Ann Hematol (2009) 88:159–166
DOI 10.1007/s00277-008-0591-8

ORIGINAL ARTICLE

Nucleophosmin mutations in Chinese adults with acute myelogenous leukemia

Guo-Rui Ruan · Jin-Lan Li · Ya-Zhen Qin · Ling-Di Li · Min Xie · Yan Chang · Yan Zhang · Yan-Rong Liu · Bin Jiang · Shan-Shan Chen · Xiao-Jun Huang

Received: 18 June 2008 / Accepted: 3 August 2008 / Published online: 23 August 2008

Abstract Recently, mutations in the nucleophosmin (*NPM1*) gene were detected in 50–60% of adult acute myelogenous leukemia (AML) patients, mainly with a normal karyotype. In this study, we detected typical *NPM1* mutations (types A, B, D) in untreated Chinese AML patients using real-time quantitative polymerase chain reaction (RQ-PCR) followed by sequence analysis. The detection rate of *NPM1* mutations in 220 AML patients was 16.4%, including 107 (14.2%) with the French–American–British (FAB) subtype M2, 43 (2.3%) with M3, and 52 (30.8%) with M4/M5. Only one case each with an *NPM1* mutation was detected in four M0, seven M1, five M6, and two M7 cases. Eight patients were followed up after treatment, and five patients in hematologic remission continued to test negative for *NPM1* mutations within 2–14 months of follow-up. Sequence analysis revealed that all the 36 positive cases were heterozygous for the mutation with 4-bp insertions at nt 959; the 36 cases included 29 (80.6%) cases with type A, four (11.1%) cases with type B, and one rare DD-3 mutation. We also detected two new mutations, namely, CTCG and CAAG insertions, named BJ-01 and BJ-02, respectively. Further, 38.9% (14/36) patients with *NPM1* mutations simultaneously exhibited internal tandem duplications in the FLT3 gene, and 66.7% (22/33) patients did not express CD34. The results demonstrated that RQ-PCR was a reliable and sensitive method for detecting *NPM1* mutations, for screening AML, and for the quantitative analysis of minimal residual diseases.

G.-R. Ruan (✉) · J.-L. Li · Y.-Z. Qin · L.-D. Li · M. Xie · Y. Chang · Y. Zhang · Y.-R. Liu · B. Jiang · S.-S. Chen (✉) · X.-J. Huang (✉)
Peking University Institute of Hematology and People's Hospital,
11 Xi-zhi-men South Street,
Beijing 100044, China
e-mail: celiar@public3.bta.net.cn
e-mail: lcs1601@yahoo.com.cn
e-mail: xjhrm@medmail.com.cn

Keywords *NPM1* mutation · Acute myelogenous leukemia · Real-time quantitative PCR

Introduction

Nucleophosmin (*NPM*) is a phosphoprotein that is mainly expressed in the nucleolus. It shuttles between the nucleus and cytoplasm and is involved in many cellular activities. Mutations in the exon 12 of the *NPM1* gene have been recently detected in 50–60% of acute myelogenous leukemia (AML) patients with a normal karyotype. AML patients with mutated *NPM1* differed from those without *NPM1* mutations in terms of clinical features, response to remission induction chemotherapy, and prognosis [1–5]. Therefore, *NPM1* study is important for the diagnostic classification, research of pathomechanisms, detection of minimal residual diseases, prognosis, and choice of treatment target. In this study, in order to detect *NPM1* gene mutations, DNA from the bone marrow (BM) of untreated adult AML patients from the Chinese mainland were examined by using the TaqMan® MGB probe-based real-time quantitative polymerase chain reaction (RQ-PCR) and sequence analysis. Simultaneously, the detection of internal tandem duplications (ITD) in the *FLT3* gene, chromosome examination, and immunophenotyping were also performed.

Materials and methods

Patient samples

This study involved untreated 220 adult AML patients. Diagnosis was established on the basis of the widely used WHO criteria. Informed consent was obtained from all the

patients prior to their enrollment in the study. The study design adhered to the principles of the Helsinki Declaration and was approved by the ethics committees of our hospital.

Analysis of *NPM1* mutations

Mononuclear cells were isolated from the BM and peripheral blood samples by using standard Ficoll-Hypaque density gradient centrifugation. DNA and RNA were extracted using DNAzol and TRIzol kits (Invitrogen, Carlsbad, CA, USA), respectively, according to the manufacturers' instructions. Complementary DNA (cDNA) was synthesized as described previously [6]. Mutations in the exon 12 of the *NPM1* gene (types A, B, and D) were detected using RQ-PCR [7]. The primers for the albumin (ALB) gene, which was the internal control, TaqMan® probes, and mutated regions of the *NPM1* gene were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA). The primers for the ABL gene, which was the internal control for cDNA template, was synthesized as described previously [8]. All the primers and probes used in the study are listed in Table 1. The PCR mixture contained 1× TaqMan® Universal PCR Master mix, 900-nM primers, 250-nM fluorescent probes, and 150–500 ng DNA. PCR was performed by using an ABI PRISM® 7500 Sequence Detection System by employing the following protocol: 50°C for 2 min, 95°C for 10 min, and 50 cycles at 95°C for 15 s and 60°C for 1 min (when cDNA was used as the template, the conditions were 62°C for *NPM1* mutation A and 59°C for *NPM1* mutation B).

Table 1 Primers and probe sequences for detecting *NPM1* mutations by real-time quantitative polymerase chain reaction

Primers and probe sequences
Genomic primer and probe sequences
g-NPM mut A-F: 5′-AGG CTA TTC AAG ATC TCT GTC TGG-3′
g-NPM mut-R2: 5′-AAG TTC TCA CTC TGC ATT ATA AAA AGG A-3′
g-NPM mut B-F: 5′-CTA TTC AAG ATC TCT GCA TGG CA-3′
g-NPM mut D-F: 5′-TAT TCA AGA TCT CTG CCT GGC A-3′
g-NPM mut-R1: 5′-AAA GGA CAG CCA GAT ATC AAC TGT T-3′
g-Probe: 5′-FAM-TTC CGT CTT ATT TCA TTT CT-MGB-3′
ALB-F: 5′-GCCCATTGTCCTGTTCTGACTT-3′
ALB-R: 5′-TTCCACTGCTGAGCCATCAC-3′
ALB-Probe: 5′-FAM- TATGATGCGGTACACAGAGCCATCCAAG-TAMARA-3′
sequencing NPM-466-F: 5′-GGTCTCTGTTCTTTCTGTTGATTTCC-3′
sequencing NPM-466-R: 5′-CAACACATTCTTGGCAATAGAACCT-3′
cDNA primer and probe sequences
cNPM- F: 5′-GAA GAA TTG CTT CCG GAT GAC T-3′
C NPM mut A-R: 5′-CTT CCT CCA CTG CCA GAC AGA-3′
C NPM mut B-R: 5′-TTC CTC CAC TGC CAT GC A G-3′
C NPM-Probe: 5′-FAM-ACC AAG AGG CTA TTC AA-MGB-3′
ABL-F: 5′-CCGCTGACCATCAATAAGGAA-3′
ABL-R: 5′-GATGTAGTTGCTTGGGACCCA-3′
ABL-Probe: 5′-FAM-CCATTTTTGGTTTGGGCTTCACACCATT-TAMARA-3′

Preparation of plasmid standards

The ALB gene and *NPM1* mutation A gene were amplified using the DNA isolated from the patients with the *NPM1* type A mutation. The PCR products were purified and subcloned into the pMD18-T vector (Takara, Dalian, China). Transformation, screening, and sequencing were then performed to obtain ALB and *NPM1* mutation A plasmid standards. The copy number was calculated based on the optical density (OD) value, and the standards were subjected to a tenfold dilution (10^1–10^6 copies) in order to plot a standard curve. For each measurement, the threshold of amplification for the curve was set at 0.08, and it included a positive control (samples from the patients with the *NPM1* type A mutation), blank control (without template), and negative control (samples from patients without *NPM1* mutation). ABL was used as the internal control when cDNA was used as the template, and the ABL plasmid standard was prepared as described previously [8].

Calculation of the *NPM1* mutation level

The copy numbers of ALB and *NPM1* mutants were calculated using the C_t value and standard curve. Our previous study revealed similar efficacies for the amplification of ALB and *NPM1* mutation A genes (the slope of standard curve was −3.45 and −3.42, respectively). In order to decrease the differences in plasmid quantification, only one ALB standard curve was plotted (Fig. 1). The *NPM1* mutant copy number in 100 ALB copies was used as the *NPM1* mutant gene content. If the ALB copy number was $\geq 3\times10^4$, the samples were considered for quantitative detection. ABL was used as the internal control gene when cDNA was used as the template. The results were analyzed based on the guidelines proposed by the European Study Group for the RQ-PCR detection of minimal residual diseases [9].

NPM1 gene sequencing

PCR was performed using DNA from the samples as the template and primers (sense: 5′-GGTCTCTGTTCTTT CTGTTGATTTCC-3′ and antisense: 5′-CAACACATT CTTGGCAATAGAACCT-3′). The 50-μl PCR mixture contained 25 μl of 2× Universal PCR Master mix (TianGen Biotech, Beijing, China), 900 nM primers, and 300 ng DNA. The PCR protocol was as follows: pre-denaturing at 95°C for 5 min, followed by 30 cycles at 95°C for 40 s, 55°C for 40 s, and 72°C for 1 min, and a final extension at 72°C for

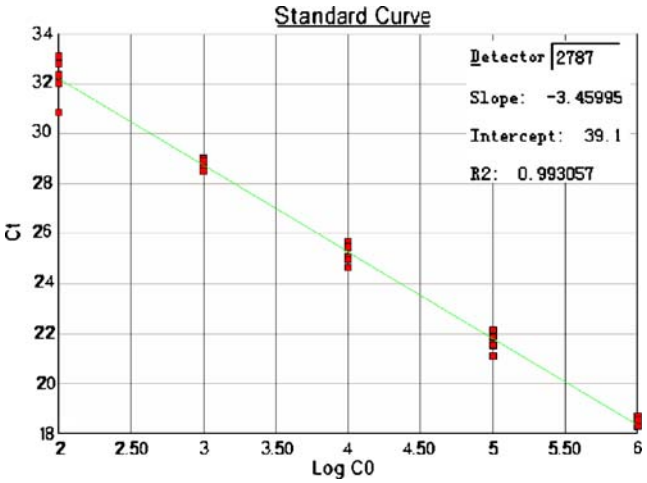

Fig. 1 Standard curve of plasmids containing the ALB gene obtained by real-time quantitative polymerase chain reaction. The standard curve shows a linear correlation between the C_t value and dilution of the ALB plasmid (correlation coefficients, >0.99)

10 min. The purified PCR products were subjected to sequence analysis with an ABI3700 DNA sequencer. The newly screened mutants were subcloned into pMD18-T vectors, followed by transformation, screening, and further sequencing.

Screening of the ITD mutations in the FLT3 gene

The presence of an FLT3/ITD was determined by amplifying a region spanning exons 14 and 15 by using the primers 14F (5′-CAA TTT AGG TAT GAA AGC C-3′) and 15R (5′-GTA CCT TTC AGC ATT TTG AC-3′) followed by 8% polyacrylamide gel electrophoresis as described previously [10].

Immunophenotyping studies

Freshly obtained BM and peripheral blood samples were analyzed using well-established methods [11] involving the four-color immunofluorescence technique for the simultaneous staining of surface and cytoplasmic antigens.

Cytogenetics

Cytogenetic G-banding analysis was performed using standard methods [12]. The definition of a cytogenetic clone and descriptions of karyotypes followed the International System for Human Cytogenetic Nomenclature.

Response to remission induction treatment

Among the 220 patients analyzed, 43 had acute promyelocytic leukemia (APL). APL has been considered as a separate disease entity among AML subtypes, and the treatment of all-trans retinoic acid has dramatically improved its clinical outcome. In addition, data of 65 patients with AML, excluding those with APL, were not analyzed due to their treatment with independent regimens or unavailable data. We therefore analyzed the response to treatment of 112 patients with AML who were treated with the protocols of daunomycin plus cytarabine or homoharringtonine plus cytarabine for induction therapy.

Statistical analysis

Statistical analysis was performed using SPSS software version 11.0 (Chicago, IL, USA). Differences between two groups were evaluated using one-way analysis of variance. Chi-square or a two-sided Fisher exact test was used to calculate the significance of association between *NPM1* mutation and other discrete variables. Values of p below 0.05 were considered to be significant.

Results

NPM1 mutation detection sensitivity

Plasmid standards of ALB and *NPM1* mutation A were prepared. Plasmid DNA concentration was determined by absorbance measurement; six serial plasmid dilutions (10^6, 10^5, 10^4, 10^3, 10^2, and 10^1 copies) were amplified by RQ-PCR to construct a standard curve for the absolute quantitative assessment of copy number. The results for amplified the ALB plasmids revealed that all correlation coefficients were above 0.99 (Fig. 1); the average C_t values for the amplified *NPM1* mutation A with copy numbers of 10^6–10^1 copies were 19.42, 22.71, 26.40, 29.24, 33.26, and 35.39, respectively, and the coefficients of variation were 1.3%, 2.9%, 0.8%, 1.1%, 1.6%, and 1.8%, respectively; the sensitivity of detection was ten copies (Fig. 2). Eight DNA samples of *NPM1*-mutation-positive patients were prepared

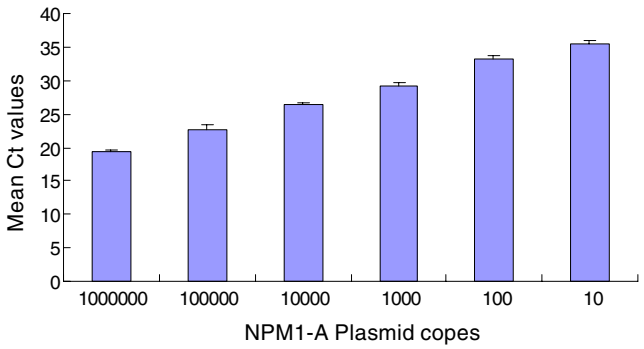

Fig. 2 Representative amplification plot of the six plasmid dilutions that were tested. The plasmid contains *NPM1* mutation A. The sensitivity for detection was ten copies. The coefficient of variation (*CV*) of the C_t value at each dilution was <3%

Table 2 Features of the amplification curves obtained with a tenfold dilution series for patients with *NPM1* mutations

No. of patients	Mutation	Maximal reproducible sensitivity	Mean C_t of undiluted diagnostic DNA	Mean C_t of maximal reproducible sensitivity	Dilution curve slope	Correlation coefficient
1	B	−4	26.7	39.5	−3.1	0.999
4	A	−4	24.0	38.0	−3.4	0.995
12	A	−5	21.9	38.1	−3.1	0.997
14	A	−4	25.0	34.8	−3.3	0.997
17	A	−5	23.4	38.8	−3.3	0.990
19	B	−5	22.1	39.9	−3.6	0.995
21	A	−4	22.4	37.7	−3.9	0.997
29	B	−4	23.2	36.6	−3.3	0.997

with a tenfold dilution series (10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}). In all cases, the C_t value was in the linear range for all dilutions. In addition, the mutations were detected with a sensitivity of 10^{-4} or 10^{-5}, and the correlation coefficient was above 0.99 (Table 2). High correlation coefficients allowed accurate assessment of the quantity of *NPM1* in unknown samples. All amplification plots were analyzed by positioning the threshold at 0.08.

Table 3 Clinical data, FAB subtype, genotypic changes, and outcome in AML patients

Variant	Total (n=220) n	Mutation (n=36, 16.4%) n (%)	Wild type (n=185, 84.1%) n (%)	P
Age (years)	39	44	38	0.045
Sex				0.900
Male	114	19 (16.7)	95 (83.3)	
Female	106	17 (16.0)	89 (84.0)	
WBC count (×10^9/l)	63.3	31.4	0.003	
BM blast (%)	59.9	56.3	0.474	
Hemoglobin (g/l)	80.3	81.8	0.796	
Platelet (×10^9/l)	72.0	55.8	0.073	
FAB				0.012
M0	4	1 (25.0)	3 (75.0)	
M1	7	1 (14.3)	6 (85.7)	
M2	107	15 (14.0)	92 (86.0)	
M3	43	1 (2.3)	42 (97.8)	
M4/M5	52	16 (30.8)	36 (69.2)	
M6	5	1 (20.0)	4 (80.0)	
M7	2	1 (50.0)	1 (50.0)	
Cytogenetics				<0.001
Abnormal	78	4 (5.1)	74 (94.9)	
t(8;21)	19	1(5.3)	18(94.7)	
t(15;17)	17	1 (5.9)	16 (94.1)	
inv(16)	8	1 (12.5)	7 (87.5)	
t(11;12)	1	0 (0.0)	1 (100.0)	
t(7;11)	1	0 (0.0)	1 (100.0)	
t(1;11)	1	0 (0.0)	1 (100.0)	
t(16;21)	1	0(0.0)	1(100.0)	
del(7)	2	0 (0.0)	2 (100.0)	
Others	28	1 (3.6)	27 (96.4)	
Normal	57	22(38.6)	35 (61.4)	
Unknown	85	10 (11.8)	75 (88.2)	
FLT3				<0.001
Mutation	22	14 (63.6)	8 (36.4)	
Wild type	198	22 (11.1)	176 (88.9)	
Outcome[a]			0.161	
CR	84	23 (85.2)	61 (71.8)	
Failure	28	4 (14.8)	24 (28.2)	

WBC white blood cell, *BM* bone marrow

[a] Excluding those with the M3 subtype and those with independent regimens or those without data to be analyzed

Detection of *NPM1* mutations with RQ-PCR

NPM1 gene mutations in 220 BM DNA samples of untreated AML patients were detected by RQ-PCR with the TaqMan® MGB probe (Table 3). The detection rate of *NPM1* mutations in AML patients (220 cases) was 16.4%, while that of M2 (107 cases), M3 (43 cases), and M4/M5 (52 cases) were 14.2%, 2.3%, and 30.8%, respectively. However, only one case each of *NPM1* mutation was detected in the M0 (four cases), M1 (seven cases), M6 (five cases), and M7 (two cases) groups. In 36 *NPM1*-mutation-positive specimens (Table 4), the average *NPM1* mutant copies/ALB copy was 19.7% (2.3–66.6%); background was amplified in eight cases whose C_t values were above 42.

Eight *NPM1*-mutation-positive patients were followed-up after treatment; among them, five continued to test negative for the *NPM1* mutation (Fig. 3) and three patients remained negative even after BM transplant. In one case, the *NPM1* mutation copies/ALB copies (%) value was 0.013% on remission, implying a decrease of 2 log as compared with the baseline. In the three cases that were not alleviated even after inductive treatment, the *NPM1* mutation copies/ALB copies (%) value was 8.8%, 7.3%, and 2.4%, thus indicating no obvious decline as compared with the baseline. Simultaneously, *NPM1* mutation A and

Table 4 Clinical features of *NPM1*-mutation-positive patients

No. of patients	Sex	Age	FAB	NPM1-mutated copes/ALB copes (%)	Types of NPM1 mutation	FLT3-ITD	CD34	Karyotype
1	F	39	M5	2.5	B (CATG	+	−	NA
2	M	35	M4	9.2	A (TCTG)	+	−	46XY
3	F	46	M5	29.8	B (CATG)	+	−	NA
4	F	44	M7	5.6	A (TCTG	−	In part +	NA
5	M	40	M4/M5	55.7	A (TCTG)	+	+	NA
6	M	38	M2	3.7	A (TCTG)	+	−	46XY
7	M	44	M2	4.1	A (TCTG)	−	NA	NA
8	F	52	M2	42.7	A (TCTG)	+	+	t(8;21)
9	M	36	M5	9.4	A (TCTG)	+	−	46XY
10	F	42	M2	41.5	A (TCTG)	+	−	NA
11	M	53	M2	9.7	A (TCTG)	−	−	NA
12	F	49	M5	27.0	A (TCTG)	−	−	46XX
13	M	69	M2	4.8	A (TCTG)	−	−	46XY
14	F	54	M5	5.3	A (TCTG)	+	+	46XX
15	M	19	M4	56.4	A (TCTG)	−	+	inv(16)
16	M	22	M3	25.1	A (TCTG)	−	−	t(15;17)
17	F	69	M2	6.9	A (TCTG)	−	−	46XX
18	F	50	M5	4.9	A (TCTG)	−	−	46XX
19	F	27	M2	32.3	B (CATG)	−	−	46XX
20	F	68	M2	23.9	A (TCTG)	+	NA	46XX
21	M	82	M2	24.5	A (TCTG)	+	In part +	46XY
22	M	19	M4	8.6	A (TCTG)	+	In part +	NA
23	M	58	M2	11.0	A (TCTG)	+	NA	46XY
24	M	22	M5	15.0	A (TCTG)	−	+	46XY
25	F	33	M2	2.3	BJ-01 (CTCG)	−	−	46XX
26	M	35	M5	4.1	A (TCTG)	−	−	NA
27	M	32	M2	18.2	A (TCTG)	−	−	46XY
28	F	65	M6	16.0	A (TCTG)	−	−	46XX
29	M	42	M4	15.3	BJ-02 (CAAG)	−	−	47XY,+8,add (9)(p13)
30	F	16	M2	14.0	B (CATG)	+	+	46XX
31	F	51	M5	45.6	A (TCTG)		−	46XX
32	F	42	M4	6.6	A (TCTG)	−	−	NA
33	M	47	M4	66.6	A (TCTG)	−	−	46XY
34	M	35	M0	NA	DD-3[a] (CAGA)	−	−	46XY
35	M	57	M2	27.1	A (TCTG)	−	−	46XY
36	F	32	M1	15.3	A (TCTG)	−	In part +	46XX

NA not applicable
[a] Detected only by sequence analysis

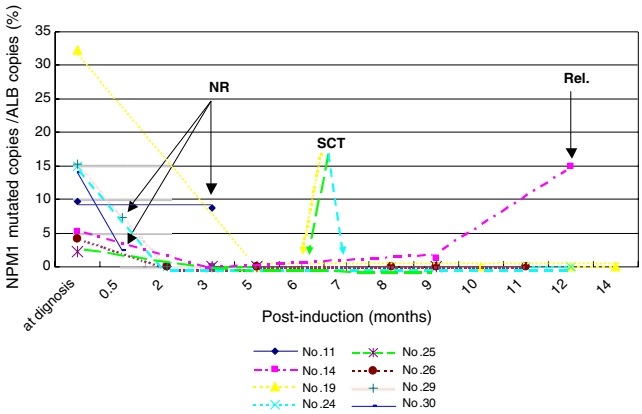

Fig. 3 *NPM1* mutated copies/ALB copies (%) at diagnosis, after induction therapy, and during follow-up in AML patients with *NPM1* mutations. *NR* no hematological remission, *Rel*. hematological relapse, *SCT* allogene haemopoietic stem cell transplantation

NPM1 mutation B had been detected in 56 cDNA specimens, and ten *NPM1*-positive patients exhibited higher levels of *NPM1* mutations (346.7–4,075.9 *NPM1* copies/100 ABL copies; average, 1,272.7). However, among 46 *NPM1*-mutation-negative patients, all *NPM1* mutation B cDNA amplifications were negative, 34 *NPM1* mutation A amplification cases were negative, while nonspecific amplification curves were observed in 12 other cases (threshold, 0.08; C_t value, 30.3–34.5; average, 31.9). Because the number of *NPM1* mutations at the DNA level represented the number of leukemia cells and the preparation of DNA samples was easier than that of cDNA, we used DNA samples for *NPM1* mutation detection in this study.

NPM1 sequencing results

Sequence analysis confirmed that all these mutations were heterozygous for a 4-bp insertion at the 959 site. Type A mutations accounted for 80.6% (29/36) of the total mutations, type B for 11.1% (4/36), type D for 0.0%, and CTCG and CAAG (BJ-01 and BJ-02, respectively) insertions were the two new mutations detected in the other two cases. Apart from the 35 cases in which the *NPM1* mutation was detected by PCR, only one case with the DD-3 mutation was detected by sequencing the other 55 normal karyotype specimens. This implies that the primer used for amplifying *NPM1* types A, B, and D mutations can also be used for screening BJ-01 and BJ-02. However, some rare *NPM1* mutations, like DD-3, require sequencing or other methods for detection.

Characteristics of *NPM1*-mutation-positive patients

NPM1 mutations were found in patients with AML of all FAB subtypes (Table 3). Patients with AML M4/M5 subtype had a higher *NPM1* mutation rate than those with M2 or M3 subtype ($p<0.05$, $p<0.001$, respectively). Cytogenetic data were available for 135 patients. The detection rates of *NPM1*-positive mutations in specimens exhibiting in the normal and abnormal karyotypes were 38.6% (22/57) and 5.1% (4/78), respectively, which was significantly different ($p<0.001$). Moreover, *NPM1* mutations have been detected in three patients with t(8;21), t(15;17) or inv(16) (Table 3). *NPM1* mutations were significantly associated with higher initial white blood cell counts in the peripheral blood and age, but not sex, blast in the bone marrow, hemoglobin and platelet counts in the peripheral blood.

Among the 36 *NPM1*-mutation-positive patients, 19 were males and 17 were females; of them, 38.9% (14/36) of the patients coherently exhibited FLT3/ITD mutations, and 66.7% (22/33) did not express CD34 on cells (the case was regarded as CD34 negative on cells when the percentage of express CD34 on blast cells is lower than 20%), as shown in Table 4. Of the 112 patients with AML, 84(75.0%) achieved complete response (CR) after induction chemotherapy. The CR rate was higher in the patients with *NPM1* mutation (23 of 27, 85.2%) than without them (61 of 85, 71.8%), but no statistical significant difference was observed ($p=0.161$; Table 3).

Discussion

NPM is a phosphoprotein with extensive expression that is present in abundance. It is involved in many cellular activities, including ribosomal biosynthesis and processing and the maintenance of genome stability. Abnormal *NPM* expression and/or its localization in cells may be involved in tumorigenesis by different mechanisms. Recent researches have indicated that mutation could occur in the exon 12 of the *NPM1* gene. Currently, more than 40 different types of *NPM1* mutations have been detected [13], and types A, B, and D are the most common. All these three types of mutations follow a similar pattern wherein a 4-bp sequence is inserted at the 959 site, causing a frame shift mutation that leads to a change in the amino acid sequence downstream of the mutation site.

By using RQ-PCR with a TaqMan® MGB probe, 220 BM DNA samples of untreated AML patients were screened for three types (A, B, and D) of *NPM1* gene mutations. The results demonstrated that the detection rate of *NPM1* mutations was 16.4% in 220 untreated AML patients and that in M2 (107 cases), M3 (43 cases), and M4/M5 (52 cases) cases was 14.2%, 2.3%, and 30.8%, respectively. Only one case each of *NPM1* mutation was detected in the M0 (four cases), M1 (seven cases), M6 (five cases), and M7 (two cases) groups. The detection rate of our study was slightly lower (16.4%, 36/220) than that of

studies conducted in China Taiwan (19.1%, 33/173) [14]. This might be because the primers we used only amplified the A, B, and D mutations and more M3 subtype in 220 AML patients which probably affects the frequency of *NPM1* mutations. The primers were thought to amplify 90–95% of the *NPM1* mutations (type A, approximately 75%; types B and D, approximately 15–20%); however, a small number of mutations were left out. Using PCR, we detected 35 cases with *NPM1* mutations, while there was one case with the DD-3 mutation that was detected by sequencing the other 55 normal karyotype samples. This implies that the primers used for amplifying the A, B, and D types of mutations could also be used for screening BJ-01 and BJ-02 [our results confirmed that the type A mutation accounted for 80.6% (29/36); type B, for 11.1% (4/36); type BJ-01, for 2.8% (1/36); and type BJ-02, for 2.8% (1/36)]. However, some rare *NPM1* mutations, like DD-3, need sequencing or other methods for their detection.

Thiede et al. [15] studied 1,485 AML patients and found that AML patients that tested positive for *NPM1* mutations often had a normal karyotype, lacked hematopoietic stem cell/progenitor cell surface markers such as CD34, and also exhibited a high frequency of FLT3/ITD mutations; these observations were in accordance with our results. Based on these observations, the AML patients were divided into four groups [15, 16]: simple *NPM1* mutation group, simple FLT3/ITD mutation group, double *NPM1* and FLT3 mutation group, and wild-type *NPM1* or FLT3 mutation group. They found that in the simple *NPM1* mutation group, the overall survival and disease-free survival rates were much higher, while the cumulative recurrence rates were lower. Therefore, the *NPM1* mutation was considered to comprise adult AML patients with common gene abnormality. In the absence of FLT3/ITD mutations, *NPM1* mutations may be one of the criteria to determine a patient's response to treatment. Suzuki et al. [3] also analyzed the prognostic value of *NPM1* mutations and found that the CR rate was significantly higher in the patients with *NPM1* mutation (42 of 49, 85.7%) than in those without (97 of 141, 68.8%; $p=0.025$). Our results showed that the CR rate was higher in the patients with *NPM1* mutation (23 of 27, 85.2%) than without them (61 of 85, 71.8%), but no statistical significant difference was observed ($p=0.161$) probably because of the small sample size.

In Italy, Gorello et al. [7] analyzed mutations in the *NPM1* gene in 13 AML patients by RQ-PCR and found that these mutations could be used to monitor and quantify the minimal residual diseases. Our results showed that the average number of the *NPM1* mutation copies/ALB copies in 35 untreated patients was 19.7% (2.3–66.6%). Of the eight patients screened for *NPM1* mutations at follow-up after treatment, five patients in remission continued to test negative for *NPM1* mutations within 2–14 months of follow-up. The average number of *NPM1* mutation copies/ALB copies (%) in one case with the ITD mutations in the FLT3 gene was 0.013% at remission, which implied a decrease of 2 log as compared with the baseline, rose, and reached 15.01% at clinical relapse, while three cases showed no alleviation even after inductive treatment. The number of *NPM1* mutation copies/ALB copies (%) was 8.8%, 7.3%, and 2.4%, with no obvious decline as compared with the baseline.

At present, *NPM1* gene mutations are one of the most extensive and specific gene mutations in a normal karyotype. In AML patients, therefore, the detection of *NPM1* mutations might be the only means to monitor AML alleviation at the molecular level, minimal residual leukemia, and recurrence [17].

We also found that RQ-PCR was a reliable and sensitive method for detecting *NPM1* mutations. It not only detects *NPM1* gene mutations in AML patients and monitor minimal residual diseases by quantitative analysis of *NPM1* mutations but also plays a role in predicting the response to treatment, in the assessment of prognosis, and research related to the pathomechanisms underlying AML.

Acknowledgments This study was supported by the grant from National Natural Science Foundation of China (no. 30670894) and supported in part by the grant from the National High Technology Research and Development Program of China (863 Program, no. 2006AA02A405).

References

1. Falini B, Mecucci C, Tiacci E, Alcalay M, Rosati R, Pasqualucci L et al (2005) Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. N Engl J Med 352:254–266. doi:10.1056/NEJMoa041974
2. Schnittger S, Schoch C, Kern W, Mecucci C, Tschulik C, Martelli MF et al (2005) Nucleophosmin gene mutations are predictors of favorable prognosis in acute myelogenous leukemia with a normal karyotype. Blood 106:3733–3739. doi:10.1182/blood-2005-06-2248
3. Suzuki T, Kiyoi H, Ozeki K, Tomita A, Yamaji S, Suzuki R et al (2005) Clinical characteristics and prognostic implications of *NPM1* mutations in acute myeloid leukemia. Blood 106:2854–2861. doi:10.1182/blood-2005-04-1733
4. Verhaak RG, Goudswaard CS, van Putten W, Bijl MA, Sanders MA, Hugens W et al (2005) 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 106 (12):3747–3754. doi:10.1182/blood-2005-05-2168
5. Boissel N, Renneville A, Biggio V, Philippe N, Thomas X, Cayuela JM et al (2005) Prevalence, clinical profile and prognosis of *NPM* mutations in AML with normal karyotype. Blood 106:3618–3620. doi:10.1182/blood-2005-05-2174
6. Qin Y-Z, Liu Y-R, Li J-L, Fu J-Y, Chang Y, Ruan G-R, Wang H, Qiu J-Y, Lu D-P, Chen S-S (2003) Follow-up Detection of M-bcr/abl and m-bcr/ abl fusion transcripts in chronic myeloid leukemia

patients after allogeneic hematopoietic stem cell transplantation. Zhongguo Shi Yan Xue Ye Xue Za Zhi 11:368–371
7. Gorello P, Cazzaniga G, Alberti F, Dell'Oro MG, Gottardi E, Specchia G et al (2006) Quantitative assessment of minimal residual disease in acute myeloid leukemia carrying nucleophosmin (*NPM1*) gene mutations. Leukemia 20:1103–1108. doi:10.1038/sj.leu.2404149
8. Qin YZ, Li JL, Zhu HH, Ruan GR, Li LD, Zhang Y et al (2006) Monitoring bcr-abl mRNA levels by real-time quantitative RT-PCR in chronic myeloid leukemia patients after hematopoietic stem cell transplantation. Zhonghua Xue Ye Xue Za Zhi 27 (8):511–514
9. Van der Velden VH, Hochhaus A, Cazzaniga G, Szczepanski T, Gabert J, van Dongen JJ (2003) Detection of minimal residual disease in hematologic malignancies by real-time quantitative PCR: principles, approaches, and laboratory aspects. Leukemia 17:1013–1034. doi:10.1038/sj.leu.2402922
10. Sheikhha MH, Awan A, Tobal K, Liu Yin JA (2003) Prognostic significance of FLT3 ITD and D835 mutations in AML patients. Hematol J 4(1):41–46. doi:10.1038/sj.thj.6200224
11. Liu YR, Yu H, Chang Y, Chen SS (2003) The application of 4-color fluorescence labeling antibodies and its significance in immunophenotyping for leukemia by flow cytometer. Zhongguo Shi Yan Xue Ye Xue Za Zhi 10(5):423–427
12. Zhang Y, He Q, Huang XJ, Jiang H, Yang SM, Lu J et al (2007) Cytogenetic study on eosinophilia. Zhongguo Shi Yan Xue Ye Xue Za Zhi 15(3):454–457
13. Falini B, Nicoletti I, Martelli MF, Mecucci C (2006) Acute myeloid leukemia carrying cytoplasmic/mutated nucleophosmin (NPMc_AML): biologic and clinical features. Blood 109(3):874–885. doi:10.1182/blood-2006-07-012252
14. Chou WC, Tang JL, Lin LI, Yao M, Tsay W, Chen CY et al (2006) Nucleophosmin mutations in de novo acute myeloid leukemia: the age-dependent incidences and the stability during disease evolution. Cancer Res 66(6):3310–3316. doi:10.1158/0008-5472.CAN-05-4316
15. Thiede C, Koch S, Creutzig E, Steudel C, Illmer T, Schaich M et al (2006) Prevalence and prognostic impact of *NPM1* mutations in 1485 adult patients with acute myeloid leukemia (AML). Blood 107:4011–4020. doi:10.1182/blood-2005-08-3167
16. Döhner K, Schlenk RF, Habdank M, Scholl C, Rücker FG, Corbacioglu A et al (2005) Mutant nucleophosmin (*NPM1*) predicts favorable prognosis in younger adults with acute myeloid leukemia and normal cytogenetics: interaction with other gene mutations. Blood 106(12):3740–3746. doi:10.1182/blood-2005-05-2164
17. Grisendi S, Mecucci C, Falini B, Pandolfi PP (2006) Nucleophosmin and cancer. Nat Rev Cancer 6(7):493–505. doi:10.1038/nrc1885