Monitoring AML1-ETO and CBFβ-MYH11 Transcripts in Acute Myeloid Leukemia

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Introduction
At diagnosis, patients with acute myeloid leukemia (AML) may have a tumor burden in excess of $10^{12}$ malignant cells. However, those achieving complete remission (CR), defined by conventional criteria of normal bone marrow morphology with a blast count of less than 5%, may still have up to $10^{10}$ leukemic cells. At present, very little is known about the kinetics of leukemia relapse, the development of drug resistance, and how the immune response and other factors affect residual leukemia. Furthermore, postremission therapy in AML does not take into account the level of residual leukemia. However, the development of molecular methodologies has enabled the sensitive detection and quantification of minimal residual disease (MRD) in acute and chronic leukemias [1•,2]. The level of MRD that was undetected by light microscopy or karyotypic analysis would now be considered evidence of significant disease with these new techniques. Studies of MRD in acute promyelocytic leukemia and other subtypes of AML have proven to be highly informative and clinically relevant [3].

Acute myeloid leukemias with t(8;21)(q22;q22) and pericentric inversion of chromosome 16, inv(16)(p13q22), account for approximately 20% of AMLs and are collectively referred to as core-binding factor (CBF) leukemias. In these leukemias there is genomic rearrangement involving alpha (CBFα or AML1) or beta (CBFβ) subunits, respectively, of the transcription factor CBF, which plays a key role in hematopoiesis. The resulting fusion genes, which characterize CBF leukemias, can be reliably and sensitively detected by polymerase chain reaction (PCR) techniques and can therefore be used as specific molecular targets for monitoring residual leukemia. Although patients with t(8;21) and inv(16) belong to the cytogenetic group of AML with a favorable prognosis, with greater than 50% of patients obtaining a long-term remission with chemotherapy, relapse is the major cause of treatment failure, occurring in up to 35% of patients [4•]. Identifying patients with high-risk disease within the group of CBF-positive AML remains a clinical priority, and monitoring MRD through quantification of the specific fusion transcripts of individual patients may be a useful approach.

Molecular Methodologies for Detecting Minimal Residual Disease
Current approaches for detecting MRD include conventional cytogenetics, fluorescence in situ hybridization, multiparameter flow cytometry, and nucleic-acid amplification (PCR). PCR strategies include qualitative amplification by nested, reverse transcription polymerase chain reaction (RT-PCR) and quantitative amplification with manual techniques, such as competitive RT-PCR, or by automation, such as real-time RT-PCR (RQ-PCR) [5]. These approaches are generally used to amplify fusion gene transcripts arising from leukemia-associated chromosomal rearrangements, including those found in CBF-positive AML. These methods are highly specific and sensitive, detecting one leukemic cell in $10^4$ to $10^6$ normal cells.

Qualitative reverse transcription polymerase chain reaction
The original qualitative RT-PCR methods relied on the assay being either positive or negative when the amplification prod-
uct was analyzed, usually by gel electrophoresis. Although typically a very sensitive assay, the level of sensitivity has been found to vary considerably from laboratory to laboratory. Consequently, a negative RT-PCR result for a particular fusion transcript does not necessarily mean absence of MRD but more specifically indicates that the MRD level is below the threshold of the test being used. The application of sensitive RT-PCR methods to detect MRD has revealed persistence of low levels of disease in many patients with leukemia, including CBF-positive AML, who are considered cured of their disease. Thus, qualitative RT-PCR methods have been of limited value in studying the kinetics of MRD and how levels change during disease progression. For these reasons, quantification of target genes as a measure of residual leukemia provides a better tool for monitoring MRD.

**Competitive quantitative reverse transcription polymerase chain reaction**

Quantification of the level of transcripts for the target gene can be carried out by end-point quantification (competitive RT-PCR) or cycle-cycle (real-time) techniques. The competitive RT-PCR approach has been successful at quantifying fusion gene transcripts and hence the level of MRD in AML with t(8;21) and inv[16] [6–8]. However, potential disadvantages of the end-point RT-PCR assay are the requirement for exponential amplification for precise quantification and the lack of reproducibility. The final amount of PCR product is very sensitive to slight variations in the reaction components that need to be rigorously controlled. To overcome these shortcomings, real-time RT-PCR techniques for quantification of target sequences have been developed and are now widely used.

**Real-time reverse transcription polymerase chain reaction**

Quantitative real-time RT-PCR (RQ-PCR) allows highly sensitive quantification of gene transcripts of interest in a few hours with minimal handling of the samples. Higuchi et al. [9] pioneered real-time PCR analysis by constructing a system that detects PCR products as they accumulate. This “real-time” system uses the intercalator ethidium bromide that binds to the increasing amounts of amplified double-stranded DNA, resulting in an increase of fluorescence. This method has been improved by the introduction of fluorescent gene-specific probes in the PCR reaction. The new methodology is based on the 5’ nuclease assay, first described by Holland and Abramson [10], which uses the 5’-3’ exonuclease activity of Taq DNA polymerase to cleave a dual-labeled probe annealed to a target sequence during PCR amplification. Briefly, cDNA is added to a PCR reaction mixture containing standard PCR components as well as a probe that anneals to the template between the two primers. This probe contains a fluorescent reporter dye at the 5’-end and a quencher dye at the 3’-end. The quencher can only quench the reporter fluorescence when the two dyes are close to each other. This is only in the case of an intact probe. Once amplification occurs, the probe is degraded by 5’-3’-exonuclease activity of the Taq DNA polymerase, and the fluorescence is detected by means of a laser integrated in the sequence detector (TaqMan ABI Prism 7700 Sequence Detection System, Perkin Elmer, Foster City, CA). The PCR cycle number at which fluorescence reaches a threshold value of 10 times the standard deviation of baseline emission is used for quantitative measurement. This cycle number is called the cycle threshold, and it is inversely proportional to the starting amount of cDNA. The increase in fluorescence intensity can be monitored during the exponential phase of each PCR cycle to give a precise and rapid quantification of PCR signal (Figs. 1 and 2). The advent of RQ-PCR allows rapid detection and quantification of gene products and will enable the study of MRD monitoring in large numbers of patients enrolled in multicenter clinical trials. Furthermore, RQ-PCR lends itself to greater standardization and quality control and ensures reliability of MRD detection within trials, thereby facilitating comparison of data between international groups.

**Predictive Value of Minimal Residual Disease with Reverse Transcription Polymerase Chain Reaction**

**AML with t(8;21) and AML1-ETO transcript**

The t(8;21) is one of the most frequent chromosomal translocations in AML, especially in the FAB-M2 subgroup. The t(8;21) fuses two genes, AML1 on chromosome 21 and ETO (MTG8) on chromosome 8 to produce the AML1-ETO fusion gene [11]. Transcripts of this fusion gene can be specifically and sensitively detected by RT-PCR in all cases of AML with t(8;21) and can therefore be used as a marker for diagnosis and monitoring of residual disease [12•,13]. AML with t(8;21) is associated with a favorable response to chemotherapy with a high remission rate and long-term disease-free survival [4•,14]. However, in spite of the relatively good prognosis, relapse remains a major problem, especially in the first 2 years of remission.

Studies of qualitative RT-PCR for detection of MRD in t(8;21)-positive AML have yielded discrepant results. Using sensitive RT-PCR assays (10^-5 to 10^-6), several groups have shown that, following chemotherapy, autologous, and allogeneic bone marrow transplantation, AML1-ETO transcripts can be detected in most patients in long-term remission [15•,16–18]. This phenomenon has been ascribed to quiescent populations of stem cells harboring the fusion gene [19•]. Furthermore, in inducible transgenic mice, AML1-ETO is not leukemogenic as such but requires the presence of additional mutations for the development of AML [20,21]. Interestingly, AML1-ETO has also been reported to be positive in a surprisingly high number of normal adult bone marrow and cord blood samples, suggesting that t(8;21) may be generated in early hematopoiesis [22]. However, the absence of AML1-ETO transcripts has also been reported in a significant number of long-term remitters [23,24,25•]. The reasons for these