A Novel Method for the Detection, Quantitation, and Breakpoint Cluster Region Determination of t(15;17) Fusion Transcripts Using a One-Step Real-Time Multiplex RT-PCR

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Key Words: Acute promyelocytic leukemia; APL; Real-time quantitative RT-PCR; Reverse transcriptase–polymerase chain reaction; all-trans retinoic acid

DOI: 10.1092/KBLQ883YXQMAFCAH

Abstract

Individuals with acute promyelocytic leukemia (APL) usually express 1 of 3 primary hybrid transcripts associated with a t(15;17). The 3 fusion transcripts are the result of heterogeneous breakpoint cluster regions (bcr) within the promyelocytic leukemia (PML) gene and are denoted bcr1 (long), bcr2 (variant), and bcr3 (short) forms. Many researchers have shown that real-time quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) of the involved transcript is a valuable tool for monitoring APL and its treatment. In addition, some research suggests that identification of a specific breakpoint region may be used to predict an individual’s likelihood of relapse and possibly their response to all-trans retinoic acid treatment. We describe the first reported 1-step multiplex RT-PCR assay capable of t(15;17) fusion transcript real-time relative quantitation and simultaneous transcript form identification in 2 reactions. This assay uses a novel dual-probe technique to achieve what has required a laborious procedure of 2 or more reactions followed by postamplification analysis. We found a correlation of 100% in detection and breakpoint determination of the long, short, and variant forms with a breakpoint 5' to nucleotide 1709 compared with results from traditional methods.

Acute promyelocytic leukemia (APL) is a distinctive subtype of acute myelogenous leukemia characterized by a translocation involving the promyelocytic leukemia (PML) gene from chromosome 15 and the retinoic acid receptor (RAR) alpha gene from chromosome 17.1-3 The breakpoint on chromosome 17 invariably is located within the second intron of the RAR alpha gene.3,4 Fusion transcript variation exists as a result of heterogeneous breakpoint cluster regions (bcr) within the PML gene and alternative splicing of the PML sequence following transcription.5-7 Approximately 37% to 45% of t(15;17)-positive cases demonstrate a breakpoint within intron 3 of the PML gene. This breakpoint region is referred to as bcr3 and results in the fusion of PML exon 3 with RAR alpha exon 3. The bcr1 is detected in approximately 45% to 55% of positive cases. It occurs within intron 6 of the PML gene and results in the fusion of PML exon 6 and RAR alpha exon 3. The most uncommon breakpoint region, bcr2, is involved in roughly 8% to 10% of t(15;17)-positive cases. Unlike bcr1 and bcr3, the breakpoint of bcr2 occurs within an exon. The bcr2 breakpoint occurs at inconsistent sites within exon 6 of the PML gene resulting in the fusion of a variable portion of PML exon 6 with exon 3 of the RAR alpha gene.8-13 The location of bcr1, bcr2, and bcr3 produces fusion transcripts of varying lengths that, as a result, also are referred to as the long, variant, and short forms, respectively. A number of researchers have reported on the clinical relevance associated with the specific type of PML-RAR alpha fusion transcript expressed in an individual. Although a subject of some debate, it has been reported that the location of the breakpoint within the PML gene may influence prognosis. Some reports suggest that APL-positive individuals
who express the short-form fusion transcript tend to have decreased periods of clinical remission compared with patients with the long form. In addition, individuals who express the variant form may have a decreased response to treatment with all-trans retinoic acid (ATRA), depending on where the break occurs within PML exon 6. Fusion transcript sequence analysis has suggested that individuals expressing the bcr2 transcript with a breakpoint 5' to nucleotide 1709 of the PML gene have reduced sensitivity to ATRA. Furthermore, individuals expressing the variant transcript with a breakpoint 3' to nucleotide 1709 may demonstrate high sensitivity to ATRA indistinguishable from individuals expressing the short or long forms.

Control Specimens

The NB4 cell line was used as a positive control for the bcr1 long form. The positive control RNA for the bcr3 form was derived from a positive patient sample that expressed the t(15;17) short form transcript. All control specimens were diluted over a range of 100 ng to 10 pg of t(15;17)-positive total RNA. The positive control specimens were brought to a final RNA concentration of 10 ng/µL using RNA extracted from the negative control cell line (HL-60).

Materials and Methods

Specimens

Peripheral blood and bone marrow samples were obtained from patients referred to IMPATH, Los Angeles, CA, for clinical diagnostic purposes. Each sample was sent to an independent laboratory for RT-PCR analysis of the t(15;17) as part of the validation process. The samples used to validate this assay were all tested by a traditional RT-PCR. Each RT-PCR–positive sample underwent additional testing, fluorescence in situ hybridization or cytogenetic analysis for confirmation of a t(15;17). Sequencing was performed only on the samples positive for the variant transcript to determine the exact location of the breakpoint involved.

Primers and Probes

All primers and probes were designed using Primer Express 1.0 (Applied Biosystems, Foster City, CA). Primers and probes are listed in Table I.

Primer and Probe Placement

One of the major obstacles in developing a real-time assay for the variant form of the t(15;17) is primer placement. False-negative results may result if a forward primer or a single probe were to be placed in the variable region of PML exon 6. The fact that the long form expresses all of exon 6 and the vast majority of variant forms contain at least an upstream portion of exon 6 permits the simultaneous detection of both forms with the same primer and probe set. The only problem with using a single probe targeted to the upstream region of exon 6 is that it would be impossible to differentiate between any variant form and a long form. To circumvent this problem, the assay was designed to incorporate 2 fluorogenic probes that target different regions of PML exon 6. The first probe is labeled with 6-carboxy-fluorescein (FAM) and hybridizes to information on the specific transcript expressed. This assay therefore may be used to quantify changes in PML-RAR alpha expression during treatment and assist in predicting the possibility of relapse.
an upstream portion of exon 6; the second probe is labeled with VIC and hybridizes to the critical downstream region of exon 6 that is deleted in the majority (approximately 60%) of variant transcripts. A probe, rather than the forward primer, was placed in the downstream region of exon 6 to assist in reducing the possibilities of false-negative results. The placement of the VIC probe essentially eliminates false-negative results because if the downstream region is deleted in a variant case, the reaction still will occur from the upstream placement of the forward primer and be detectable by the signal from the upstream FAM probe. If a long form or a variant with a break 3' to nucleotide 1709 is present, 2 signals are generated, indicating the presence of their intended target. A variant form with a break 5' to nucleotide 1700 would be identified by the presence of a signal in the FAM dye layer and no signal in the VIC layer.

The short-form transcript is identified in a second reaction. The forward primer and a FAM-labeled probe target exon 3 of the \( PML \) gene and were designed to use the same reverse primer as the long or variant reaction.

### RNA Extraction

Total RNA was isolated from 300 µL of whole blood or bone marrow using the RNA Mini Blood Kit (Qiagen, Chatsworth, CA) followed by DNase treatment directly on the RNA binding column using the RNase-free DNase kit (Qiagen). The RNA was quantified using a SpectraMax Plus spectrophotometer (Molecular Devices, Sunnyvale, CA). All RNA samples were brought to a final concentration of 10 ng/µL. Each sample was determined to be free of contaminating genomic DNA by using a standard beta-actin (-RT) PCR before RT-PCR analysis (data not shown). Ten microliters (100 ng) of each sample were used in a 1-step RT-PCR reaction using the EZ TaqMan RT-PCR kit (Applied Biosystems).

### One-Step Multiplex Real-Time RT-PCR

The procedure is performed in 2 separate reactions that are set up and performed simultaneously. The first reaction multiplexes the \( bcr3 \) primers and FAM-labeled probe with the beta-actin primers and a VIC-labeled probe. The second reaction detects the \( bcr1 \) and \( bcr2 \) transcripts using a single
pair of primers, a FAM-labeled fluorogenic probe and a VIC-labeled probe to distinguish between the long and variant forms with a break 5' to nucleotide 1709. The reaction conditions were the same for both the bcr3/beta-actin and the bcr1/bcr2 reactions. Each reaction was performed in a 25-µL reaction volume consisting of a 3-mmol/L concentration of Mn(OAc)₂, 1x EZ RT-PCR buffer, a 0.2-µmol/L concentration of deoxynucleoside triphosphate, a 0.1-µmol/L concentration of all PML-RAR primers and probes, a 0.025-µmol/L concentration of beta-actin primers and probe, 0.25 U of uracil N-glycosylase, and 2.5 U of Tth polymerase. The reaction mixtures were placed in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) and subjected to the following conditions: hold 2 minutes at 50.0°C (contamination control), hold 30 minutes at 60.0°C (primer-specific reverse transcription), followed by 45 cycles of 15 seconds at 95.0°C (denaturation) and 1 minute at 60.0°C (anneal/elongation).

The ABI PRISM 7700 uses a charged coupled device camera to collect emission data generated from the cleavage of a sequence-specific fluorogenic probe labeled with a reporter and a quencher dye. The dual-labeled fluorogenic probe is hydrolyzed using the 5’ nuclease activity of Tth polymerase during the extension phase of PCR. The fluorescent emission data is then analyzed by the ABI PRISM 7700 software and displayed as an amplification plot of normalized reporter signal (Rₙ) vs cycle number. The software uses background data collected from cycles 3 to 15 to establish a baseline. A threshold then is set at 10 SD above the baseline. If a sample’s Rₙ exceeds the threshold, it is given a threshold cycle (Cₜ) value. The Cₜ of an unknown sample is used to determine the input amount of a specific target relative to a standard curve generated from known input amounts of the same target.

Results

This 1-step multiplex RT-PCR procedure was evaluated for sensitivity, specificity, reproducibility, and correlation with traditional t(15;17) detection methods. The assay then was used to characterize the translocation forms in 25 cases of APL. The sensitivity of this assay was determined by diluting positive control total RNA into negative control total RNA from the HL-60 cell line. Each positive control was brought to a final concentration of 10 ng/µL with negative control RNA to maintain consistent quantities of background RNA throughout the dilution series. The positive control specimens consisted of 100, 10, 1.0, 0.1, and 0.01 ng of t(15;17)-positive RNA per 10 µL. These dilutions were based on recommendations in a recent report that determined by real-time quantitative RT-PCR that there are approximately 10⁴ copies of the long-form fusion transcript per 1 µg of total RNA contained in the NB4 cell line. Therefore, the dilution series roughly represents a range of 1,000 copies to less than 1 copy of the t(15;17) long-form transcript per 10 µL of input RNA. The assay consistently detected the 100-pg control in both reactions. A slight degree of variation was observed between the Cₜ values generated from the upstream FAM probe and those from the downstream VIC probe in the NB4 dilution series, but overall, the amplification plots from both probes demonstrated a high degree of similarity. The addition of multiple fluorescent signals in the reaction tube seemed to have little or no effect on the curve, as no shift in the Cₜ values was observed without the addition of the multiplexed probes (data not shown). The bcr3/beta-actin and bcr1/bcr2 reactions were capable of maintaining reproducible linearity over the dilution series with slopes of approximately −3.3 and correlation coefficients of more than 0.99.

In addition, the interassay and intra-assay coefficient of variation (CV) was calculated for each reaction to evaluate the reproducibility of the assay. The interassay reproducibility, determined by comparing Cₜ values from 100- and 0.5-ng positive control specimens for 10 different runs, demonstrated CVs of 1.5% and 1.9%, respectively, for the long-form reaction. The interassay reproducibility of the short-form reaction had CVs of 1.6% and 1.8% for the 100- and 0.5-ng controls, respectively. The intra-assay CVs for the long and short reactions, calculated from the Cₜ values of 10 replicates of the 100- and 0.5-ng positive control specimens from a single run, were 0.6% and 1.1% and 0.7% and 0.9%, respectively.

Each sample was evaluated by validated methods to confirm a t(15;17). RT-PCR was performed on each sample, and the results were confirmed by a secondary method including fluorescence in situ hybridization, cytogenetic analysis, or sequencing, if required. This assay had a correlation of 100% compared with a traditional RT-PCR procedure used for detection of the 3 fusion transcripts associated with a t(15;17) and sequencing methods used to identify the location of bcr2 breakpoints 5’ to nucleotide 1709. Amplification plots generated from patient samples expressing the long-form fusion transcript demonstrated consistent Cₜ values between the upstream and downstream fluorogenic probes, indicating the presence of an intact PML exon 6. When this procedure was applied to variant samples that had a breakpoint 3’ to nucleotide 1709, the signal was indistinguishable from that of a signal generated from a long-form transcript. Variant samples with a break 5’ to nucleotide 1709 showed a complete loss of the VIC signal, which demonstrated the loss of a portion of PML exon 6, 5’ to nucleotide 1709.
Quantitation of the involved transcript was determined in relation to the positive control series in this assay and normalized to beta-actin expression. The assay uses the standard curve method for multiplexed samples in a single tube to determine relative quantitative values. For practical clinical laboratory purposes, beta-actin has proven thus far to be an acceptable endogenous control (data not shown).

During development and validation, 25 positive t(15;17)-positive samples were evaluated. The assay successfully identified the long, short, and variant forms with a breakpoint 5' to nucleotide 1709 in all cases. One case was determined to be a variant form with a breakpoint 3' to nucleotide 1709 by RT-PCR using the same method as described with the substitution of a probe that targets the PML exon 6 region 3' to nucleotide 1709 (data not shown).

Of the 25 t(15;17)-positive samples, 12 (48%) were identified as expressing the long-form transcript, 10 (40%) expressed the short-form transcript, and 3 (12%) expressed the variant transcript. The 3 variant samples included 2 cases with a breakpoint 5' to nucleotide 1709 and 1 with a break 3' to nucleotide 1709. Fusion transcript sequence analysis of the 3 variant samples was performed to determine the exact breakpoint involved. The 2 variant cases with a breakpoint 5' to nucleotide 1709 contained breakpoints at nucleotide 1685, whereas the variant case with a break 3' to nucleotide 1709 contained a breakpoint at nucleotide 1718. The variant breakpoints included in this report support the findings of previous research, which identifies nucleotide 1685 as the most common breakpoint found in variant samples.
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Discussion

APL is a life-threatening disease, and the diagnosis must be made as quickly and accurately as possible to facilitate prompt treatment. The standard diagnosis of APL traditionally has been morphologic findings confirmed by cytogenetic analysis. The microgranular variant form, in particular, usually requires cytogenetic analysis. While molecular techniques also are available, detection of the PML-RAR alpha translocation by RT-PCR traditionally has been a laborious and time-consuming process. Many procedures perform reverse transcription and PCR in 2 separate reactions, which may add unnecessary time and risk of contamination to the procedure. In addition, some procedures require secondary reactions to distinguish between bcr1 and bcr2 transcripts, while others use nested reactions to attain high sensitivity.

Real-time RT-PCR provides a platform by which many obstacles of the traditional methods may be eliminated. For example, by placing a probe instead of the forward primer in the critical breakpoint region of a variant, amplification still will occur, and the possibility of a false-negative result is eliminated. The inclusion of a FAM-labeled probe in the upstream region of PML exon 6 permits the determination of
a successful reaction even if the variant break falls within the sequence of the downstream VIC-labeled probe. The only limitation is that a long form and a variant with a breakpoint 3' to nucleotide 1709 are indistinguishable. As some research suggests, a 3' break to nucleotide 1709 may not affect ATRA sensitivity, and, therefore, it may be clinically insignificant to differentiate between the long form and a variant with a break 3' to nucleotide 1709. Additional assays were performed on all of the long-form positive cases with a probe targeting the 3' region of nucleotide 1709. Only 1 variant sample was detected with a break 3' to nucleotide 1709 using this method. It may be possible in the future to include 3 probes in a single reaction to determine whether the breakpoint of a variant form occurs 5' or 3' to nucleotide 1709, but the present limitations of dyes available to the ABI PRISM 7700 Sequence Detection System platform does not yet permit including 3 probes.

Although APL is highly curable, it also is fatal if not detected at a very early stage, regardless of the fusion transcript present. Microgranular variants are especially difficult to identify and may be misdiagnosed as acute myelomonocytic leukemia, resulting in inappropriate treatment. The major advantage of this system is that it is performed in a minimal amount of time (about 4 hours), which is critical to patient care. It minimizes turnaround time by using 1-step chemistry and a multiplexed endogenous control to normalize input RNA and serve as a determinant of the reaction success. It permits the simultaneous detection of the 3 t(15;17) fusion transcripts associated with APL and provides information about the critical breakpoints in the variant cases, eliminating the need to sequence inconclusive variant cases. It provides relative quantitative data, which may be used to monitor the course of the disease and evaluate treatment efficacy. It demonstrates a level of sensitivity comparable to that of methods that use a traditional 2-step RT-PCR and nested reactions.

Previous research suggests that, although the majority of people with PML respond well to treatment with ATRA, the response to treatment may depend on which form of fusion transcript is involved. People expressing either the long- or short-form transcript tend to have shorter periods of clinical remission than people with the long-form translocation. In addition, in some reports, the location of the breakpoint involved in variant cases has been shown to affect ATRA sensitivity, while in other reports, the breakpoint location seems to be less of a factor for ATRA sensitivity. We currently are collecting outcome data about the samples included in this report. The fact that variant cases represent only 12% of t(15;17) cases makes it difficult to gain a substantial number of samples for study. Verbal communications with the treating physicians of the 3 people with variant-form translocations included in this report support the findings of Gallagher et al. The 2 people with breaks 5' to nucleotide 1709 demonstrated minimal response to ATRA and died shortly after induction. The patient with a break 3' to nucleotide 1709 demonstrated high sensitivity to ATRA and was in clinical remission at last follow-up. Further study is required to gain sufficient data on the relationship between ATRA sensitivity and the breakpoint location of the involved fusion transcript.

This 1-step, multiplex, real-time RT-PCR is sensitive and specific and can identify the long, short, and variant transcripts with a break 5' to nucleotide 1709 associated with APL. It provides relevant quantitative information about the involved transcript. In addition, it provides more information about the t(15;17) translocation in substantially less time than traditional methods. We believe that it represents a critical frontline assay to assist in the urgent diagnosis of suspected APL.

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Acknowledgments: We thank Robert Gallagher, MD, for helpful discussions and variant form sequence information, and Long Luu for technical assistance. We also thank the IMPATH Molecular Pathology and Hematopathology staff and Adrian Choppa for advice and support.

References


