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Quantitative monitoring of single nucleotide mutations by allele-specific quantitative PCR can be used for the assessment of minimal residual disease in patients with hematological malignancies throughout their clinical course

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Abstract

Background: Monitoring of minimal residual disease (MRD) in patients with hematological malignancies is important for evaluating the patients' therapeutic response and risk of relapse. Single nucleotide mutations associated with leukemogenesis can be considered as applicable MRD markers.

Methods: We developed an allele-specific quantitative polymerase chain reaction (AS-qPCR) for *FLT3* 2503G > T, *KIT* 2446G > T, and *KIT* 2447A > T and compared the change in the expression levels of the *FLT3* or *KIT* mutations assessed by AS-qPCR to those of the *RUNX1-RUNX1T1* fusion gene and *WT1* by conventional quantitative PCR.

Results: The AS-qPCR using primers including template-mismatched nucleotide or template-mismatched nucleotide plus locked nucleic acid substituted nucleotide provided higher selectivity for mutant nucleotides. The change in the expression levels of the *FLT3* or *KIT* mutations at the time of relapse and just after hematopoietic stem cell transplantation correlated well with that of the *RUNX1-RUNX1T1* fusion gene and *WT1*. Moreover, during complete remission, only AS-qPCR could detect low-level expression of residual mutations.

Conclusions: The AS-qPCR for analyzing single nucleotide mutations contributes to the monitoring of MRD in patients without recurrent fusion gene throughout the clinical course and thus broadens the spectrum of patients in whom MRD can be monitored.

1. Introduction

In patients with hematological malignancies, minimal residual disease (MRD) status is correlated with the clinical outcome, and monitoring of MRD during chemotherapy and after hematopoietic stem cell transplantation (HSCT) is important in evaluating the patients' therapeutic response and the risk of relapse. The targets frequently used for MRD detection are fusion gene transcripts such as *RUNX1-RUNX1T1, CBFB-MYH11*, and *PML-RARA* resulting from t(8;21)(q22;q22), inv(16)(p13q22) or t(16;16)(p13;q22), and t(15;17)(q22;q12) [1]. In addition to the recurrent reciprocal translocations associated with leukemogenesis, a number of genetic alterations such as insertion, interstitial/partial tandem duplication, and single nucleotide mutation have been reported to be involved in the pathogenesis of leukemia and associated with the prognosis of the affected patients [2,3]. Recently, several groups have performed quantitative assessment of *NPM1* or *FLT3* mutations and used them as MRD markers in patients with acute myelogenous leukemia [4–10]. *NPM1* mutations are mainly characterized by tetranucleotide insertion. The quantitative assessments of the *NPM1* mutation were performed by using TaqMan systems or LightCycler assays using primers or probes specific for the duplicated tetranucleotide. *FLT3* mutations consist of 2 major types: internal tandem duplication and a missense point mutation in the tyrosine kinase domain. Quantitative assessments of *FLT3*- internal tandem duplication have been reported using the assay similarly to that for assessment of *NPM1* mutations. According to the review by Renneville et al. [3], single nucleotide mutations of genes such as *KIT*, *FLT3*, *RAS*, and *PTPN11*; *RUNX1* and *CEBPA*; and *TP53* are involved in proliferative advantage, impairment of hematopoietic

differentiation, and regulation of cell cycle and apoptosis, respectively. These single nucleotide mutations can be considered as applicable targets for monitoring MRD. However, a few studies have reported the quantitative assessments of single nucleotide mutations as compared to those that have reported insertions/duplications. Recently, several studies have reported that single nucleotide mutations of *JAK2* and *MPL*, or *BCR-ABL* were quantitatively monitored in myelofibrosis patients following transplantation [11–13], or in chronic myelogenous leukemia patients with imatinib resistance, respectively [14,15]. The detection of single nucleotide mutations in excess amount of wild-type nucleotides is more complicated than that of insertions/duplications involving several nucleotides. Allele-specific quantitative polymerase chain reaction (AS-qPCR) based on the amplification refractory mutation system (ARMS) or mismatch amplification mutation assay (MAMA) has been developed for quantification of single nucleotide mutations [16–18]. To obtain high specificity and sensitivity of the AS-qPCR, improvements, including the modification of primers and/or probes and additional procedures with restriction enzymes to digest residual wild-type nucleotides are required [19–21]. The AS-qPCR for single nucleotide mutations seems to be a useful method for the assessment of MRD.

In the present study, to evaluate whether AS-qPCR for single nucleotide mutations can be used for precise monitoring of MRD, we developed AS-qPCRs for *FLT3* 2503G > T, *KIT* 2446G > T, or *KIT* 2447A > T and compared the change in the expression levels of the *FLT3* or *KIT* mutations assessed by AS-qPCR to those of *RUNX1-RUNX1T1* fusion gene and *WT1*, which were assessed using conventional quantitative PCR (qPCR).

2. Materials and methods

2.1. Patients and controls

We performed the present study using bone marrow (BM) cells obtained from 4 t(8;21)-positive acute myeloid leukemia (AML) patients carrying gene mutations (1 with *FLT3* 2503G > T, 1 with *KIT* 2446G > T, and 2 with *KIT* 2447A > T) (Table 1). Ten BM samples from persons without hematological malignancies were used as normal controls. This study was approved by the institutional review board of the Shinshu University. Informed consent was obtained from the patients or guardians of the patients following institutional guidelines.

2.2 Total RNA extraction and complementary DNA (cDNA) synthesis

Total RNA was extracted from 4 t(8;21)-positive AML patients with gene mutations using the QIAamp RNA blood mini kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. The first-strand cDNA was synthesized from 1 μg total RNA in 20 μL reaction buffer containing 10 mM of deoxynucleotide triphosphate, 0.1 M of dithiothreitol, 25 μM of random hexamer primers, and 200 U of Moloney murine leukemia virus reverse transcriptase. The reverse transcription reaction was incubated at 42°C for 1 h.

2.3. Construction of plasmids carrying wild-type or mutant FLT3 and KIT

To construct plasmids carrying the wild-type *FLT3* or *KIT*, PCR products obtained by amplification of cDNA samples from normal controls were cloned into a pCR2.1 vector using the TA cloning kit (Invitrogen, Paisley, UK). Plasmids carrying the *FLT3* 2503G > T, *KIT* 2446G > T, or *KIT* 2447A > T mutation were then synthesized from the

wild-type plasmids by oligonucleotide-directed mutagenesis using a site-directed mutagenesis kit (Stratagene, La Jolla, CA). The nucleotide sequences of the wild-type and mutant plasmids were confirmed by direct sequencing from both directions on an automatic DNA sequencer (ABI3100 Genetic Analyzer, Applied Biosystems, Foster City, CA).

2.4. Design of the primers for the FLT3 or KIT mutations in 3 types of AS-qPCR

Three different mutation-specific primers for the *FLT3* or *KIT* mutations were designed as follows: mutant AS-qPCR primers including only a mutant-matched nucleotide in the 3′-end; mismatched AS-qPCR primers including a mutant-matched nucleotide in the 3′-end and a template-mismatched nucleotide at the penultimate 3′-end; locked nucleic acid (LNA)-AS-qPCR primers including a mutant-matched nucleotide in the 3′-end, a template-mismatched nucleotide at the penultimate 3′-end, and LNA at the -2 position from the 3′-end (Table 2) [19,21].

2.5. AS-qPCR

TaqMan probes, including fluorescein amidite (FAM) at the 5′-end nucleotide and a quencher (tetramethylrhodamine, TAMRA) at the 3′-end nucleotide, were exploited to assess the specificity and sensitivity of the AS-qPCR. The AS-qPCR reaction mixture contained cDNA (corresponding to 100 ng RNA), 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 0.5 μmol/L of each primer, and 0.25 μmol/L of TaqMan probe in a total of 50 μL. The AS-qPCR was performed using an ABI PRISM 7900 Sequence Detection System (Applied Biosystems) at 50°C for 2 min, 95°C for 10 min, followed by 50 cycles at 95°C for 15 sec and 60°C for 1 min. To examine the

specificity of the 3 types of AS-qPCR to discriminate between wild-type and mutant plasmid DNA, the difference of the threshold cycles (ΔCt) was calculated as follows: (Ct for mutant plasmid) - (Ct for wild-type plasmid) in each AS-qPCR assay. To investigate the sensitivity for mutant plasmid DNA of the mismatched AS-qPCR and LNA-AS-qPCR, the delay in Ct (delay Ct) was calculated as follows: (Ct for mutant plasmid in mismatched AS-qPCR or LNA-AS-qPCR) - (Ct for mutant plasmid in mutant AS-qPCR) [19,21]. The expression levels of the *FLT3* or *KIT* mutations, *RUNX1-RUNX1T1* fusion gene, or *WT1* were normalized with respect to the expression of the *abelson* (*ABL*) gene, and expressed as copy numbers every 10^4 copies of *ABL* [22]. Each AS-qPCR assay for an individual patient was performed in triplicate.

2.6. Detection limit of the AS-qPCR for FLT3 and KIT mutations or qPCR for RUNX1-RUNX1T1 fusion gene, and normal expression level of WT1

To determine the detection limit of the AS-qPCR, cDNA obtained from patients at diagnosis were serially 10-fold diluted in pooled cDNA from healthy controls, and the AS-qPCR was performed using the diluted samples [23]. In patient 2, the sample at diagnosis was not available and thus, the BM sample after chemotherapy was used to determine the detection limit. The normal expression level of *WT1* was determined using BM samples from persons without hematological malignancies.

2.7. Quantification of RUNX1-RUNX1T1 fusion gene and WT1

The expression levels of the *RUNX1-RUNX1T1* fusion gene or *WT1* were quantified by conventional qPCR using the cDNA of the same time point as that used for the AS-qPCR [1,24].

2.8. Statistics

Analysis of variance (ANOVA) was used for the comparisons of ΔCt among the 3 types of AS-qPCR, and unpaired t-test was used for the comparisons of delay Ct between mismatched AS-qPCR and LNA-AS-qPCR; $p < 0.05$ was considered to be significant.

3. Results

3.1. Characterization of mutant AS-qPCR, mismatched AS-qPCR, and LNA-AS-qPCR

Using equal copy numbers of the constructed wild-type and mutant plasmids, the ΔCt was examined in the mutant AS-qPCR, mismatched AS-qPCR, and LNA-AS-qPCR (Figure 1). Compared with the mutant AS-qPCR, the other 2 types of AS-qPCR (mismatched AS-qPCR and LNA-AS-qPCR) showed significantly higher specificity for discrimination between the wild-type and mutant plasmid in all 3 gene mutations (Fig. 1, $p < 0.01$). Next, the delay Ct was calculated to evaluate the sensitivity for mutant plasmids by mismatched AS-qPCR or LNA-AS-qPCR (Fig. 1). The delay Ct in the mismatched AS-qPCR for *FLT3* 2503G > T, *KIT* 2446G > T, and *KIT* 2447A > T were 0.12, 0.00, and 0.69 cycles, respectively. The delay Ct in the LNA-AS-qPCR for *FLT3* 2503G > T, *KIT* 2446G > T, and *KIT* 2447A > T were 1.59, 0.17, and 4.63 cycles, respectively. We adopted the LNA-AS-qPCR for *FLT3 2503*G > T and *KIT 2446*G > T except *KIT 2447*A > T. For *KIT 2447*A > T, we adopted the mismatched AS-qPCR because the delay Ct in the LNA-AS-qPCR was 4.63 cycles which meant a more than 10 ($2^{3.33}$)-fold reduction of sensitivity.

3.2. Detection limit of the AS-qPCR for FLT3 and KIT mutations or qPCR for the RUNX1-RUNX1T1 fusion gene, and normal expression level of WT1

The expression levels of *FLT3* 2503G > T or *KIT* 2447A > T at diagnosis in patient 1, 3, and 4 were 2.04×10^4, 3.02×10^4, and 4.43×10^4, respectively (Fig. 2a, c, and d). The detection limits of *FLT3* 2503G > T or *KIT* 2447A > T in patient 1, 3, and 4 were 2.04×10, 3.02×10, and 4.43×10, respectively (Fig. 2a, c, and d). As compared with the expression level at diagnosis, the mutations could be detected at a 10^3-fold lower expression level. In patient 2 (Fig. 2b), a sample at diagnosis was not available; therefore, a BM sample after chemotherapy was used to determine the detection limit. The expression level of *KIT* 2446G > T in the BM sample after chemotherapy was 3.85×10^3 and the detection limit was 3.85×10; thus, the mutation could be detected at a 10^2-fold lower expression level. The expression levels of the *RUNX1-RUNX1T1* fusion gene in patient 1, 2, 3, and 4 were 3.75×10^4, 3.03×10^2, 2.02×10^4, and 2.71×10^4, respectively (Fig. 2). The detection limits in patient 1, 2, 3, and 4 were 3.75, 3.03, 2.02, and 2.71, respectively (Fig. 2). As compared with the expression level at diagnosis, the *RUNX1-RUNX1T1* fusion gene could be detected at a 10^4-fold lower expression level, except in patient 2. In patient 2, the *RUNX1-RUNX1T1* fusion gene could be detected at a 10^2-fold lower expression level. The expression levels of *WT1* in patient 1, 2, 3, and 4 were 1.59×10^3, 3.03×10, 3.39×10^2, and 3.90×10^3, respectively (Fig. 2). The mean expression level of *WT1* in 10 normal controls was $(5.96 \pm 4.63) \times 10$ (Fig. 2).

3.3 Comparison of the change in the expression levels of FLT3 2503G > T, KIT

2446G > T, or KIT 2447A > T assessed by AS-qPCR with that of the RUNX1-RUNX1T1 fusion gene and WT1 by qPCR

Using samples obtained from 4 t(8;21)-positive patients with *FLT3* 2503G > T, *KIT* 2446G > T, or *KIT* 2447A > T, the clinical significance of the AS-qPCR was investigated by comparison of the change in the expression level of these mutations with that of the *RUNX1-RUNX1T1* fusion gene and *WT1*. In patient 1 (Fig. 2a), the expression level of *FLT3* 2503G > T and the *RUNX1-RUNX1T1* fusion gene decreased to an undetectable level soon after induction therapy and retained this level during complete remission (CR). In contrast, the expression level of *WT1* did not show more than 10^2-fold reduction after induction therapy and the levels did not further decline during CR. In patient 2 (Fig. 2b) whose diagnostic sample was not available and a sample obtained after chemotherapy was used as the first time point of the follow-up, the expression level of *KIT* 2446G > T showed an increase before and at the time of relapse, which was in accordance with that of the *RUNX1-RUNX1T1* fusion gene and *WT1*. In the follow-up during continuous CR after HSCT, the expression levels of *KIT* 2446G > T and the *RUNX1-RUNX1T1* fusion gene were detected at the level of 10- and 10^2-fold reduction. The expression level of *WT1* maintained at steady levels close to that of normal controls. In patient 3 (Fig. 2c) who underwent HSCT twice, the increase in the expression level of *KIT* 2447A > T at the time of the first and second relapse, and the decrease in the expression level following the first and second HSCT agreed well with the change in the expression levels of the *RUNX1-RUNX1T1* fusion gene and *WT1*. During CR following the first HSCT, the expression levels of *KIT 2447*A > T were marginally detectable, while that of the *RUNX1-RUNX1T1* fusion gene were below the detection limit. Seven months after the second HSCT, the expression

level of *KIT* 2447A > T was below the detection limit and retained at that level until now. In patient 4 (Fig. 2d), after HSCT, the expression levels of *KIT* 2447A > T and the *RUNX1-RUNX1T1* fusion gene decreased 10^3-fold and that of *WT1* decreased 40-fold. However, the expression of both *KIT* 2447A > T and the *RUNX1-RUNX1T1* fusion gene have been detected at the level of 10 to 10^2 at 11 months after HSCT.

4. Discussion

In the present study, we developed AS-qPCRs to quantify leukemia-associated single nucleotide mutations in genes such as *FLT3* and *KIT*, and evaluated the AS-qPCR to monitor MRD during chemotherapy and post-HSCT by comparison with the qPCR for the *RUNX1-RUNX1T1* fusion gene and *WT1*.

Several allele-specific PCR methods based on ARMS or MAMA have been developed for the detection of single nucleotide mutations [16,17]. The primers included a mutant-matched nucleotide at the 3′-end and a template-mismatched nucleotide near the 3′-end. Subsequently, an AS-qPCR based on ARMS or MAMA was developed to quantify low levels of mutant nucleotides in the presence of high levels of the counterpart wild-type nucleotides [18]. To increase the selectivity of the AS-qPCR, our present methods utilized primers including template-mismatched nucleotides or template-mismatched nucleotides plus LNA-substituted nucleotides which increased the binding affinity compared to standard nucleotides [19,21,25–27]. For all mutations evaluated in this study, the ΔCt in the LNA-AS-qPCR was more than 15 cycles and that in the mismatched AS-qPCR was more than 9 cycles (Fig. 1). The LNA-AS-qPCR provided the highest selectivity for the 3 mutations. The LNA-AS-qPCR was used to monitor the *FLT3* 2503G > T and *KIT* 2446G > T

mutations, and the mismatched AS-qPCR was used to monitor the *KIT* 2447A > T mutation. Accordingly, we developed adequate AS-qPCRs with high specificity and sensitivity for each mutation.

Throughout the clinical course, monitoring of MRD in patients with hematological malignancies is important for the early detection of hematological and/or extramedullary relapse. Quantitative assessments of MRD can be achieved by qPCR techniques which target immunoglobulin/T-cell receptor gene rearrangements and fusion gene transcripts and by fluorescence *in situ* hybridization techniques which analyze for numerical and constructional chromosome abnormalities. For the purpose of clinical validation, the present AS-qPCR assay was used for monitoring of MRD in 4 patients of whom sequential samples were available.

The qPCR analyses for fusion genes have been widely used and are regarded as reliable methods to evaluate MRD. In comparison between the present AS-qPCR and qPCR for fusion genes, we used *RUNX1-RUNX1T1* as an adequate MRD marker because the *KIT* or *FLT3* mutations have been detected frequently in patients with the *RUNX1-RUNX1T1* fusion gene. Out of the 4 patients with the *RUNX1-RUNX1T1* fusion gene available for screening of the gene mutations, 1 had the *FLT3* mutation and 3 patients had *KIT* mutations. The *RUNX1-RUNX1T1* fusion transcripts are detected persistently by non-quantitative PCR analysis, so that the quantitative analysis of fusion gene transcripts should be performed to evaluate MRD [28,29]. At relapse, the increases in the expression of the *FLT3* or *KIT* mutant gene by AS-qPCR were in accordance with that of the *RUNX1-RUNX1T1* fusion gene by qPCR (Fig. 2a and b). In some measurements during complete remission (CR) in patient 3, the expression of *KIT* 2447A > T was detected at quantifiable levels, while that of the *RUNX1-RUNX1T1*

fusion gene was below the detection limit (Fig. 2c). The recent studies have shown that, in patients with t(8;21)-positive leukemia, the expression of both wild-type *KIT* and mutated *KIT* was significantly higher than that in patients with t(8;21)-negative leukemia [30,31]. Therefore, during CR in patient 3, the over-expressed mutated *KIT* gene could be detected by the present AS-qPCR. The observation that patient 3 experienced a second relapse indicates that the AS-qPCR may be useful to monitor MRD for prediction of imminent relapse in patients with only a single nucleotide mutation as available MRD marker. In patient 4, although no clinical and hematological abnormalities have been observed after HSCT, both *KIT* 2447A>T and *RUNX1-RUVX1T1* have been detected at quantifiable levels persistently, and there is a possibility of relapse at high risk (Fig. 2d). Therefore, close follow up of patient 4 with AS-qPCR is requisite. To develop a single gene mutation as a MRD marker, the stability of the gene mutation itself during follow-up is needed to be validated.

WT1 transcripts have been reported as MRD marker in patients with no recurrent chromosomal or genetic abnormalities. There have been controversies regarding the significance of *WT1* transcripts as MRD marker, because *WT1* transcripts were detected not only in tumor cells but also in normal cells [32,33]. *RUNX1-RUX1T1*-positive patients showed significantly lower *WT1* expression than patients with other types of AML and normal individuals [34,35]. In the present cases with the *RUNX1-RUX1T1* fusion gene, the *ABL*-normalized *WT1* expressions levels were in the range 10^2–10^4. Although no decreases in the *WT1* expression of more than 10^2-fold were observed during CR, increases in the *WT1* expression at relapse were paralleled to that of *KIT* 2446G > T or *KIT* 2447A > T and the *RUNX1-RUX1T1* fusion gene (Fig. 2b and c). A quality-controlled standardized approach has been developed for an accurate and

reproducible quantification of *WT1* expression [36]. For the wide application of the present AS-qPCR to monitor MRD in patients without recurrent chromosomal and genetic abnormalities, it should be required to compare the alteration in the quantity of the mutant gene to that of *WT1* in a large number of patients.

The percentage of autologous cells (chimerism) analyzed by short tandem repeat-PCR (STR-PCR), which characterizes the origin of post-transplant hematopoiesis, has been used as a surrogate marker for MRD [37]. The change in the quantity of the mutation detected by AS-qPCR using genomic DNA corresponded with the change of chimerism by STR-PCR [21]. The AS-qPCR was more sensitive than the STR-PCR. In our patients, the mutated gene could be quantified significantly by AS-qPCR even when the chimerism by STR-PCR was less than 5% or negative (data not shown).

In conclusion, the AS-qPCRs for single nucleotide mutations had comparable accuracy to qPCR for the *RUNX1-RUNX1T1* fusion gene and *WT1* and were applicable to monitor MRD throughout the clinical course including prior to transplantation and post-transplantation. The AS-qPCR for single nucleotide mutations may permit us to monitor MRD in patients that lack recurrent chromosomal abnormalities and the specific fusion gene, which broadens the spectrum of patients in whom MRD can be monitored.

Abbreviations: MRD, minimal residual disease; AS-qPCR, allele-specific quantitative polymerase chain reaction; HSCT, hematopoietic stem cell transplantation; ARMS, amplification refractory mutation system; MAMA, mismatch amplification mutation assay; AML, acute myeloid leukemia; LNA, locked nucleic acid; FAM, fluorescein amidite; TAMRA, tetramethylrhodamine; *ABL*, *abelson*; CR, complete remission.

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Nonstandard abbreviations: AS-qPCR, allele specific-quantitative PCR; q-PCR, quantitative PCR; LNA, locked nucleic acid; ARMS, amplification refractory mutation system; MAMA, mismatch amplification mutation assay; STR-PCR, short-tandem repeat PCR; BM, bone marrow; .

Figure Legends

Figure 1. Comparison of 3 types of AS-qPCRs

Three types of AS-qPCRs (1, mutant AS-qPCR; 2, mismatched AS-qPCR; 3, LNA-AS-qPCR) were compared in terms of difference of threshold cycles (ΔCt) and delay in Ct (delay Ct). The ΔCt was calculated as follows: (Ct for mutant plasmid) - (Ct for wild-type plasmid) in each AS-qPCR assay. The delay Ct was calculated as follows: (Ct for mutant plasmid in mismatched AS-qPCR or LNA-AS-qPCR) - (Ct for mutant plasmid in mutant AS-qPCR). Data are expressed as means ± S.D. *, $p < 0.05$; **, $p < 0.01$ in comparisons of ΔCt among the 3 types of AS-qPCRs. $^{\#\#}$, $p < 0.01$ in comparisons of delay Ct between mismatched AS-qPCR and LNA-AS-qPCR. N.S., not significant.

Figure 2. Comparison of the quantity of *FLT3* 2503G > T, *KIT* 2446G > T, and *KIT* 2447A > T transcripts by AS-qPCR with that of *RUNX1-RUNX1T1* fusion and *WT1* transcripts by qPCR throughout the clinical course

The expression of the mutated genes, the *RUNX1-RUNX1T1* fusion gene, and the *WT1* gene was evaluated in 4 patients. The expression of the target genes using cDNA was normalized with respect to the expression of the *abelson* (*ABL*) gene, and expressed as copy numbers every 10^4 copies of *ABL*. To determine the detection limit of the AS-qPCRs for *FLT3* 2503G > T, *KIT* 2446G > T, and *KIT* 2447A > T, and that of the qPCR for the *RUNX1-RUNX1T1* fusion gene, cDNA obtained from patients at diagnosis were serially 10-fold diluted in pooled cDNA from healthy controls and the AS-qPCR and qPCR were performed using the diluted samples. Dotted lines in *FLT3* 2503G > T,

KIT 2446G > T, *KIT* 2447A > T, and *RUNX1-RUNX1T1* indicate the detection limit of the AS-qPCRs. The normal expression of the *WT1* gene was determined using 10 BM samples from persons without hematological malignancies. Dotted line in *WT1* indicates the mean expression level of normal controls. CR, complete remission; R, relapse; HSCT, hematopoietic stem cell transplantation

Table 1

Table 1. Summary of the 4 patients

Patient	Age	Diagnosis	Type of gene mutation	Karyotype
1	5	AML (M2)	*FLT3* 2503G > T	46,XX,t(8;21)(q22;q22)[6]/46,XX[5]
2	8	AML (M2)	*KIT* 2446G > T	45,X,-Y,t(8;21)(q22;q22)[20]
3	2	AML (M2)	*KIT* 2447A > T	46,XY,der(4)t(4;8)(p16;q21),der(8)t(8;21)(q22;q22)[12]/46,XY[7]
4	27	AML (M2)	*KIT* 2447A > T	46,XX,t(8;21)(q22;q22)[20]

Table 2

Table 2. Sequences of primers and probes for AS-qPCR

Target genes	Primers (5′ - 3′)	Probes (5′ - 3′)
FLT3 2503G > T	F: gatatgtgactttggattggctcG*c*t*	FAM-gcaatgcccgtctgcctgtaaaatggatgg-TAMRA
	R: ggtgtagatgccttcaaacaggc	
KIT 2446G > T	F: gatttgtgattttggtctagccaG*c*t	FAM-ccatccacttcacaggtagtcgagcgtt-TAMRA
	R: ccataggaccagacgtcactttc	
KIT 2447A > T	F: gatttgtgattttggtctagccagac*t*	FAM-ccatccacttcacaggtagtcgagcgtt-TAMRA
	R: ccataggaccagacgtcactttc	
RUNX1-RUNX1T1	F: cacctaccacagagccatcaaa	FAM- aacctcgaaatcgtactgagaagcactcca-TAMRA
	R: atccacaggtgagtctggcatt	
WT1	F: caggctgcaataagagatattttaagct	FAM-cttacagatgcacagcaggaagcacactg-TAMRA
	R: gaagtcacactggtatggtttctca	
ABL (abelson)	F: tggagataacactctaagcataactaaaggt	FAM-ccatttttggtttgggcttcacaccatt-TAMRA
	R: gatgtagttgcttgggaccca	

* The LNA base is depicted in uppercase; template-mismatched base in lowercase in italics; mutant-matched base specific base in lowercase underlined. LNA, locked nucleic acid; FAM, fluorescein amidite; TAMRA, tetramethylrhodamine. The primers and probes for *RUNX1-RUNX1T1*, *WT1*, and *ABL* were designed according to the previous reports [1,22,24].

Figure 1

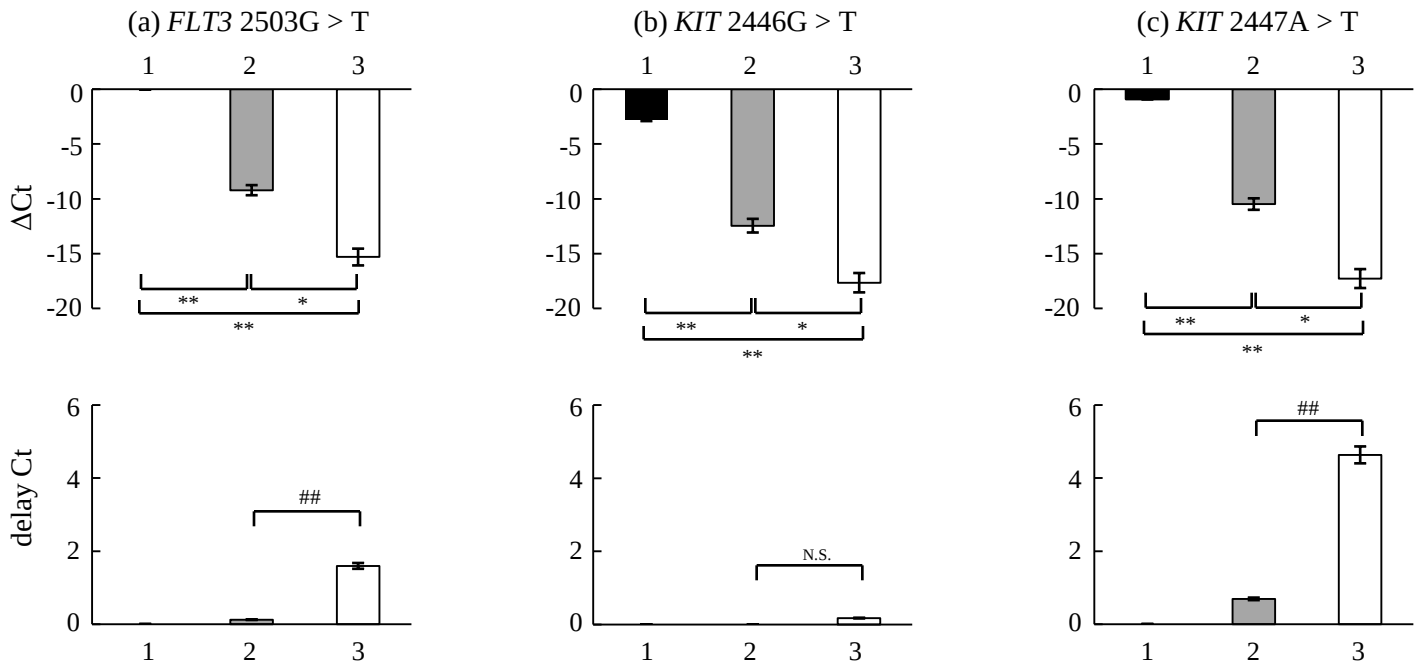

Fig 1.

Figure 2

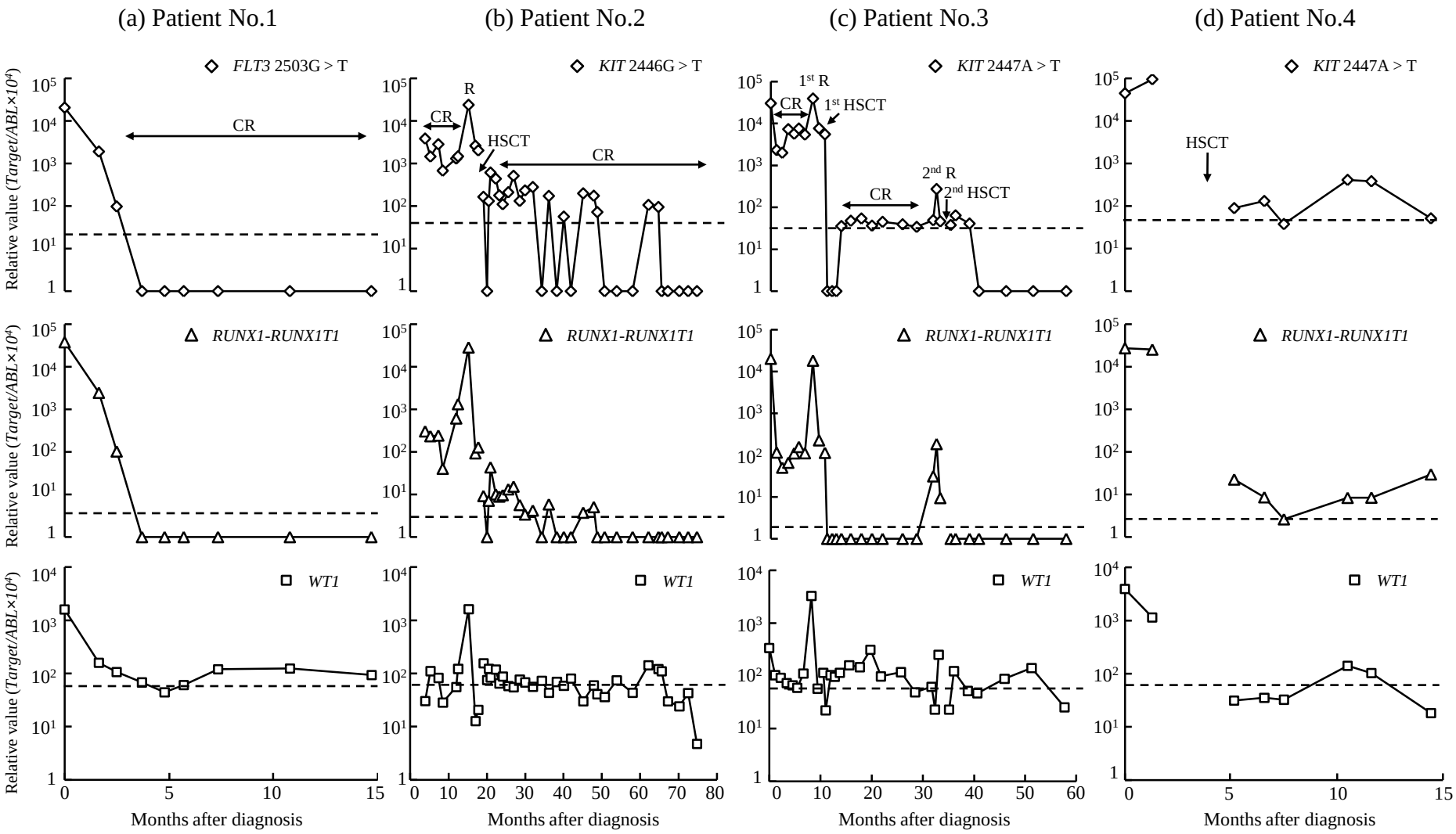

Fig2.