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MINI-REVIEW

Detection and quantification of residual disease in chronic myelogenous leukemia

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The degree of tumor load reduction after therapy is an important prognostic factor for patients with CML. Conventional metaphase analysis has been considered to be the 'gold standard' for evaluating patient response to treatment but this technique normally requires bone marrow aspiration and is therefore invasive. The frequency of cytogenetic analyses can be considerably reduced if patients are also monitored by molecular methods, which can be performed on peripheral blood specimens. Of the various techniques available, most attention has been paid to RT-PCR for BCR-ABL mRNA since this is by far the most sensitive. Simple, non-quantitative RT-PCR analysis gives only limited information on patients after treatment. Quantitative RT-PCR assays have been developed to monitor the kinetics of residual BCR-ABL transcripts over time. Variables in the quantitative PCR assay may be controlled for by quantification of transcripts of a normal gene (eg ABL or glucose-6-phosphate dehydrogenase, G6PD) as an internal standard. After allogeneic stem cell transplantation, most patients become RT-PCR negative, often after a period of low level positivity that may persist for several months. Those patients destined to relapse are characterized by the reappearance and/or rising levels of BCR-ABL transcripts. In contrast, for patients treated with interferon- (IFN) residual disease is rarely, if ever, eliminated. The actual level of minimal residual disease in complete cytogenetic responders to IFN correlates with the probability of relapse. New quantitative real time procedures promise to simplify the protocols that are currently in use, but standardization and the introduction of rigorous, internationally accepted controls are required to enable RT-PCR to become a robust and routine basis for therapeutic decisions.
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Introduction

Chronic myelogenous leukemia (CML) constitutes a clinical model for molecular detection and therapy surveillance of malignant disease since this entity was the first leukemia shown to be associated with a specific chromosomal rearrangement, the Philadelphia (Ph) translocation t(9;22)(q34;q11),[1–3] which generates two chimeric genes: BCR-ABL[4,5] on the derivative chromosome 22, and ABL-BCR on the derivative chromosome 9. BCR-ABL is transcribed and translated in most patients into a 210 kDa fusion protein with deregulated tyrosine kinase activity. ABL-BCR is expressed in about 60% of patients with CML, but probably lacks any biological function[6] (review, see Ref. 7).

The degree of tumor load reduction is an important prognostic factor for patients with CML on therapy.[8] Response is expressed at three levels: (1) hematological response, defined as the normalization of the peripheral blood values and of spleen size; (2) cytogenetic response defined as the proportion of residual Ph-positive metaphases; and (3) molecular response defined according to the method used as the proportion of the residual BCR-ABL gene, transcript, or protein. The principle aim of residual disease analysis in patients with CML is to determine patient response to treatment and to enable early diagnosis of relapse.

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Detection and quantification of BCR-ABL-positive cells

Cytogenetic analysis of bone marrow metaphases

The Ph chromosome is present in about 90% of patients with a clinical presentation consistent with CML. Three to five percent of patients show a normal chromosome 22 but molecular evidence of the BCR-ABL translocation. At presentation cytogenetic analysis usually reveals the Ph chromosome in 100% of cells analyzed with standard 20–30 cell analysis. Conventional cytogenetics is still considered the 'gold standard' for evaluating this response.

Major drawbacks of cytogenetics are the requirement of bone marrow cells in mitosis, and the analysis of relatively small numbers of metaphases, resulting in significant sampling errors.[9] The major advantage of cytogenetics is the early detection of clonal evolution consistent with acceleration of the disease.[10,11]

The frequency of cytogenetic analysis can be reduced if patients are monitored by other methods, such as quantitative Southern blot, fluorescence *in situ* hybridization (FISH), quantitative Western blot, or quantitative RT-PCR. In CML molecular methods can be performed on peripheral blood specimens and are therefore less invasive than conventional cytogenetic analysis of bone marrow metaphases. Furthermore, these techniques are applicable to Ph-negative, BCR-ABL positive cases.

Fluorescence in situ hybridization (FISH)

FISH analysis is typically performed by co-hybridization of a BCR and an ABL probe to denatured metaphase chromosomes or interphase nuclei. Probes are large genomic clones, such as cosmids or YACs, and are labeled with different fluorochromes. Dual-color FISH using probes for BCR and ABL genes allows the specific detection of the BCR-ABL gene fusion in interphase and/or metaphase nuclei. Most cells exhibit four distinct signals, two of each color corresponding to the two normal BCR and ABL alleles. CML cells are recognized by the juxtaposition of one of the BCR and ABL signals.[12] FISH analysis does not depend on the presence of the typical Ph chromo-

some and will detect rare BCR and ABL variant fusions.[12–15] The lineage of positive and negative cells can be determined in combination with conventional May–Grünwald staining or immunocytochemistry.[16–17]

A limitation of interphase FISH method is the background of a variant proportion of false positive cells, depending on the probe/detection system used.[18–21] In practice, the limit of detection of CML cells is typically 1–5% and depends in part on which probes are used, the size of the nucleus, the precise position of the breakpoint within the ABL gene and the criteria used to define co-localization.[22] The advantage of FISH over conventional cytogenetics is the analysis of a larger number of nuclei, resulting in smaller sampling errors.[23] Therefore, FISH is applicable for quantification of residual disease in partial, minor, and nonresponders to IFN- [24–26] and for determination of the BCR-ABL positivity of individual cell colonies,[27] or the proportion of BCR-ABL-positive cells in small samples, such as highly enriched cell fractions.[28]

The specificity of interphase FISH can be increased by introducing an additional probe that permits identification of both the Ph chromosome and the derivative 9 chromosome in Ph+ cells, thus lowering the rate of false positivity.[29–32] In many cases, however, large genomic deletions around the Ph-translocation breakpoints preclude the use of these techniques.[32]

The development of a 'hypermetaphase FISH' based on improved culture techniques and computerized analysis of a large number of metaphases made it possible to distinguish different levels of Ph chromosome positivity at presentation. Where readings can be obtained on 500 cells, one can reliably estimate parameters which characterize MRD. However, hypermetaphase FISH is evaluating only cycling cells and cannot count Ph+ cells that do not enter division.[33–35]

Southern blot analysis

Southern blotting exploits the fact that the breakpoint within the BCR gene in most cases falls in a limited area, the 5.8 kb major breakpoint cluster region (M-bcr).[4] Genomic DNA extracted from leukocytes from a patient is digested by a set of appropriate restriction enzymes (eg *Bgl*II, *Xba*I, *Hin*dIII, *Eco*RI, *Bam*HI), fractionated on an agarose gel, transferred to a nylon membrane and hybridized to two labeled DNA probes derived from the 3 and 5 portion of M-bcr. After autoradiography, a band corresponding to the unrearranged BCR allele is visible; for patients with CML, one or two additional bands may reveal the rearranged BCR allele. Using this technique, a BCR rearrangement is detectable in about 98% of Ph-positive patients and in a significant proportion of Ph-negative cases.[36] For routine use, *Bgl*II and *Xba*I are sufficient, which are informative in almost all cases. However, since there are rare restriction site polymorphisms in the M-bcr, the finding of a rearrangement with at least two restriction enzymes is generally considered necessary to exclude a false-positive result in any new patient.[37,38] False-negative results may arise because the rearranged band is too large, too small or coincidentally exactly the same size as the normal allele, as a result of partial deletion of BCR sequences on the translocated allele[39] or of rare variants that have breakpoints outside the M-bcr.[14,40,41] False-positive or -negative results are, however, rare.[36]

After therapy Southern blot analysis allows quantification of the proportion of cells with BCR rearrangement compared to all cells investigated. The proportion of CML cells is determined by twice the intensity of the rearranged band divided by the sum of the intensities of the rearranged plus germline bands (BCR ratio). This is because each CML cell contributes signals from one normal and one rearranged chromosome 22, whereas the normal cells contribute identical signals from two normal chromosomes.[36,42] The level of disease detected in contemporaneous peripheral blood and bone marrow samples is essentially identical[42,43] and therefore peripheral blood is usually used for analysis. Quantitative Southern blot allows the detection and quantification of down to 1–5% leukemic cells.[36,43] To evaluate the response to treatment, the knowledge of the initial restriction pattern and intensity of the rearranged band is mandatory. Complete cytogenetic responses are associated with disappearance of rearranged BCR bands in most cases.[36,44,45]

The BCR ratio is usually lower than the proportion of Ph-positive metaphases. The reason for this is that Southern blotting analyses a large number of dividing and resting cells, including BCR-ABL-negative lymphocytes and cytogenetic analysis provides information only on a small number of dividing myeloid cells.[36,42] Empirically derived cut-off points in the BCR ratio were introduced in order to define molecular response groups: a BCR ratio of 0% was defined as complete response, and ratios of 1–24%, 25–50%, and 50% were defined as partial, minor, and no molecular response, respectively. Using these cut-off points, a major cytogenetic response could be predicted or excluded in more than 90% of cases.[36] The main advantage of Southern blotting over cytogenetics is the independence from dividing cells which permits the use of peripheral blood instead of bone marrow.

Western blot analysis

Western blotting can be used to detect BCR-ABL proteins directly in cell extracts qualitatively and quantitatively both in bone marrow and in peripheral blood. Leukocytes are lysed in the presence of potent protease inhibitors, fractionated on a polyacrylamide gel, transferred to a nylon membrane and probed with an anti-ABL antibody. Different types of BCR-ABL proteins (p190, p210, p230, rare variants) can be distinguished from p145 ABL by differences in migration.[14] The limit of sensitivity is about 0.5–1%. A quantitative Western blot assay found a linear correlation between BCR-ABL/ABL protein ratios and contemporaneous conventional cytogenetics.[46,47]

Reverse transcriptase polymerase chain reaction (RT-PCR)

In 1989, first encouraging results concerning detection of MRD by PCR in CML patients after allogeneic bone marrow transplantation were reported.[48] However, conflicting data from a comparative multicenter study revealed serious problems of the method with a high rate of false-positive results and provoked an open discussion.[49,50] Over the past 10 years, PCR has been optimized and developed. Specificity has been considerably increased by the partial standardization of methodology and the introduction of rigorous precautions to avoid contamination.[51] Sensitivity has been improved by using nested primer pairs and performing two consecutive PCR steps. In view of the limited value of qualitative PCR for monitoring CML patients after therapy, quantitative BCR-ABL PCR assays were developed to monitor patients after stem cell

transplantation[52,53] or treatment with interferon (IFN)[54,55] and are now in routine clinical use.

Screening for BCR-ABL mRNA transcripts at diagnosis

For diagnostic samples, the use of *multiplex PCR* has been suggested to detect simultaneously several kinds of BCR-ABL and BCR transcripts as internal controls in one reaction[56] by using three BCR and one ABL primer. This method allows the reliable detection of typical BCR-ABL transcripts, such as b2a2 or b3a2, and atypical types, eg transcripts lacking ABL exon a2 (b2a3 and b3a3), transcripts resulting from BCR breakpoints outside M-bcr, such as e1a2, e6a2 or e19a2,[14,15,57] or transcripts with inserts between BCR and ABL exons.[58]

Detection of minimal residual disease, 'nested' RT-PCR

Since patients with leukemia at presentation or relapse usually have a total burden of more than 10^{12} malignant cells,[59] and cytogenetics, Western blot, and conventional FISH have a sensitivity of maximum 1%, a patient with negative results may harbor as few as zero or as many as 10^{10} residual leukemic cells. At this point, the patient is judged to be in clinical and hematologic remission, although the term 'remission' refers only to a arbitrary point of a continuum of residual leukemic cell numbers.[60]

RT-PCR for BCR-ABL mRNA is by far the most sensitive assay in the context of residual disease analysis and can detect a single leukemia cell in a background of 10^5–10^6 normal cells. Therefore, PCR is up to four orders of magnitude more sensitive than conventional methods. However, patients who have no residual disease detectable by RT-PCR may still harbor up to a million malignant cells that could contribute to subsequent relapse. The sensitivity with which residual disease can be detected will be limited by the amount of peripheral blood or bone marrow that can be analysed.

By *nested* RT-PCR with two pairs of ('nested') primers corresponding to appropriate BCR and ABL exons used in two rounds of amplification residual CML cells after treatment can be specifically detected with a sensitivity of up to 1 in 10^6 cells.[61]

This qualitative method has been used for MRD detection after allogeneic stem cell transplantation. Most transplant centers have demonstrated that the majority of patients is PCR positive in the first 6 months after transplantation, two thirds of patients become PCR negative during follow-up due to the graft-versus-leukemia effect, persistent PCR negativity after 1 year is a marker for good prognosis, and PCR-positive patients more than 6 months after BMT have a great risk of relapse (Figure 1).[62] However, qualitative PCR cannot predict relapse in the individual patient.[52,63–65] In the majority of cases after transplantation, RT-PCR and DNA-PCR (using patient specific primers) results are concordant, ie that patients in remission do not generally harbor a substantial pool of CML cells that do not express BCR-ABL mRNA.[66]

Nested PCR is essentially useless in patients after IFN-therapy even in cytogenetic remission, since almost all patients remain repeatedly positive.[67]

If the RT-PCR method is pushed to extreme, BCR-ABL mRNA can be detected at a very low level of 1 to 10 transcripts per 10^8 cells in many normal individuals with a frequency that is age dependent (Figure 2).[68,69] It has been suggested that BCR-ABL, and probably several other fusion genes, are being continuously formed in mitotic cells in the normal bone marrow, but only the combination of an in frame BCR-ABL fusion in the correct primitive hematopoietic progenitor would have the selective advantage to become functional as an expanding clone.[70] In addition, it is possible that BCR-ABL alone is not sufficient to result in the expansion of myeloid cell numbers, and that other co-operating genetic events may be required.

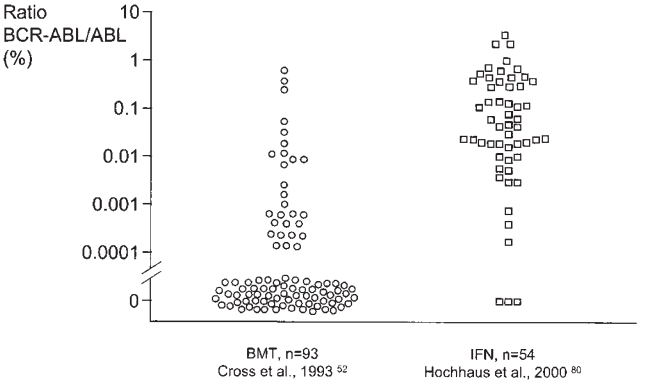

Figure 1 Quantitative PCR analysis for the ratio BCR-ABL/ABL in complete cytogenetic responders after allogeneic stem cell transplantation and interferon (IFN) therapy. Two thirds of patients after allogeneic bone marrow transplantation (BMT) are after 6 months RT-PCR negative,[52] whereas almost all patients after IFN therapy are persistently RT-PCR positive. Only three out of 54 patients showed transient PCR negativity.[80]

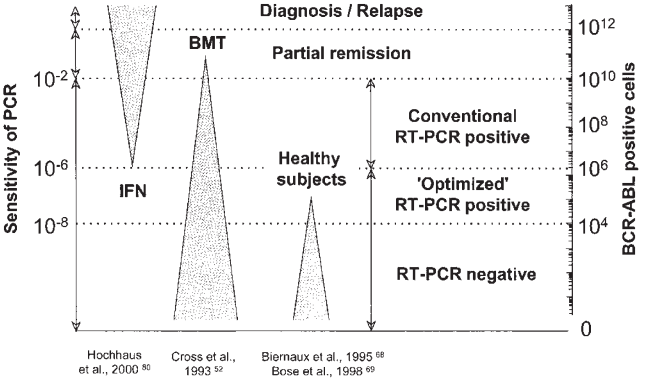

Figure 2 Schematic illustration of therapeutic response of CML patients on molecular level. Almost all interferon (IFN)-treated patients remain RT-PCR positive,[80] the majority of patients after allogeneic stem cell transplantation become RT-PCR negative.[52] Healthy donors may be RT-PCR positive for BCR-ABL transcripts using an optimized, very sensitive PCR strategy.[68,69]

Quantitative PCR

In view of the very limited value of qualitative PCR, several groups have developed quantitative PCR assays to estimate the amount of residual disease in positive specimens. Most groups have initially used *competitive PCR strategies* that can effectively control for variations in amplification efficiency and reaction kinetics.[52,54,71–73]

In general, nested PCR is performed using serial dilutions of a BCR-ABL competitor construct added to the same volume

of patients' cDNA. The equivalence point at which the competitor and sample band would be of equal intensity is determined by densitometry.[52,55] In order to standardize results for both quality and quantity of blood, RNA, and cDNA quantification of transcripts of normal housekeeping genes, such as ABL or glucose-6-phosphate dehydrogenase (G6PD) has been employed. The standardized results are expressed as the ratios BCR-ABL/ABL or BCR-ABL/G6PD in per cent. The quantification of the transcript level of control genes is of particular importance if different RNA qualities are expected, ie in particular if samples are mailed, eg in multicenter trials.

In patients after allogeneic stem cell transplantation, rising or persistently high levels of BCR-ABL mRNA can be detected prior to cytogenetic or hematologic relapse. Low or falling BCR-ABL transcript levels are associated with continuous remission, whilst high or rising BCR-ABL transcript levels predict relapse.[52,53,63,74–76]

Quantitative PCR is the method of choice to determine the best time point for therapeutic interventions in the case of relapse after stem cell transplantation. Quantitative PCR data has been used to determine the optimum time point to initiate donor lymphocyte transfusions[77] and to monitor its response.[78] The great majority of patients who respond to donor lymphocyte infusions achieve durable molecular remission (RT-PCR negativity) with a median follow-up of more than 2 years.[78]

Quantitative RT-PCR for BCR-ABL has been shown to be a reliable method for monitoring residual leukemia load in mobilized peripheral blood stem cells, particularly in Ph-negative collections. Quantitative RT-PCR allows selection of the best available collections for reinfusion into patients after myeloablative therapy (autografting).[79]

Almost all patients after IFN- therapy are persistently positive for BCR-ABL transcripts. The median ratios of complete, partial, minor, and nonresponders differ significantly. The results of IFN- nonresponders and patients at diagnosis are not different.[55,67] Cytogenetic response to IFN- (complete, partial, minor/none) was compared to molecular response by introducing cut-off points for the BCR-ABL/ABL ratio. Optimum cut-off points were 2% and 14%, ie a complete response is associated with a ratio up to 2%, a partial response with a ratio of 2 and 14%, and a minor or nonresponse with a ratio of 14%.[55]

All 54 patients investigated who had achieved complete response to IFN- treatment had molecular evidence of residual disease during complete remission, although three patients were intermittently negative by RT-PCR. It should be noted that this level of sensitivity can be reached only if at least 5×10^7 cells (10–20 ml of peripheral blood) are processed and that the actual level of sensitivity for each specimen can only be determined by quantification of a control gene. The levels of residual disease in complete responders spans a range over four orders of magnitude.[67] In general, BCR-ABL transcript numbers were inversely related to the duration of CR. The median ratio of BCR-ABL to ABL transcripts at the time of maximal response for each patient was 0.045% (range 0–3.6%). During the period of observation 14 patients relapsed – 11 cytogenetically to chronic phase disease and three directly to blastic phase. The median ratio of BCR-ABL/ABL at maximal response was significantly higher in patients who relapsed than in those who remained in CR.[80] The findings also show that the level of residual disease falls with time in patients who maintain their cytogenetic response to IFN, but molecular evidence of disease is rarely if ever eliminated. In other series, the frequency of PCR negativity in complete cytogenetic responders is higher in a long-term follow-up using a RT-PCR strategy with a lower sensitivity.[81]

RT-PCR analysis of CFU-GM colonies grown from bone marrow of eight complete cytogenetic responders demonstrated that residual disease resides in myeloid colony-forming cells which may have the potential to repopulate the bone marrow and contribute to relapse.[82] Therefore, it is unlikely that CML can be cured by IFN- therapy. However, since the actual level of residual BCR-ABL transcripts is related to the probability of relapse, molecular monitoring may identify a subset of patients for whom treatment may be safely withdrawn.[80]

Recently, novel *real time PCR procedures* have been developed that promise to simplify existing protocols. Several procedures for quantification of BCR-ABL mRNA using the TaqMan system have been developed.[83–86] The assay is based on the use of the 5 nuclease activity of *Taq* polymerase to cleave a nonextendible dual-labeled hybridization probe during the extension phase of PCR. One fluorescent dye serves as a reporter and its emission spectra is quenched by the second fluorescent dye. The nuclease degradation of the probe releases the quenching resulting in an increase of fluorescent emission. The fluorescence is monitored by a sequence detector in real time. C_T (threshold cycle) values are calculated by determining the point at which the fluorescence exceeds a threshold limit. C_T corresponds to the amount of target transcripts in the sample.[87]

An alternative real time RT-PCR approach for detection and quantification of BCR-ABL fusion transcripts has been established using the LightCycler technology,[88,89] which combines rapid thermocycling with online fluorescence detection of PCR product formation as it occurs. Fluorescence monitoring of PCR amplification is based on the concept of fluorescence resonance energy transfer (FRET) between two adjacent hybridization probes carrying donor and acceptor fluorophores. Excitation of a donor fluorophore (fluorescein) with an emission spectrum that overlaps the excitation spectrum of an acceptor fluorophore results in nonradioactive energy transfer to the acceptor (Figure 3). Once conditions are established, the amount of fluorescence resulting from the two probes is proportional to the amount of PCR product. Due to amplification in glass capillaries with a low volume/surface ratio PCR reaction times have been reduced to less than 30 min.

A pair of probes was designed that was complementary to ABL exon 3, thus enabling detection of all known BCR-ABL variants and also normal ABL as an internal control. Conditions were established to amplify less than 10 target molecules/reaction, and to detect one CML cell in 10^5 cells from healthy donors, and one K562 cell in 10^7 HL60 cells. To determine the utility of the assay, BCR-ABL and ABL transcripts in a series of 254 samples from 120 patients with CML after therapy were quantified. The level of residual disease was expressed as the ratio of BCR-ABL/ABL. A highly significant correlation was seen between the BCR-ABL/ABL ratios determined by the LightCycler and (1) the BCR-ABL/ABL ratios obtained by nested competitive RT-PCR performed with the same cDNA samples; (2) the proportion of Philadelphia chromosome-positive metaphases determined by contemporaneous cytogenetics; and (3) the BCR ratio determined by Southern blot analysis.[90]

Real time PCR approaches are reliable and sensitive methods to monitor CML patients after therapy. The major advantages of the methodology are (1) amplification and product analysis are performed in the same reaction vessel,

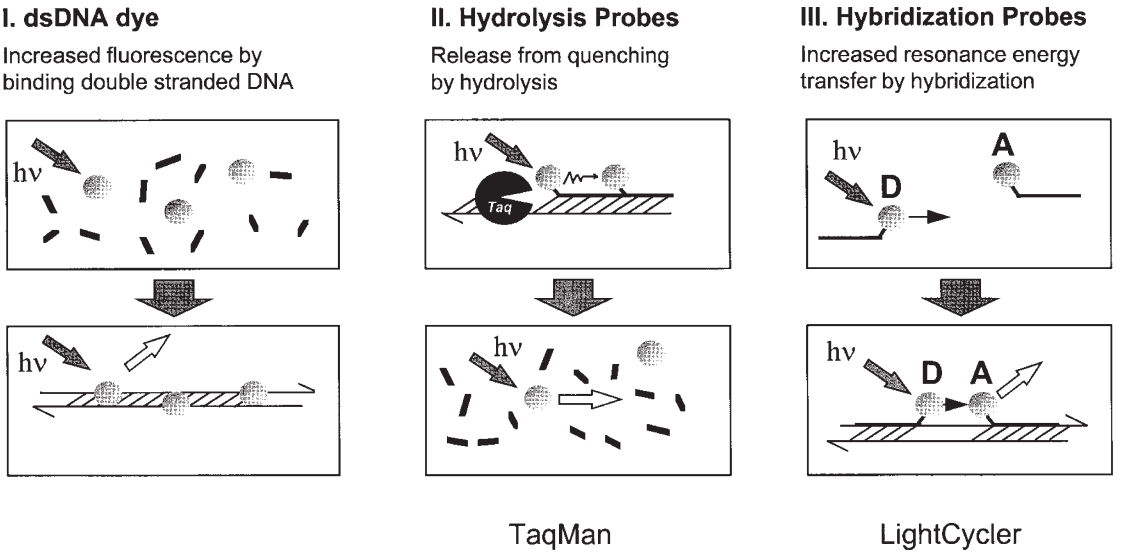

Figure 3 Real time fluorescence detection of PCR products during the amplification phase (Real-time PCR). Fluorescence labeling can be performed (I) by nonspecific binding of an appropriate dye to double stranded DNA (eg SYBR Green), (II) by hybridization of TaqMan probes carrying quencher and reporter dyes which are cleaved during elongation, or (III) by hybridization to a pair of probes labeled with a donor (D) and an acceptor (A) dye with increased fluorescence in juxtaposition (LightCycler method).

avoiding the risk of contamination, and (2) the results are standardized by the quantification of housekeeping genes.

Conclusion

Quantitative determination of residual disease levels after treatment for patients with CML may be achieved by various methods. However, there is a need to standardize methodologies and interpretation of results in order to reduce interlaboratory variation. The development of new real time RT-PCR procedures offers a unique opportunity for this to be achieved and we believe that quantitative real time PCR will shortly become a routine and robust basis for clinical decision making, not only in CML but also in other leukemias with specific molecular markers.

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