Mutant Enrichment with 3'-Modified Oligonucleotides (MEMO)-Quantitative PCR for Detection of *NPM1* Mutations in Acute Myeloid Leukemia

Sang-Yong Shin, Chang-Seok Ki, Hee-Jin Kim, Jong-Won Kim, Sun-Hee Kim, and Seung-Tae Lee*

Department of Laboratory Medicine & Genetics, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea

Background: Detection of *NPM1* mutations in acute myeloid leukemia (AML) is important for risk stratification, treatment decision, and therapeutic monitoring. We have designed a real-time PCR method implementing the Mutant enrichment with 3'-modified oligonucleotides (MEMO) technique to detect *NPM1* mutations and validated its utility in clinical samples. **Methods:** Sensitivity and linearity were evaluated using serially diluted *NPM1*-positive samples. Clinical usefulness was assessed by measuring the levels of mutant alleles in 29 patients at diagnosis and in ten patients after induction chemotherapy. **Results:** Excellent linear relationships between the mutant allele proportion and the threshold cycle (Ct) values ($r = 0.999$) were observed in a range of $1:1-1:10^3$. MEMO-PCR was able to detect *NPM1* muta-

tions regardless of mutant type and also detected novel mutants (964_967delTG-GAinsATGATGTC, 957_959delCTGinsATGCATG, 960insTAAG, and 960insTCAG). The concentrations of *NPM1* mutant alleles decreased after induction chemotherapy in accordance with the reduction of tumor cells, and in one case, *NPM1* mutant alleles were detectable about 7 months before morphological relapse. **Conclusion:** MEMO-quantitative PCR was shown to detect virtually all types of *NPM1* mutants with high sensitivity and specificity. This novel method may be useful in the diagnosis of AML with an *NPM1* mutation, the detection of minimal residual disease, and the monitoring of treatment response. J. Clin. Lab. Anal. 29:361–365, 2015. © 2014 Wiley Periodicals, Inc.

Key words: *NPM1*; AML; real-time PCR

INTRODUCTION

Acute myeloid leukemia (AML) is the second most common leukemia in adults (1), and the prognosis is generally poor. Several recurrent chromosomal translocations are identified in AMLs, defining the pathological and clinical nature of each subgroup and enabling sensitive diagnosis by molecular assays directed to the specific translocations. However, about half of AML cases are cytogenetically normal (CN-AML), and in this group, molecular markers other than cytogenetic markers are needed for the diagnosis, prognostic stratification, and therapeutic monitoring. The cancer gene mutations, which have been identified in AML thus far include *FLT3*, *KIT*, *KRAS*, *NRAS*, *NPM1*, *CEBPA*, *MLL*, *WT1*, *TP53*, *TET2*, *IDH1*, *IDH2*, and many others (2). Some of these gene mutations are shown to be important for risk stratification, management of AML as well as monitoring of

minimal residual diseases (3, 4). Among these mutations, European LeukemiaNet strongly recommended that the *NPM1*, *CEBPA*, and *FLT3* genes be tested at the initial work-up of AML patients (4, 5). Furthermore, the World Health Organization classified AML with mutations of *NPM1* and *CEBPA* as separate provisional entities (6).

Grant sponsors: Basic Science Research Program and National Research Foundation of Korea (NRF), Grant number: NRF-2012R1A1A2043879.

*Correspondence to: Seung-Tae Lee, Department of Laboratory Medicine & Genetics, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50 Irwon-dong, Gangnam-gu, Seoul 135-710, Korea. E-mail: nb.seungtae.lee@gmail.com

Received 23 November 2013; Accepted 29 April 2014

DOI 10.1002/jcla.21779

Published online in Wiley Online Library (wileyonlinelibrary.com).

Fig. 1. Principles of MEMO-quantitative PCR for *NPM1* mutant detection. Blocking primers complementary to the normal sequence anneal to normal DNAs, hampering PCR amplification. In the presence of a 4 bp insertion mutation, the blocking primers can limitedly bind to mutant DNAs due to the mismatch and so enable amplification.

NPM1 is the most commonly mutated gene in AML, especially in CN-AMLs (~50% of cases) (7). The clinical utility of *NPM1* mutation testing is well demonstrated for its strong prognostic implications (8) and usefulness in minimal residual disease monitoring (9). The molecular basis of the *NPM1* mutation is the insertion of one or two tetranucleotides at exons 11 or 12, with the most common type being the 960insTCTG (type A) mutation. There is no clinical significance according to the type of mutation (10). Several methods of detecting the *NPM1* mutation have been introduced but have not satisfactorily met clinical needs (11). Sanger sequencing reliably detects any type of base change but can only detect a mutation when the mutant alleles exist at a proportion greater than 20% of the wild-type alleles. Other sensitive methods, such as the amplification-refractory mutation system and Taqman assays, show high sensitivities but can only detect a limited set of mutations at which mutant-specific primers are directed. Based on these findings, we designed a new quantitative PCR assay based on Mutant enrichment with 3'-modified oligonucleotides (MEMO) (12), which can detect virtually all types of mutants, and evaluated its performance in clinical samples.

METHODS AND MATERIALS

Patient Samples and DNA Extraction

Bone marrow samples at initial diagnosis, during follow-up, and at relapse were obtained from 29 AML patients with *NPM1* mutations, which were first confirmed

by conventional Sanger sequencing. Genomic DNA was isolated using the Wizard Genomic DNA Purification kit (Promega, Madison, WI). The concentration of the extracted DNA was adjusted to 200 ng/μl for each sample. To assess analytical performance, a DNA sample from an AML patient containing about 50% of *NPM1* mutant alleles (960ins TCTG) was serially diluted in equimolar normal DNAs (1:1, 1:10, 1:10², 1:10³, 1:10⁴, and 1:10⁵).

MEMO-Quantitative PCR and High-Resolution Melting Curve Analysis

For the detection of *NPM1* mutations, a MEMO-quantitative PCR and high-resolution melting curve analysis based on the MEMO-PCR technique was developed with a modification of the previous method (12). Briefly, one blocking primer (5'-TTCAAGATCTCTGGCAGTGGAGGAAGTCT-[C3]-3') was designed to be complementary to wild-type sequences and encompass the mutation site around the middle of the primer, partially overlapping the forward primer (5'-TTTTTCCAGGCTATTCAAGA-3'). The 3'-end of the blocking primer was modified with a C3 spacer so that the blocking primer would not serve as an amplification primer and hinder the PCR reaction of wild-type DNAs (Fig. 1). In the reaction tube containing AccuPower[®] HF PCR PreMix (Bioneer Corp., Daejeon, Korea) and BEBO dye (TATAA Biocenter, Goteborg, Sweden), 50 pmol of blocking primers, 10 pmol of forward primers, and 10 pmol of reverse primers (5'-TAAACAGGCATTTTGGACA-3') were added along

Fig. 2. Analytical performance of MEMO-quantitative PCR for serially diluted *NPM1* mutant samples. An amplification plot (A) and standard curve (B) show low variability and strict linear correlation ($r = 0.999$). Melting curve analysis shows mutant peaks around 79°C down to a 1:10³ dilution (C).

with 200 ng of testing DNA. The cycling conditions were as follows: 94°C for 5 min, 50 cycles of the main reaction (94°C for 30 sec, 55°C for 30 sec, and 72°C for 60 sec), and 72°C for 7 min, followed by melting temperature identification by 0.2°C steps. Data were analyzed using the Corbett Rotor-Gene 6000 Application Software version 1.7 (QIAGEN, Valencia, CA). Standard curves were generated using diluted samples with duplicate runs in each dilution factor. Mutant allele concentrations in the test samples were calculated from the fitted standard curves. After melting curve analysis to disclose the presence of the mutation, every product was sequenced to verify the results.

RESULTS

Analytical Performance of MEMO-Quantitative PCR

A strict linear correlation was observed in dilutions ranging from 1:1 to 1:10³, and the correlation coefficient (r) in this range was around 0.999 (Fig. 2). In the duplicate runs, differences in threshold cycle (Ct) values were less

than 0.6, which was an acceptable range. Melting curve analysis identified melting peaks around 79.2°C in the mutant DNAs, which was identifiable down to 1:10³ dilutions. Subsequent sequencing analysis of the PCR product confirmed the presence of mutant alleles in all of the samples that showed mutant melting curves.

Clinical Utility in Initial Diagnosis and Minimal Residual Disease Monitoring

Among the 29 patients with *NPM1* mutations, 24 (82.8%) had a type A mutation (960insTCTG), one had a type B mutation (960insCATG), and the remaining four had novel mutations including 964_967delTGGGinsATGATGTC, 957_959delCTGinsATGCATG, 960insTAAG, and 960insTCAG (Table 1). MEMO-quantitative real-time PCR detected mutations in all samples, regardless of the mutation type. All cases showed melting peaks around 79°C. We defined a cutoff value of 0.01 for clinical decision considering that clones with <1% proportion may have less significant clinical importance, and although rare, the chance of

TABLE 1. Clinical Characteristics and Mutant Allele Concentrations of 29 Cases Enrolled in This Study

Case no.	Age, (years)	Hb, (g/dL)	WBC, (/μL)	PLT, (10 ³ /μL)	Blast% (marrow)	<i>NPM1</i> mutation type	<i>NPM1</i> mutant concentration	
							At diagnosis	After induction
1	35	11.1	12,560	35	85	960insTCTG (type A)	4.9226	0.0003
2	64	10.6	271,490	59	90	960insTCTG (type A)	0.2232	0.0003
3	24	9.3	112,060	42	100	960insTCTG (type A)	2.3723	0.0003
4	44	9.2	34,180	183	88.9	960insTCTG (type A)	1.3057	0.0050
5	34	4.3	6,230	165	47.62	964_967delTGGinsATGATGTC (novel)	0.5309	0.0024
6	37	10.2	6,450	63	93.1	960insTCTG (type A)	1.1360	0.0012
7	49	10.5	12,920	61	64.33	960insTCTG (type A)	3.1816	0.0004
8	3	7.1	20,690	27	64.8	957_959delCTGinsATGCATG (novel)	0.4849	0.0017
9	65	8.2	11,920	29	40.8	960insTCTG (type A)	1.2095	0.0003
10	35	9.4	47,250	83	85	960insTCTG (type A)	2.1170	0.0156
11	64	8.2	66,910	144	20.66	960insTCTG (type A)	4.8500	NA
12	50	9.8	58,790	38	88.45	960insTAAG (novel)	4.8347	NA
13	36	7.8	26,200	591	29.8	960insTCTG (type A)	5.8690	NA
14	35	7.9	4,620	132	43.8	960insTCAG (novel)	3.7214	NA
15	35	7.4	84,440	27	80.4	960insCATG (type B)	2.1170	NA
16	59	6.7	111,713	141	72.3	960insTCTG (type A)	2.9322	NA
17	59	8	650	56	26	960insTCTG (type A)	1.1108	NA
18	44	8.1	8,110	64	70	960insTCTG (type A)	1.0371	NA
19	29	9.7	3,690	14	34.94	960insTCTG (type A)	4.9940	NA
20	33	9.5	4,530	44	50.91	960insTCTG (type A)	1.5542	NA
21	70	9.5	18,120	19	32.54	960insTCTG (type A)	2.5805	NA
22	50	6.6	1,850	34	17	960insTCTG (type A)	0.8243	NA
23	73	7.9	30,290	129	36.4	960insTCTG (type A)	2.4797	NA
24	73	9.7	99,690	40	51.8	960insTCTG (type A)	0.9600	NA
25	63	8.7	16,080	35	95.63	960insTCTG (type A)	2.0611	NA
26	55	9.4	148,560	52	97	960insTCTG (type A)	1.4535	NA
27	57	8.7	37,100	74	77.8	960insTCTG (type A)	1.3744	NA
28	51	9	13,870	26	11.5	960insTCTG (type A)	2.0430	NA
29	56	9.4	205,620	97	90	960insTCTG (type A)	0.7525	NA

Hb, hemoglobin; NA, not available; WBC, white blood cell.

technical artifacts may increase in amplifying a very low proportion of targets (Fig. 2C).

In ten of the 29 patients, we tested bone marrow samples obtained after induction chemotherapy. All ten cases achieved complete hematologic remission, and mutant allele concentrations decreased with the decrease of leukemic blast proportion (Fig. 3A). Two cases relapsed during the follow-up period, and among them, samples during this period were available in one case (case no. 6 in Table 1). At the time of full relapse, a high concentration of mutant *NPM1* alleles was observed again. The mutant allele concentration started to rise above 0.01 about 7 months before the full-blown relapse (Fig. 3B). The concentration of mutant DNAs in case no. 10 did not decrease below 0.01 after induction chemotherapy, and the patient expired due to sepsis after allogeneic bone marrow transplantation.

DISCUSSION

Considerations in selecting the detection method of the *NPM1* mutation may include the sensitivity, reliability,

and ability to detect various types of mutations. Type A (ins 960TCTG), B (ins960CATG), and D (ins960CCTG) mutations comprise about 90% of cases, with the remaining 10% of cases being heterogeneous with more than 30 different types of rare mutants (13). Therefore, methods universally applicable to common mutations as well as rare mutations are of greater clinical utility (14). Sanger sequencing and PCR fragment analysis meet such needs, but their sensitivities (~20% and ~5%, respectively) are not sufficient for minimal disease monitoring (15). Many sensitive methods such as allele-specific quantitative PCR assays designed for patient-specific mutants have been developed but lack “universal applicability” (9, 16, 17). Our MEMO-PCR method has a similar mechanism with that of peptide nucleic acid (PNA) or locked nucleic acid (LNA) based PCR clamping (18, 19) and shares the strengths of such methods, including high sensitivity, low false-positivity, quantitative measurement, and applicability to most variants on specific sites. By replacing the expensive PNA or LNA techniques with inexpensive and easily designable oligonucleotides, the mutations still could be detected with high sensitivity and reliability. In a

Fig. 3. The concentrations of mutant alleles tested by MEMO-quantitative PCR were decreased after induction chemotherapy in accordance with the reduction of leukemic blasts (A). In one patient, the mutant allele was detected 7 months before morphologic relapse (B).

relapsed case, our method could predict clinical relapse 7 months before the morphologic relapse, like other allele-specific quantitative real-time assays have previously developed. One limitation of the MEMO-quantitative PCR may be difficulties in implementing specific probes such as Taqman probe. In this study, we used an intercalating dye with cycle threshold measurement and melting curve analysis. Such methods can be less specific than probe-based methods and be affected by DNA concentrations in some cases, especially in those with a very low proportion of mutants.

In conclusion, we developed a MEMO-quantitative PCR assay for the detection of *NPM1* mutations, and this novel method could be easily and efficiently used in clinical laboratories.

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