RAPID COMMUNICATION

Monitoring of Minimal Residual Disease by Quantitative Reverse Transcriptase-Polymerase Chain Reaction for AML1-MTG8 Transcripts in AML-M2 With t(8;21)

By Khalid Tobal and John A. Liu Yin

We have developed a quantitative reverse transcriptase-polymerase chain reaction method for the quantification of AML1-MTG8 transcripts in patients with AML-M2 and t(8;21) in different phases of the disease. Using this method, we have tested sequential samples from 13 patients to monitor minimal residual disease and were able to show a significant increase in AML1-MTG8 transcripts level in two patients 2 and 4 months before clinical relapse. In five patients tested at presentation and then sequentially at remission, we detected a marked decrease in the level of AML1-MTG8 transcripts as the treatment progressed. Patients in long-term remission of their disease had a level of up to 1 x 10^6 AML1-MTG8 molecules/μg RNA. Two patients tested 2 and 4 months before hematologic relapse showed a level of 0.71 x 10^6 molecules/μg RNA and this level increased further during relapse to 0.71 x 10^6 and 2.27 x 10^6 molecules/μg RNA, respectively. Our results show that quantification of AML1-MTG8 transcripts by competitive polymerase chain reaction is valuable in predicting early relapse in AML with t(8;21). Identification of at-risk patients may allow treatment to be modified to include additional or alternative therapy such as bone marrow transplantation.

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MATERIALS AND METHODS

Patients and samples. Initial diagnosis of AML-M2 was made morphologically and cytochemically. The presence of t(8;21)(q22;q22) was confirmed by karyotypic analysis. All 13 patients underwent intensive induction chemotherapy with daunorubicin, cytosine arabinoside (Ara-C), thioguanine, or etoposide. Postremission therapy consisted of two further courses of intensive chemotherapy (daunorubicin, Ara-C, and Thioguanin/etoposide and M-Amsa, Ara-C, and etoposide) followed by allogeneic or autologous bone marrow transplantation (BMT) in two patients (Table I). Four patients received a fourth course of chemotherapy with mitoxantrone and intermediate-dose Ara-C (MIDAC). All patients went into complete remission after one course of induction therapy.

BM samples were collected from patients at presentation and at different intervals during remission and after BMT. The patients studied had been in remission from 1 to 103 months. Approval for these studies was obtained from the Hospital Ethical Committee. Informed consent was provided according to the declaration of Helsinki. Mononuclear cells (MNCs) from BM samples were obtained by Ficoll-Hypaque density gradient centrifugation and either used immediately for RNA isolation or stored at -80°C. RNA was isolated from MNC samples by the guanidium-phenol-chloroform method of Chomczynski and Sacchi with minor modifications. RNA was quantified by spectrophotometry at 260 and 280 nm and used immediately for cDNA preparation.

RT. Ten micrograms of total RNA was used for the synthesis of cDNA in a 20 μL reaction containing 0.25 μg random hexamers, 200 μ mol/L Moloney's murine leukemia virus (MMLV) RT, and 40 μg RNAsin. RT reaction was performed at 37°C for 1 hour followed by 45°C for 30 minutes and 75°C for 5 minutes.

RNA quality assessment. Two microliters of cDNA was subjected to a PCR amplification of ABL in a 25 μL reaction containing primers CA3 and A2. Primer sequences and PCR parameters were as described previously. AML1-MTG8 RT-PCR. Two microliters of cDNA was subjected to two rounds of PCR amplification for the AML1-MTG8 transcripts. The first-round PCR was performed in a 50 μL reaction containing primers 11 and 12 at 95°C for 2 minutes (1 cycle); 93°C for 50 seconds, 56°C for 50 seconds, and 72°C for 1 minute (40 cycles); and 72°C for 5 minutes (1 cycle). First round product (2.5 μL) was used in a second-round PCR in a 50 μL reaction containing primers TS and 24 at 95°C for 2 minutes (1 cycle); 93°C for 50 seconds, 56°C for 50 seconds, and 72°C for 1 minute (30 cycles); and 72°C for 5 minutes (1 cycle). Second-round products were electrophoresed on a 2% agarose gel. This PCR protocol is a transcript-specific.

THE (8;21) IS ONE OF THE most common chromosomal translocations in acute myeloid leukemia (AML), especially in French-American-British (FAB) subgroup M2, occurring in about 20% of adult and 40% of pediatric AML-M2. The t(8;21)(q22;q22) fuses two genes, AML1 on chromosome 21 and MTG8 (ETO) on chromosome 8, to produce the AML1-MTG8 (AML1-ETO) fusion gene on the derivative chromosome 8. Despite the relatively good prognosis of AML with t(8;21), relapse of leukemia remains the commonest cause of treatment failure. Monitoring minimal residual disease (MRD) in leukemia has two main aims: to assess the effectiveness of treatment and to detect early signs of relapse. Using qualitative reverse transcriptase-polymerase chain reaction (RT-PCR) methods, we and other groups have shown the persistence of detectable levels of AML1-MTG8 transcripts in patients in long-term remission of AML with t(8;21). These methods are therefore not suitable for monitoring MRD in those patients. For corresponding situations in other leukemias such as chronic myeloid leukemia (CML), in which qualitative RT-PCR methods have limited value in monitoring MRD, quantitative RT-PCR methods were found to be valuable in predicting relapse. We have developed a sensitive quantitative RT-PCR method for the quantitation of AML1-MTG8 fusion transcripts and assessed the level of MRD in sequential bone marrow samples from patients in remission of AML with t(8;21).

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amplification (TSA) designed to amplify the main (in-frame) AML1-MTG8 transcript that is detected in all patients with t(8;21).16-12

Negative and positive controls were used in all tests. All necessary precautions were taken to eliminate contamination.

RT-PCR sensitivity analysis. The sensitivity of the transcript-specific (TSA) RT-PCR was assessed on a serial dilutions of Kasumi-1 cell line.13

Competitor construction. We have developed a method for the preparation of a competitor for the AML1-MTG8 transcript based on the principle of splicing by overlap extension (SOE)14 (Fig 1). The competitor produced was 44 bp smaller than the main (in-frame) AML1-MTG8 transcript. Dilutions of this competitor were made in a range of 1 to 10^9 molecules/2 μL, with a dilution at every half an order of magnitude on a logarithmic scale.

Primers used. The primers used were as follows: 11, 5' AGC-CATGAAAGACCGGAG3'; 12, 5' AGGCTGTAGGAGAATGG3'; TS, 5' CCCCGAGAACCCCTGAAATCGT3'; 24, 5' GTTGTCCGG-1

GTAAATGAA3'; Runt15, 5' GCTGAGCTGAGAAATGCTAC3'; Runt16, 5' TGAAGCCATTGGGTTGAG3'; RTS, 5' TACGAT-TTCAGAGGTTGTCCGGAGCC3'; CTS, 5' CGAGAACCTCGGA-1

AATGCAACCCAGAATCTAGGCTGAGT3'.

Competitive RT-PCR. Two microliters of samples' cDNA was mixed with 2 μL of competitor and subjected to two rounds of PCR amplification of the AML1-MTG8 as described above. Samples were quantified at every order of magnitude and then at every half order. The point of equivalence was assessed by gel densitometry. Because of the size difference between the AML1-MTG8 transcript band (152 bp) and the competitor band (108 bp), the number of competitor molecules at the point of equivalence was multiplied by 0.71 (the ratio of the size of competitor and transcript).

Serial dilutions of Kasumi-1 cells were made into normal cells, and competitive RT-PCR amplifications were performed.

RESULTS

RT-PCR. Sensitivity analysis of the RT-PCR method performed with serial dilutions of Kasumi-1 cell line found

Table 1. Cytogenetics, Treatment, and RT-PCR Results of Patients Included in This Study

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<th>Karyotype</th>
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the sensitivity to be $10^{-7}$. Sequential samples from 13 patients with AML and t(8;21) were analyzed first by qualitative RT-PCR. Six presentation samples were available and all were found positive. All remission samples except two were positive (Table 1 and Fig 2).

**Linearity and reproducibility of results.** Serial dilutions of Kasumi-1 cell line were made with normal MNCs and were subjected to competitive RT-PCR analysis to assess the accuracy and reproducibility of results. Results of this analysis show this method to be accurate, with the point of equivalence corresponding well with the cell line dilution. The points of equivalence for $10^{-3}, 10^{-4}, 10^{-5}$ and $10^{-6}$ cells, were $2.27 \times 10^2, 2.27 \times 10^3, 2.27 \times 10^4$, and $2.27 \times 10^5$ AML1-MTG8 molecules/\mu g of total RNA, respectively (Fig 3). This test was repeated twice and similar results were obtained. These results show the linearity of the method and equal efficiency of amplification for both the AML1-MTG8 transcript and the competitor. It also established the reproducibility of the results achieved.

**Competitive RT-PCR.** Quantitative RT-PCR analysis on six presentation samples found the level of AML1-MTG8 transcript to be in a range of $0.71 \times 10^7$ to $2.27 \times 10^7$ molecules/\mu g RNA. Remission samples, including those in long-term remission, showed a level of AML1-MTG8 transcripts of up to $1.5 \times 10^7$ molecules/\mu g RNA (Fig 4). BM samples from two patients 2 and 4 months before relapse showed an increase in the level of AML1-MTG8 transcript to $0.71 \times 10^5$ molecules/\mu g of RNA (Figs 4 and 5) while remaining morphologically and cytogenetically normal. This level increased further to $0.71 \times 10^4$ and $2.27 \times 10^5$ molecules/\mu g RNA at relapse, respectively.

**DISCUSSION**

We have developed a sensitive competitive RT-PCR method that allows the quantitation of the number of AML1-MTG8 transcripts and, by inference, the number of leukemic cells present in samples from AML patients with t(8;21) at different phases of their disease.

The t(8;21) translocation is closely associated with AML-FAB M2 and is generally accepted as a marker with a favorable prognosis. Most patients will achieve complete hematologic remission; the t(8;21) is then no longer detectable in the BM using cytogenetic techniques. Indeed, a high cure rate (~60%) can be achieved with chemotherapy alone, and this modality is now being offered as first-line treatment for AML with t(8;21).

However, leukemic relapse remains

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**Fig 1. Diagrammatic description of the preparation of AML1-MTG8 competitor construct using the SOE methodology.**

**First round PCR: primers 11+12 : 176 bp**
**Second round PCR: primers TS+24 : 108 bp**

**First round PCR: primers 11+12 : 220 bp**
**Second round PCR: primers TS+24: 152 bp**
the major cause of treatment failure, although relapses after more than 2 years in remission are uncommon. Thus, a method that predicts relapse at an early stage is potentially very useful, because these patients could be offered additional or alternative treatment such as BMT.

Although AML1-MTG8 transcripts could be detected by qualitative RT-PCR, we and others have shown6-5 that this method has little value in monitoring MRD, because most patients continue to express detectable levels of fusion transcripts even during long-term remission. The quantitative RT-PCR method described here, on the other hand, was able to distinguish between samples from patients in remission with a level of up to $1.5 \times 10^5$ molecules/µg of RNA and samples from patients before relapse with a level of $0.71 \times 10^5$ molecules/µg of RNA. It is worth stressing that, in the two patients who relapsed, BM examination was both morphologically and karyotypically normal at the time of detecting the marked increase in AML1-MTG8 transcripts level. Recently, we and others have described the presence of diverse forms of transcripts for the AML1-MTG8 fusion gene in patients with AML M2 and t(8;21) in different phases of the disease.6-12 The use of TSA directed toward the sole amplification of the main in-frame transcript of AML1-MTG8 eliminates the possible interference of the amplification of the other AML1-MTG8 transcripts with the accurate quantitation of this fusion gene mRNA. Tests performed on serial dilutions of the Kasumi-1 cell line showed equal efficiency of the amplification of AML1-MTG8 and the competitor and showed the method to be linear; these results were reproducible.

The persistence of leukemic cells expressing the AML1-MTG8 in patients in long-term remission indicates that long-term disease-free remission is not inconsistent with the presence of small number of leukemic cells. However, this persistence of leukemic cells makes a strong case for developing a quantitative RT-PCR method for monitoring MRD in those patients. Such an approach (quantitation of BCR-ABL transcripts) has been particularly useful in predicting relapse in CML patients after BMT.6-7

Our results show a marked decrease in the number of AML1-MTG8 transcripts, implying a reduction in leukemic cells as the course of treatment progresses. These results show induction chemotherapy to produce approximately a 2 to 3-log reduction in leukemic cells from presentation and
Fig 4. (A) Serial quantitation of AML1-MTG8 transcripts in (A) patients no. 1 through 7 and (B) patients no. 8 through 13 during presentation and/or remission and relapse.
a further reduction of approximately 1 log reduction after consolidation treatment. A high level (eg. 2.27 × 10^3 molecules/μg of RNA) during early stages of treatment is not inconsistent with response to chemotherapy as long as there is a gradual reduction in the level of AML1-MTG8 transcript as the treatment progresses. This must be distinguished from patients who show an increasing level of fusion transcripts after completion of chemotherapy, because this may represent an early relapse.

Our results also show that a level of up to 1.5 × 10^3 AML1-MTG8 molecules/μg of RNA is consistent with a durable remission, with two patients in long-term remission having a level of up to 1 × 10^3. However, in the first few years, BM sampling at 3-month intervals from patients in remission may be necessary to allow detection of an increased level of transcripts before relapse, because it is clear from our preliminary results in the two relapsed patients that there is a window of approximately 6 months between the last remission samples with a level less than 1.5 × 10^3 molecules/μg of RNA and clinical relapse.

In summary, our results in this small series of patients clearly show the value of quantitative RT-PCR for AML1-MTG8 transcripts in predicting early leukemic relapse. However, more data are required in prospective studies involving larger numbers of AML patients with t(8;21) not only to validate the usefulness of quantitative RT-PCR in predicting early relapse, but also to establish the optimal frequency required for BM sampling during remission.

ACKNOWLEDGMENT

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Monitoring of minimal residual disease by quantitative reverse transcriptase-polymerase chain reaction for AML1-MTG8 transcripts in AML-M2 with t(8; 21)

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