1 to 3 (Courses 3, 4, 10, etc.), 75 mg/m^2 etoposide by intravenous infusion on Days 1 to 5 (Courses 5, 6, 11, etc.), and 12 mg/m^2 aclarubicin by continuous infusion over 2 h on Days 1 to 7 (Courses 7, 8, 12, etc.). Patients who failed to obtain complete remission received second line induction regimes, such as mitoxantrone and cytarabine (MA), and aclarubicin, cytarabine and etoposide (AAE). If a cumulative dose of 540 mg/m^2 daunorubicin was achieved, thioguanine would take place of daunorubicin.

2.3 DNA isolation and polymerase chain reaction

Genomic DNA was extracted from approximately 10^6 mononuclear cells (Gentra Puregene Blood DNA kit, Minneapolis, MN, USA). A multiplex polymerase chain reaction (PCR) procedure was used to detect *FLT3*/*ITD* and *NPM1* mutations (Huang *et al*., 2008). The 20 μl PCR reaction solution consisted of 200 ng DNA template, 10× PCR buffer 2 μl, 25 mmol/L $MgCl_2$ 1 μl, 10 mmol/L deoxyribonucleoside triphosphate (dNTP) 0.5 μl, 200 nmol/L primer for *FLT3* and *β-globin*, 350 nmol/L primer for

NPM1, and 1.5 U *Taq* DNA polymerase (Promega, USA). Samples were amplified using the following PCR conditions: 95 °C for 2 min, 35 cycles at 95 °C for 30 s, 60 °C for 40 s, and 72 °C for 40 s, the final cycle at 72 °C for 30 min.

2.4 Capillary gel electrophoresis

PCR products were diluted 1:5 (v/v) in distilled water and analyzed using 3130 genetic analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. A peak equal to or above 50 relative fluorescence units (RFU) in the electropherogram was defined as positive. The results were analyzed with GeneMapper software Version 3.2 (Applied Biosystems). The NB4 cell line was used as a normal control, and the sample from a known positive patient was used as a positive control.

2.5 Sequencing analyses of *FLT3* and *NPM1*

To validate the results from capillary gel electrophoresis, 10 *FLT3*/*ITD*- and 10 *NPM1*-mutated samples were amplified for sequencing analysis. PCR products from *FLT3*/*ITD* samples were resolved on a 3.5% (w/v) agarose gel stained by ethidium bromide. Each sample displayed an additional PCR product (>330 bp). The longer PCR products were purified by the standard methods and directly sequenced with the same primers used for amplification. PCR products from *NPM1* mutant were cloned into pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA). At least four recombinant colonies were selected, and the plasmid DNA was sequenced by the ABI377 sequencer (Applied Biosystems).

2.6 Statistical analysis

The Fisher extract test was used to compare mutation status with dichotomous variables, and the Mann-Whitney *U* test was used to compare the mutation status with continuous variables. Survival curves for OS and RFS were calculated according to Kaplan-Meier and compared using two-sided log rank test. OS was defined as the time from diagnosis to death owing to any causes. RFS was defined as the time from achieving complete remission (CR) to the first event of either relapse or death. $P<0.05$ was considered statistically significant. SPSS Version 16.0 software (Chicago, IL, USA) was used for statistical analysis.

3 Results

3.1 Frequencies of *FLT3* and *NPM1* mutations

The fluorescently labeled multiplexing PCR products from 76 samples were used to screen for the prevalence of *FLT3* and *NPM1* mutations by capillary gel electrophoresis. Wild type *FLT3* and *NPM1* genes were 329 and 167 bp (Fig. 1a). *FLT3*/*ITD*-positive samples showed an additional peak at a range from 350 to 410 bp (Fig. 1b). *NPM1* mutation showed a double peak at positions of 167 and 171 bp (Fig. 1c). The control gene (*β-globin*), which was used to verify DNA quality and PCR reaction, had a peak around 469 bp. *FLT3*/*ITD* mutation was detected in 15 (19.7%) of 76 subjects, and *NPM1* mutation in 20 (26.3%) subjects. Seven (9.2%) cases were positive for both *FLT3*/*ITD* and *NPM1* mutations. In the *FLT3*/*ITD*-positive cases, the *NPM1* mutation was significantly more frequent than in *FLT3*/*ITD*-negative cases (46.7% vs. 21.3%). Ten randomly chosen *NPM1* mutants were confirmed by sequencing analysis. *NPM1* mutation variant *A*, a "TCTG" insertion at position nucleotide (wild type) 959 was detected in 8 (80%) cases. The mutation variants *B* and O_M were detected, respectively, in one case.

3.2 Gene mutations and clinical characteristics

Both *FLT3* and *NPM1* mutations in CN AML subjects were associated with FAB subgroups. Frequencies of *NPM1* mutation were significantly higher in FAB M5 (47.1%) than non-M5 (20.0%) ($P<0.001$), and lower in M2 (11.1%) than non-M2 (34.7%) ($P=0.03$) (Fig. 2b). Frequency of *FLT3*/*ITD* was significantly higher in FAB M1 (42.8%) than in other FAB subgroups (14.5%) ($P<0.001$) (Fig. 2a). *FLT3*/*ITD*-positive subjects had statistically higher WBC counts in peripheral blood and blast counts in bone marrow compared with the *FLT3*/*ITD*-negative group ($P=0.006$ and $P=0.05$, respectively). In the *NPM1* mutation group, higher WBC counts were found (mean 81.3×10^9 L^{-1}, median 54.9×10^9 L^{-1}) compared with mutation-negative group (mean 64.5×10^9 L^{-1}, median 34.6×10^9 L^{-1}, $P=0.022$). Multivariate analysis showed *FLT3*/*ITD* and *NPM1* mutations were significantly associated with lower CD34 antigen expression ($P=0.029$ and $P=0.002$, respectively). None of the 20 cases of *NPM1* mutations showed CD7 aberrant expression, whereas 15 of 56 cases of wide

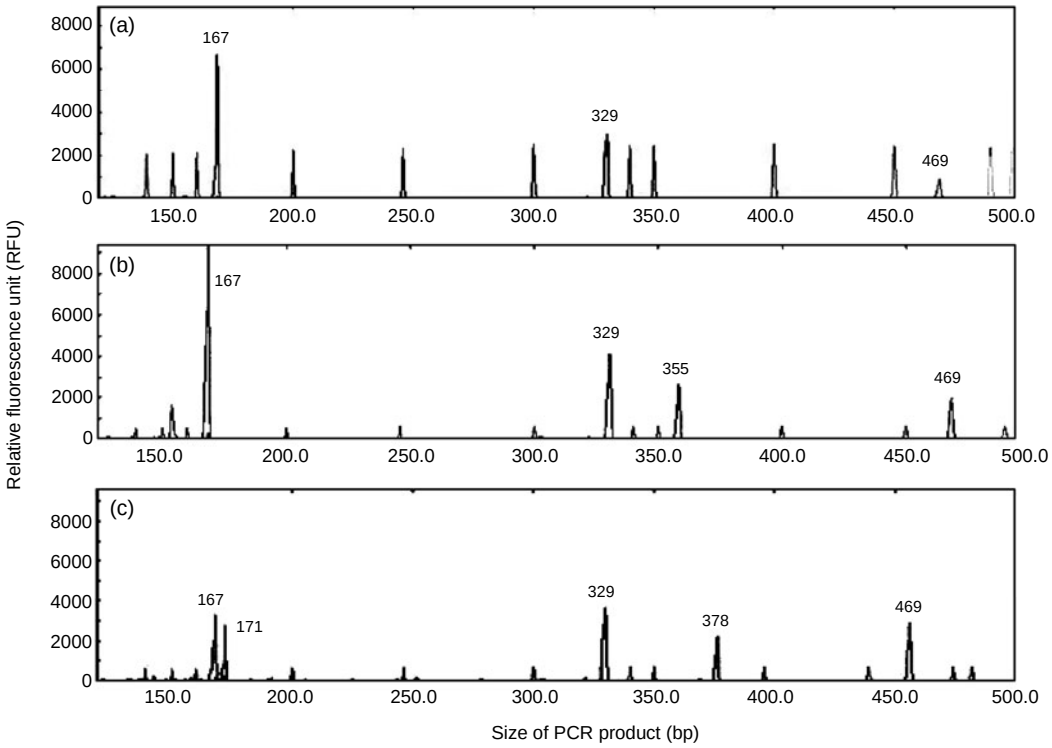

Fig. 1 Capillary gel electrophoresis for *FLT3* and *NPM1* genes in three cases

(a) A case with wide type *NPM1* (167 bp) and wide type *FLT3* (329 bp); (b) A case with *FLT3*/*ITD* positive; (c) A case with *NPM1* mutant and *FLT3*/*ITD* positive simultaneously

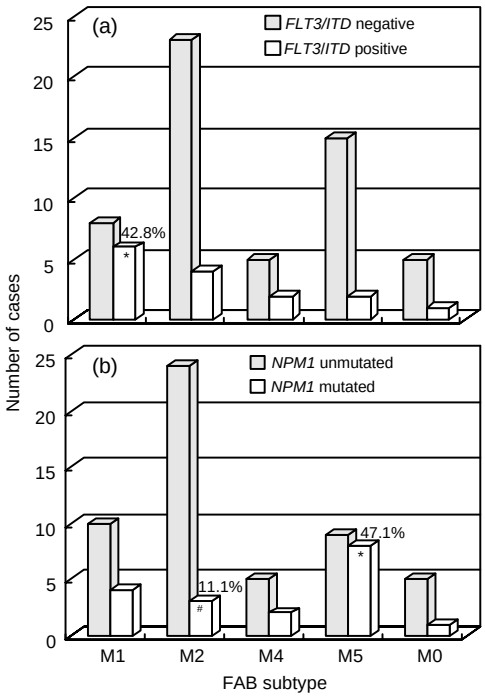

Fig. 2 Distribution of *FLT3* (a) and *NPM1* (b) mutations in morphologic subtypes

* $P<0.001$, # $P<0.05$, compared with other FAB subtypes

type *NPM1* expressed CD7 antigen (P=0.004). The frequencies of myelomonocytic markers, such as CD11, CD13, CD14, and CD15, did not significantly differ between the four groups.

3.3 Response to induction therapy

The total CR rate after induction chemotherapy was 77.6% in all 76 AML subjects. The *FLT3*/*ITD*-positive group, with or without *NPM1* mutation, showed a statistically lower CR rate (53.3%) compared to the *FLT3* wide type group (83.6%) (χ^2=6.356, P=0.019). The subjects with *NPM1* mutation-positive showed a similar CR rate to *NPM1* mutation-negative subjects (70.0% and 80.3%, respectively). When the two gene mutations were simultaneously analyzed, the highest CR rate was achieved in subjects with *FLT3*/*ITD*-negative/*NPM1* mutation-positive (84.6%), followed by the *FLT3*/*ITD*-negative/*NPM1* mutation-negative group (83.3%). The CR rate in subjects with *FLT3*/*ITD*-positive/*NPM1* mutated-positive (42.9%) was significantly lower than those in the other three groups (χ^2=7.193, P=0.015) (Table 2). Subjects harboring *FLT3*/*ITD*-negative/*NPM1* mutation-positive

Table 2 Complete remission survival according to *FLT3*/*ITD* and *NPM1* mutation statuses

	Number of patients[#]			
	FLT3/*ITD*-negative (*n*=61)		*FLT3*/*ITD*-positive (*n*=15)	
	NPM1 unmutated (*n*=48)	*NPM1* mutated (*n*=13)	*NPM1* unmutated (*n*=8)	*NPM1* mutated (*n*=7)
CR	40 (83.3%)	11 (84.6%)	5 (62.5%)	3 (42.9%)[*]
CR=1	33 (68.8%)	10 (76.9%)	4 (50.0%)	3 (42.9%)
CR>1	7 (14.6%)	1 (7.7%)	1 (12.5%)	0
RD	8 (16.7%)	2 (15.4%)	3 (37.5%)	4 (57.1%)
OS at four years	16 (33.3%)[*]	4 (30.8%)	0	1 (14.3%)
RFS at four years	14 (29.2%)[*]	2 (15.4%)	0	0

[#] Data are expressed as number (percentage); [*] $P<0.05$. CR=1: complete remission with a single induction therapy; CR>1: complete remission after more than one course of induction therapy; RD: resistant disease; OS: overall survival; RFS: relapse-free survival

had a higher CR rate (76.9%), achieved by the first induction therapy, than the other three groups, but these differences had no statistical significance (χ^2= 3.425, $P>0.05$).

3.4 Survival analysis

The median follow-up time was 20 months. The median duration of remission showed a statistically significant difference between *FLT3*/*ITD*-negative and *FLT3*/*ITD*-positive subjects (18 months vs. 7 months, P=0.017). *FLT3*/*ITD*-positive subjects showed a trend of worse median OS (10.5 months vs. 20 months in *FLT3*/*ITD*-negative subjects), but this difference was not significant (P=0.073). No statistical difference on median OS or RFS was found between *NPM1*-unmutated and *NPM1*-mutated subjects (Fig. 3b). The four-year rates of OS and RFS for subjects with *FLT3*/*ITD* without *NPM1* mutation were zero, and for those without *FLT3*/*ITD* were 33.3% and 29.2%, respectively. Kaplan-Meier survival curves of the effects of *FLT3*/*ITD* status on OS and RFS showed significantly shorter OS and RFS in subjects harboring *FLT3*/*ITD* (log rank=5.729, P=0.017 for OS and log rank=5.488, P=0.019 for RFS) (Fig. 3a). Survival analysis of the four genotypes revealed a worse OS for the *FLT3*/*ITD*-positive/*NPM1* mutation-negative group than for both the *FLT3*/*ITD*-negative/*NPM1* mutation-negative group and the *FLT3*/*ITD*-negative/*NPM1* mutation-positive group (P=0.002 and P= 0.015, respectively), whereas no difference on OS emerged between the *FLT3*/*ITD*-positive/*NPM1* mutation-negative group and the *FLT3*/*ITD*-positive/*NPM1* mutated-positive group (P=0.069) (Fig. 4a). The same pattern was found for RFS (Fig. 4b). Another factor impacting OS and RFS was the number of induction therapy course required to reach CR. Subjects who achieved CR after more than one course of induction chemotherapy were revealed to have significantly worse OS and RFS (log ranks were 36.284 and 36.556, respectively, $P<0.001$).

4 Discussion

FLT3/*ITD* and *NPM1* mutations have been shown to be the most prevalent somatic alterations in AML, especially in CN AML. In our study, the incidences of *FLT3*/*ITD* and *NPM1* mutations were 19.7% and 26.3%, respectively, in 76 subjects with CN AML, which were obviously lower than those reported in Germans (31%, 53%) (Döhner *et al*., 2005; Schnittger *et al*., 2005; Thiede *et al*., 2006) and in Japanese (28.0%, 47.4%) (Suzuki *et al*., 2005; Schlenk *et al*., 2008), but approximated to those reported by Colovic *et al*. (2007). The lower detection rate may be due to a higher background of wild type allele, or a lower percentage of *FLT3*/*ITD* or *NPM1* mutation-positive cells in some cases. In addition, *FLT3*-TKD mutation was difficult to detect by capillary gel electrophoresis.

Certain associations between the two gene mutations and clinical characteristics have been reported in these past years. We also found a significantly increased leukocyte count in subjects with *FLT3*/*ITD* (P=0.006) or with *NPM1* mutation (P=0.05). There was a significant difference in blast count observed only between *FLT3*/*ITD*-positive subjects and *FLT3*/*ITD*-negative subjects (P=0.022). These findings were consistent with some previous reports (Fröhling *et al*., 2002; Colovic *et al*., 2007; Huang *et al*., 2008; Schlenk *et al*., 2008). Although the effect of *FLT3*/*ITD*

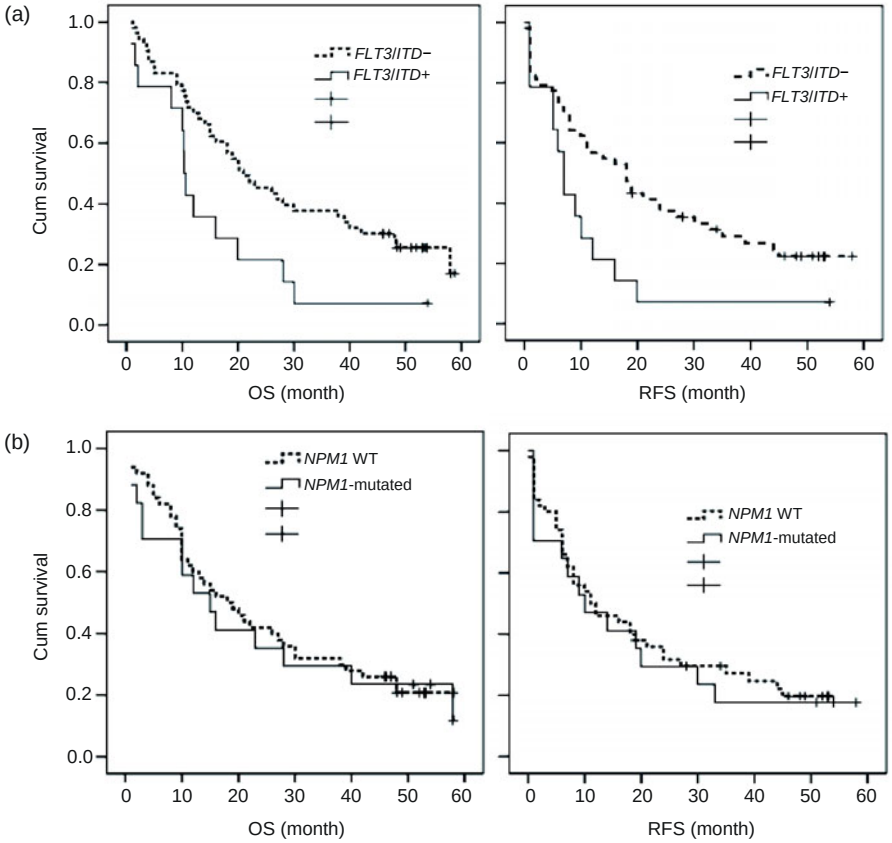

Fig. 3 Kaplan-Meier analysis results according to the gene mutation status in CN AML patients
(a) OS and RFS for *FLT3*/*ITD*; (b) OS and RFS for *NPM1*

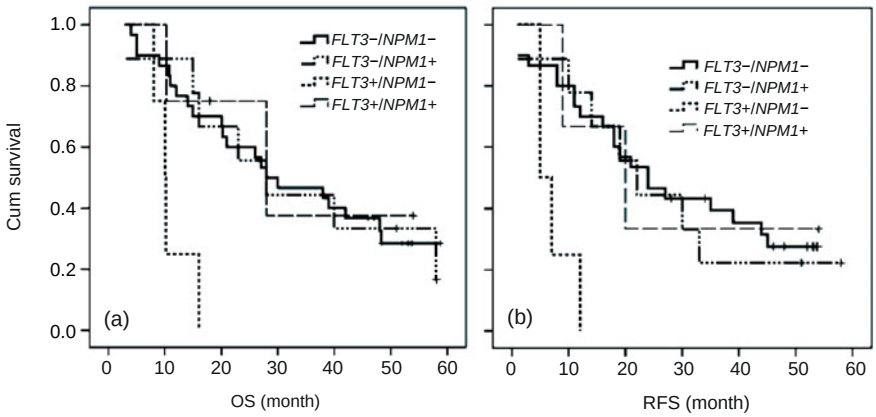

Fig. 4 Survival analysis estimates for probabilities of OS (a) and RFS (b) according to the four mutation statuses

on inducing leukemogenesis was not directly proved, the ligand-independent constitutive activation of *FLT3* induced by *ITD* mutation could activate some downstream signal molecules including mitogen-activated protein (MAP) kinase, signal transducer and activator of transcription 5 (STAT5), and serine-threonine kinases Akt, which contribute to cell proliferation and survival advantages (Hayakawa *et al*., 2000; Kiyoi *et al*., 2002; Tse *et al*., 2002; Grundler *et al*., 2005; Rocnik *et al*., 2006). Kelly *et al*. (2002) induced a myeloproliferative disease in a murine bone marrow transplant model by transforming *FLT3*/*ITD*

mutants, but did not observe hematologic disorders. These findings might partially explain the close relationship between *FLT3*/*ITD* and higher WBC count. CD34 has been regarded as a distinct surface marker on immature hematopoietic precursor cells. As shown previously (Falini *et al*., 2005; Schnittger *et al*., 2005), significantly lower CD34 antigen expression was observed in *FLT3*/*ITD*- and *NPM1*-mutated subjects in our present study, but unlike the findings in other studies (Schnittger *et al*., 2002; Thiede *et al*., 2002; Colovic *et al*., 2007), more frequent *FLT3*/*ITD* (42.8%) was found in FAB M1 subtype. Zheng *et al*. (2002; 2004) reported that *FLT3*/*ITD* blocked granulocytic differentiation through suppression of CCAAT/enhance binding protein alpha (*C*/*EBPα*) expression in 32D cells transfected with *FLT3*/*ITD*. These effects could be inhibited by an *FLT3* inhibitor, lestaurtinib (CEP-701). The high frequency of *FLT3*/*ITD* in M1 subtype might be explained by the theory that *FLT3* aberrant activation blocked the differentiation of myeloblastic cells, most probably granulocytic differentiation. Higher blast count, absent CD34 expression in *FLT3*/*ITD*, and higher frequency in M1 project the implication that *FLT3*/*ITD* may be associated with the differentiation stasis of granulocytic lineage and the proliferation of leukemic cells. Meanwhile, a statistically higher frequency of *NPM1* mutation was identified in subjects with AML M5 and lower frequency in AML M2, which was consistent with findings from previous studies (Fröhling *et al*., 2002; Schnittger *et al*., 2005; Suzuki *et al*., 2005; Verhaak *et al*., 2005; Thiede *et al*., 2006; Huang *et al*., 2008). Mori *et al*. (2007) provided the evidence that the expression of CD11b and CD14 antigens was significantly associated with *NPM1* mutation, suggesting a close association between *NPM1* mutation and monocytic features of AML. More frequent *NPM1* mutation in AML M5 and a higher frequency of monocytic marker expression in *NPM1*-mutated subjects indicated a participation of *NPM1* mutations in inducing leukemic development towards monocytic features. The relation of *NPM1* mutations and higher WBC counts may be due to an accompanying occurrence of *NPM1* mutations and *FLT3*/*ITD*.

CD7, as a T/NK cell associated antigen, is aberrantly expressed on blast cells of AML. Its function in AML blast remains unclear. Some previous studies have suggested CD7 may participate in the early development of myelopoiesis due to its association with some known immature antigens, such as CD34 and human leukocyte antigen-DR (HLA-DR) (Rabinowich *et al*., 1994; Baarcenai *et al*., 1995). Rausei-Mills *et al*. (2008) reported a higher frequency (73%) of aberrant CD7 co-expression in *FLT3*/*ITD*-positive AML subjects with normal karyotype. In comparison, only 4 of 15 *FLT3*/*ITD*-positive AML patients displayed CD7 co-expression in the present study. Interestingly, all 20 *NPM1*-mutated AML subjects showed no aberrant CD7 co-expression. The absent CD7 expression in *NPM1*-mutated cases coincided with the lower CD34 expression and highlighted the potential effect of mutated *NPM1* on blocking the differentiation of hematopoietic precursor cell at monoblastic stage.

We also analyzed the clinical outcomes of 76 Chinese adults with AML and normal cytogenetics, except for FAB M3 subtype. In *FLT3*/*ITD*-positive subjects, CR rate was statistically lower than that in *FLT3* wide type subjects. However, no significantly higher CR rate was observed in subjects with *NPM1* mutation. When the two gene mutations were analyzed together, the significantly lower CR rate was observed in *FLT3*/*ITD*-positive/*NPM1* mutation-positive subjects. These results were similar to the findings of Döhner *et al*. (2005). 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 (CRD), lower CR rate, and worse disease-free survival (DFS) and OS (Yanada *et al*., 2005; Baldus *et al*., 2006; Thiede *et al*., 2006; Colovic *et al*., 2007). In our study, *FLT3*/*ITD*-positive subjects showed a poorer median duration of remission, a trend towards worse median OS and lower four-year rates of OS and RFS. Survival analyses of the four genotypes revealed that subjects with *FLT3*/*ITD* had worse OS and RFS due to the unfavorable outcome of *FLT3*/*ITD*-positive/*NPM1* mutation-negative subjects. However, subjects with *NPM1* mutation showed no better impact on OS or RFS in absence of *FLT3*/*ITD*, which was different from other reports (Fröhling *et al*., 2002; Schnittger *et al*., 2005; Verhaak *et al*., 2005). Although there was no statistical difference on OS or RFS between the *FLT3*/*ITD*-positive/*NPM1* mutation-negative group and the *FLT3*/*ITD*-positive/*NPM1* mutation-positive group in present study, *NPM1* mutation might be a favorable

prognostic factor for OS and RFS in the presence of *FLT3*/*ITD*. Our results indicate that Chinese AML patients with *FLT3*/*ITD* seem to have a worse prognosis. Unlike the findings in Caucasians, *NPM1* mutation did not suggest a favorable prognosis in our study. This might be due to a relatively low response rate to chemotherapy in the *NPM1* mutation-positive group. Of course, a different genetic background and chemotherapy regimen might also affect the results. Thus, further studies with larger sample sizes are warranted.

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