Multiplex RT-PCR for the Detection of Leukemia-Associated Translocations

Validation and Application to Routine Molecular Diagnostic Practice

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The aim of this study was to validate the application of a commercially available multiplex reverse transcription polymerase chain reaction (RT-PCR) assay [Hemavision-7 System] for the seven most common leukemia translocations for routine molecular diagnostic hematopathology practice. A total of 98 samples, comprising four groups, were evaluated: Group 1, 16 diagnostic samples molecularly positive by our existing laboratory-developed assays for *PML-RAR α /t (15;17)* or *BCR-ABL/t (9;22)*; Group 2, 51 diagnostic samples negative by our laboratory-developed assays for *PML-RAR α /t (15;17)* or *BCR-ABL/t (9;22)*; Group 3, 21 prospectively analyzed diagnostic cases, without prior molecular studies; and Group 4, 10 minimal residual disease (MRD) samples. Analysis of the two previously studied cohorts (Groups 1 and 2) confirmed the diagnostic sensitivity and specificity of the multiplex assay with regard to these two translocations. Additionally, however, in the "negative" Group (Group 2) the assay revealed three unanticipated translocations (*CBF β -MYH11*, *BCR-ABL*, and *MLL-AF4*), two of which were confirmed on cytogenetics. Analysis of the prospective cohort demonstrated that the assay was cost-effective and amenable to standard laboratory practice, with an identically sensitive MRD detection rate to that of our laboratory-developed tests. Virtually all of the results obtained were consistent with the phenotype and karyotype by conventional methods. This study illustrates the utility of a kit-based multiplex RT-PCR assay for the molecular diagnosis and monitoring of leukemias and reinforces the complementary roles of molecular testing

and cytogenetics in diagnostic hematopathology. (*J Mol Diagn* 2003, 5:231–236)

The current pathological approach to the diagnosis of acute leukemia is a multifaceted one involving morphology, cytochemistry, immunophenotyping, cytogenetic and molecular diagnostic studies. Each of these components is central to appropriate diagnostic evaluation; however, there is increasing evidence that major disease-defining, prognostically relevant, and therapy-determining data are provided by the latter two genetic analyses. Although conventional cytogenetic studies remain the cornerstone of genetic testing, molecular-based technologies have emerged as a most useful tool for the detection of disease-defining genetic lesions.¹ Most translocations, when evaluated at the molecular level, are detected by reverse transcription-polymerase chain reaction (RT-PCR) in the routine diagnostic setting. RT-PCR detection of the major leukemia translocations has numerous advantages over conventional cytogenetics, including shorter turn-around time, no requirement for dividing cells, detection of translocations that may be missed by conventional cytogenetics ("cryptic" translocations),^{2–7} and providing a sensitive marker for subsequent minimal residual disease testing.⁸ A number of studies, comparing tandem RT-PCR and conventional cytogenetic studies, have reported a quite variable frequency (ranging from <1% to >35%, even when evaluating the same genetic fusion) of false-negative cytogenetic analyses.^{2–7} This occurrence of false-negative conventional cytogenetics suggests that molecular-

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Table 1. Preanalytic Features of the 98 Cases Studied

Group	Analysis	N	Disease phase	Molecular status	Features
1	Retrospective	16	Diagnosis	Positive	PML-RAR α + (n = 9) BCR-ABL + (n = 7)
2	Retrospective	51	Diagnosis	Negative	PML-RAR α - (n = 24) BCR-ABL - (n = 27)
3	Prospective	21	Diagnosis	Unknown	Newly diagnosed acute leukemias
4	Retrospective	10	MRD	Variable	PML-RAR α (n = 5; 2+, 3-) BCR-ABL (n = 5; 2+, 3-)

based testing is likely to assume an increasing role in the initial evaluation of most, if not all, leukemia patients.

Although a plethora, and ever-expanding menu, of molecular diagnostic assays exists, RT-PCR of t(15;17) and t(9;22) account for the bulk of the leukemia molecular cytogenetic testing in our molecular pathology laboratory (and in many others), with other translocations being relatively low-volume tests. Nevertheless, the ability to screen for and detect some of the other common, and clinically/therapeutically relevant, leukemia translocations at the molecular level would expand the advantages stated above to a larger number of leukemia specimens. In some instances, a negative RT-PCR result for either the t(15;17) or t(9;22) translocations might inadvertently be clinically misinterpreted; however, exposure to a broader test menu may uncover an unanticipated translocation, thus placing such cases into another specific, molecularly defined category (as will be illustrated by some of our data). Additionally, the concomitant evaluation of multiple leukemia translocations in the same specimen, and correlation with standard cytogenetic analysis, serves as a powerful internal quality control for the assays' analytic specificities and sensitivities. As recognized by the recent World Health Organization classification,⁹ the genetic analysis of leukemias has prognostic and therapeutic implications. This, together with the advent of targeted therapy, underscores the need for an accurate genetic diagnosis that can be rapidly obtained by molecular approaches, and which is key to rational risk stratification and institution of appropriate therapy.

A major advantage of conventional cytogenetic analysis is its ability to globally determine the presence of abnormalities, both balanced and numeric. By contrast, RT-PCR assays are designed to detect specific fusions, and it would be extremely labor intensive to evaluate leukemias via a panel of individual monoplex assays. This can be circumvented by the use of multiplex RT-PCR assays. A number of multiplex RT-PCR assays have been described.^{2-3,10-16} However, their development is extremely labor-intensive, and the existence of a variety of different assays, independently developed in different laboratories, is potentially confounding, with the lack of interlaboratory standards. Accordingly, the aim of this study was to evaluate the use of a commercially available and standardized multiplex RT-PCR system for the simultaneous detection of the seven most common leukemia translocations, and address the issues of diagnostic accuracy and laboratory efficiency inherent to the establishment of any new test in diagnostic laboratory practice.

Materials and Methods

Diagnostic Samples

A total of 98 adult patient samples were analyzed, which are characterized in four distinctive patient groups, as detailed below and in Table 1. The material was obtained as dictated clinically. It was procured and analyzed in accordance with the guidelines of the Institutional Review Board of the University of Pennsylvania.

Group 1 represented the stored RNA from 16 diagnostic leukemia samples that were positive by our existing laboratory-developed assays for t(15;17) [n = 9] or t(9;22) [n = 7]. Our laboratory-developed assays are fully validated for clinical testing using published primers and PCR conditions^{2,17} and have a reproducible sensitivity of 10^{-3} to 10^{-4} . In our archive of clinical specimens, these samples were chosen based on a positive RT-PCR result for one of these two translocations, sufficient RNA to perform both the multiplex assay, and the ability to perform additional assays as warranted for the clinical follow-up of each individual patient.

Group 2 was composed of the stored RNA from 51 diagnostic samples that were negative for our laboratory-developed assays for t(15;17) [n = 24] or t(9;22) [n = 27]. Of these, 39 samples were from patients with a clinical diagnosis of acute leukemia, and the remaining 12, all submitted for t(9;22) RT-PCR, were from cases submitted as "rule out CML" (chronic myeloid leukemia). The specimens were selected from our archive of RT-PCR-negative clinical specimens using criteria similar to those used for Group 1.

Group 3 comprised 21 submitted samples, with no previous molecular studies, and a clinical diagnosis of acute leukemia. The fresh material from these "prospective cases" included blood and bone marrow samples directly collected and submitted at the time of diagnosis (n = 15), or viably frozen cells retrieved from the Leukemia Tumor Bank at the University of Pennsylvania (n = 6).

Group 4 was composed of 10 cases originally submitted to our laboratory for minimal residual disease (MRD) assessment, including two cases positive for t(15;17), two cases positive for t(9;22), and six negative cases, three for each of these two translocations.

For most of these samples, although there was prior knowledge of the differential diagnosis based on the submitted clinical information, they were analyzed and scored as positive or negative without the knowledge as to their prior (where appropriate) molecular results. Con-

Figure 1. Principle of the multiplex RT-PCR reaction system. Following the generation of cDNA, two separate master mix (MM) multiplex PCR reactions are run that have the ability to detect, but not distinguish, the five different fusions in each MM reaction. A positive band in step I will lead to the appropriate step II, in which the five fusions are evaluated in five separate simplex reactions.

ventional cytogenetics studies were performed in only 53% ($n = 47$) of the diagnostic cases ($n = 88$), and none of the 10 MRD cases.

RNA Isolation

RNA was extracted with a silica gel-based membrane system, the RNeasy Mini Kit (Qiagen Inc., Valencia, CA), according to the manufacturer's specifications, and following the recommended protocol adjustments for fresh samples or viably frozen cells, as appropriate.

RT-PCR Reaction

The Hemavision-7 System by DNA Technology A/S (Aarhus, Denmark) allows for the detection of the following seven translocations/inversions, including the most commonly occurring variants thereof: t(1;19), t(12;21), inv(16), t(8;21), t(4;11), t(15;17), and t(9;22). The system was used with the reagents, concentrations and volumes indicated in the manufacturer's specifications. cDNA was synthesized using 1.6 μ g (16 μ l) RNA, 7 μ l cDNA primer solution, 8 μ l 5X Moloney murine leukemia virus reverse transcriptase (MMLV-RT) buffer solution, 4 μ l 100 mmol/L dithiothreitol (DTT), 4 μ l deoxynucleotide triphosphates (dNTP) mix (10 mmol/L of each), and 1 μ l MMLV-RT (200U/ μ l), followed by two PCR steps [see Figure 1]. Step I is a screening step that will allow for the detection of a translocation, without specifically identifying the translocation, while step II distinguishes which specific translocation is present. Step II includes two parallel multiplex PCR amplifications, each containing six primer pairs (including one pair for a proprietary internal control product of 984-bp to assess the integrity of the RNA and the adequacy of the various reactions). Each amplification involves the use of a master mix that includes HotStart Taq polymerase and buffer (Qiagen, Valencia, CA), distilled water, dNTPs, and a master primer solution as

Figure 2. Illustration of two cases. Case 7 shows no translocation fusion band in either reaction, illustrating only the internal RNA control; thus, it has been screened for all of the translocations and is scored as negative. For case 8, the first PCR reaction yields a positive band with the first master mix (MM1); hence, the split-out reaction that is targeted in this step I is used in step II to further characterize the specific translocation. A *CBFβ-MYH11* fusion is present, and all other fusions in this analysis are negative. Note that while MM2 also contains primers for a *CBFβ-MYH11* fusion, these are distinct from those used in MM1, to allow for the detection of variant breakpoints; hence, no band is seen in MM2 for case 8.

specified in the manufacturer's instructions. The PCR conditions are also optimized by the manufacturer so that they are the same for the first and the second PCR steps: 15 minutes at 95°C; 15 cycles of 0.5 minutes at 95°C, 1 minute at 65°C $-0.2^{\circ}\text{C}/\text{cycle}$ and 1.5 minutes at 72°C; 22 cycles of 0.5 minutes at 95°C, 0.5 minutes at 62°C and 1.5 minutes at 72°C; hold at 4°C. The presence of a specific band on an ethidium bromide-stained 1.5% agarose gel in one of these two master mix reactions in step I determines which subsequent "split-out" analysis is to be performed in step II. In step II, each split-out analysis entails six individual monoplex reactions: five test reactions and one positive control, for a total of 12 (6×2) possible individual reactions (three of the seven translocations are analyzed with two alternative primer pairs, to enable the detection of variant breakpoints leading to a total of 10 possible monoplex reactions and two controls, including a known fusion gene-positive specimen and a cDNA-free PCR contamination control). Positive controls for the individual translocations are provided with the kit. The monoplex reactions are similarly performed according to the manufacturer's specifications. Two cases following this principle are illustrated in Figure 2.

Interpretation of the RT-PCR Results

The assay was considered satisfactory for interpretation when the internal control 984-bp PCR product was

present, the external fusion-gene positive control was positive, and the water control showed no amplified product. A band was considered positive when it was in the expected size range and its intensity was similar to that of the internal control band in that specific lane. Occasionally (~2% of reactions overall), much fainter bands were identified; however, these were well outside the expected size range, in addition to their faintness. Thus, they were easy to distinguish from bona fide bands and were rejected.

Cytogenetic Analysis

Cytogenetic analysis was performed on unstimulated 24-hour cultures of bone marrow or peripheral blood cells. Trypsin/Giemsa-banded metaphases were prepared and analyzed using standard techniques of colchicine arrest, hypotonic treatment, and 3:1 of methanol:acetic acid fixation.

Results

Concordance of Positive Results (Group 1)

All 16 of the *PML-RAR α* fusions/t(15;17) and *BCR-ABL* fusions/t(9;22) that were detected with our laboratory-developed assay were also discerned with the multiplex RT-PCR. Complete concordance was also achieved when discriminating among the different *PML-RAR α* (short and long; there were no variant breakpoints in this series) and *BCR-ABL* (M-bcr and m-bcr) breakpoints/fusion products. Furthermore, a total of 10 of these 16 cases had accompanying conventional cytogenetic studies, and each correlated with the RT-PCR results. The assay detected all of the translocations and, despite the use of multiple primers in a multiplexed PCR scenario, there were no bands produced in the split-out reactions, for the fusions other than *PML-RAR α* and *BCR-ABL* that could have been misinterpreted as positive. Accordingly, this facet of the study validated the diagnostic accuracy and diagnostic sensitivity of the multiplex assay.

Concordance of Negative Results (Group 2)

None of the 24 *PML-RAR α* fusion cases that were negative with our laboratory-developed assay were positive for this fusion in the multiplex PCR reaction system, validating the diagnostic specificity of the *PML-RAR α* assay. However, two of the 24 were positive for other fusions: one for *CBF β -MYH11*/inv(16) and one for m-*BCR-ABL*/t(9;22). Both of these molecular results were subsequently validated by conventional cytogenetics that had been performed at diagnosis and, on further review, the hematopathologic data were most consistent with these findings (with the diagnoses being acute non-lymphoblastic leukemia M4Eo and CD13+, CD33+ adult precursor B-cell acute lymphoblastic leukemia, respectively).

Of the 27 cases submitted for *BCR-ABL* testing, 12 were submitted as "rule out CML", and 15 were from patients with acute leukemia. All of the former cases, with

a submitted clinical diagnosis of "rule out CML" that were *BCR-ABL* negative with the laboratory-developed assay, were also negative (for all fusion transcripts) with the multiplex PCR system. Furthermore, none of the 15 acute leukemia cases that were *BCR-ABL* negative with the laboratory-developed assay were *BCR-ABL* positive in the multiplex assay. However, one of these 15 had an *MLL-AF4* fusion/t(4;11). The tandem conventional, but technically suboptimal, cytogenetic evaluation failed to show a t(4;11) translocation; importantly, however, this was evident cytogenetically when the patient subsequently relapsed.

The Use of a Multiplex RT-PCR Assay in Routine Diagnostic Practice (Group 3)

The 21 cases in Group 3 represented a prospective cohort, in that none had previous molecular analysis and no karyotypic information was available at the time of molecular evaluation. Of the 15 cases that were submitted to our laboratory, five had detectable fusions: two with *CBF β -MYH11*/inv(16), one with *CBF α -ETO*/t(8;21), and two with *BCR-ABL*/t(9;22). Both of the *BCR-ABL* fusion transcripts were also evident with the laboratory-developed test. All five of these results correlated well with the hematopathologic data, and perfectly with the karyotyping by conventional cytogenetics. Five out of the 10 remaining negative RT-PCR cases had conventional cytogenetic studies; these were either negative or showed a complex karyotype with none of the specific translocations included in the multiplex RT-PCR test. Of the six acute leukemia cases from the tumor bank (on which we had no prior cytogenetic or molecular genetic results), one had a *PML-RAR α* fusion/t(15;17) by the multiplex assay and which was also detected by our laboratory-developed test. Subsequent review of the tandem conventional cytogenetic results confirmed the presence of this translocation, and the hematopathological diagnosis was indeed acute promyelocytic leukemia. There were no cytogenetic results on the remaining five cases.

The Use of a Multiplex RT-PCR Assay for Detection of Minimal Residual Disease (Group 4)

The sensitivity of the Hemavision system ranges between 10^{-2} to 10^{-4} for each of the seven translocations, as reported by the manufacturer; however, we did not formally evaluate this. These sensitivities are similar to those of our laboratory-developed assays for t(9;22) and t(15;17). Indeed, all of the MRD cases that were positive with our laboratory-developed assays were similarly positive with the multiplex assay. There was also complete concordance for the negative MRD samples. No cytogenetic analysis was performed in any of the 10 MRD samples. The results of all four groups are summarized in Table 2.

Discussion

The data derived from the various arms of the study (laboratory-developed monoplex RT-PCR assay, kit-

Table 2. Summary of Results (See Text for Details)

In-house test result	N	Positive by multiplex	Negative by multiplex	Cytogenetics concordance
<i>Group 1</i>				
t(15;17) pos	9	9/9	0/9	5/5
t(9;22) pos	7	7/7	0/7	5/5
<i>Group 2</i>				
t(15;17) neg	24	2/24	22/24	15/15*
t(9;22) neg, AL	15	1/15	14/15	9/10†
t(9;22) neg, rule out CML	12	0/12	12/12	1/1
<i>Group 3</i>				
No previous diagnosis	21	6/21	15/21	11/11‡
<i>Group 4</i>				
MRD pos	4	4/4	0/4	Not done
MRD neg	6	0/6	6/6	Not done

Abbreviations: AL, acute leukemia; CML, chronic myeloid leukemia; MRD, minimal residual disease.

*, including the two positive cases with the multiplex assay.

†, the one "discordant" case was positive with the multiplex assay, and was cytogenetically validated at relapse.

‡, including five out of the six cases positive for the multiplex assay (conventional cytogenetics not performed in one of these six cases).

based multiplex RT-PCR assay and cytogenetics, when available) indicate that these approaches are of similar diagnostic sensitivity and specificity, with one single possible exception, as detailed below. Importantly, however, this study illustrates how the availability of a broad spectrum RT-PCR assay may improve clinical diagnostic accuracy. This is clearly evident by evaluating the results from Group 2, in which 3 of the 39 cases (8%) of the acute leukemias submitted to evaluate for the presence of a t(15;17) or t(9;22) had different, and initially unanticipated, fusions/translocations (*CBFβ-MYH11*/inv(16), *BCR-ABL*/t(9;22), and *MLL-AF4*/t(4;11)). This is also illustrated in the 15 prospectively submitted cases (Group 3), in which one-third (5 of 15) had a positive translocation, with the result expected from and/or corroborating the hematopathological impression. Although not specifically addressed in this study, it is clear that fluorescence *in situ* hybridization (FISH) analysis is also an integral complement to such evaluations.

The notion that RT-PCR methods and conventional cytogenetics are complementary tests is supported by our results. The major advantage of the former technology is to discern cryptic (submicroscopic) translocations. However, essentially no such cryptic translocations were present in our study, probably because of the relatively small numbers in general and the paucity of those translocations (t(8;21), and inv(16)), which are more likely to be missed cytogenetically.⁴⁻⁷ Furthermore, there were none of less common cryptic *BCR-ABL*/t(9;22) fusions, which occur in ~5% of CML and ALL, present in this study. The single exception was the only t(4;11) in our series, that was already suspected hematopathologically, based on the "characteristic" CD15+, CD10- early pro-B-cell immunophenotype. This was only detected by RT-PCR, while conventional cytogenetics could not definitively identify this lesion at diagnosis, due to a specimen of suboptimal quality. Since the translocation was clearly evident cytogenetically at relapse, this might not be considered a truly cryptic translocation, but rather a discrepancy due to poor specimen quality. It should be noted that the only MLL translocation detectable by this

multiplex assay is the t(4;11) that creates an *MLL-AF4* fusion; other MLL translocations will be missed.

A single positive case out of 47 with tandem cytogenetics (~2%) indicates a much lower proportion of discrepant translocations as compared with what others have reported (as high as 30%⁴⁻⁵), and is in keeping with the low rate observed in another study.⁷ This may well reflect the overall quality of specimens and conventional karyotyping diagnostic services, often related to single-versus multi-institutional studies, which may play a role in this apparent discrepancy between series. Nevertheless, this single discrepant case reinforces the diagnostic appropriateness of using both conventional cytogenetics and RT-PCR in the initial evaluation of leukemias. Furthermore, the multiplex RT-PCR typically has a more rapid turn-around time of ~24 hours, affording the ability to make crucial therapeutic decisions, without having to wait for cytogenetic studies, that typically take up to 72 hours to complete. Conventional cytogenetics and FISH are the current methodologies of choice for detecting numeric abnormalities, until other molecular techniques (for example, comparative genomic hybridization) enter into the realm of clinical practice, and they remains important in the genetic determination of disease progression/clonal evolution.

Another aim of this study was to validate an assay that would be most useful to a routine molecular pathology laboratory with a broad referral practice, ensuring diagnostic accuracy, covering the most relevant translocations for both adult and pediatric populations, and maintaining cost-effectiveness. While the labor and reagent cost of the full analysis for seven translocations (10 to cover all of the breakpoints) with this multiplex system is ~\$500, and thus ~\$70/translocation, the current in-house cost for running these separate reactions individually would be at least four times greater (~\$280/translocation). These estimates exclude physician interpretation, and the latter estimate does not incorporate the added cost of send-out tests that would be required for assays not already established in-house. This significant difference would be even greater if indi-

rect costs such as the expense of maintaining the technologist's expertise in 10 different reactions *versus* a single one, and quality control/quality assurance expenses associated with 10 independent tests, are taken into account. An additional important facet of this study is to highlight how the use of a commercial kit may provide standardization that is crucial in achieving diagnostic accuracy and precision that can be robustly compared between institutions and studies. This is superior to relying on a multitude of heterogeneous, non-standardized, internally developed, individual laboratory generated, so-called "home-brew" assays. While the purpose of this study was to validate the screening multiplex diagnostic use of this test, if clinically indicated the laboratory can use monoplex reactions where appropriate, for example for *PML-RAR α /t(15;17)* and *BCR-ABL/t(9;22)*

Although neither an aim nor formally evaluated in this study, we had an opportunity to appraise the utility of the multiplex assay, as compared with our laboratory-developed monoplex assay, for MRD detection. We demonstrated 100% concordance in the results of these two assays (that have a similar 10^{-2} to 10^{-4} level of sensitivity) for MRD specimens. However, in an era where more sophisticated and accurate real time methods are actively being validated for a more meaningful quantitative determination of residual disease,⁸ the role of qualitative tests for MRD detection is anticipated to significantly diminish. Thus, while we foresee a role for this multiplex assay in screening for disease-specific fusions at diagnosis that can then be exploited for subsequent MRD testing, this assay *per se* is not likely to be useful for MRD testing.

In summary, we have demonstrated that molecular-based screening, via a multiplex RT-PCR system evaluated here, is still extremely valuable in clinical practice, given the quite high false-negative rate of conventional cytogenetics reported by others, in particular for the commonly occurring t(8;21) and inv(16) abnormalities. The benefits of identifying such cases are at least twofold, with regard to therapeutic decision making: the recognition of patients who will not benefit from bone marrow transplantation in first complete remission, thus rendering this a cost-effective approach given the significant cost of this procedure, and the documentation of a marker that can be used for MRD testing, which has prognostic relevance, allowing for more rational, tailored therapy, with the potential to improve patient care.

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