

Evaluation of Relationship between Minimal Residual Disease (MRD) and Relapse in Childhood Acute Lymphoblastic Leukemia (ALL) Using Quantitative Fluorescent PCR

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Abstract

Acute leukemia is the most common malignancy in children accounting for approximately one-third of all childhood cancers. Modern treatment protocols lead to complete remission (CR) in a considerable proportion of patients with lymphoproliferative disorders. However, many of these patients ultimately relapse, showing that in spite of clinical CR still significant amounts of residual malignant cells persist. Various protocols like cytogenetic, molecular and immunological techniques that are more sensitive than morphology are increasingly used to assess and quantify minimal residual disease (MRD). These techniques produce different levels of sensitivity allowing detection of MRD between 10^{-2} - 10^{-6} . In this study we have assessed the feasibility of fluorescent PCR in MRD detection and quantification using immunoglobuline heavy chain (IgH) gene rearrangement. Bone marrow samples obtained from 40 patients with precursor-B cell ALL after induction, consolidation, reconsolidation and intensification therapy. Analysis of clonally rearranged IgH gene carried out by CDR3 amplification in presence of an internal competitor with known copy number. After induction therapy 53% of the patients who were considered to be in clinical remission phase still had detectable MRD. After consolidation therapy 43% of the patients were MRD positive. Subsequent to maintenance-2 or intensification therapy still in 30% of patients MRD was observed.

Keywords: MRD; IgH; Fluorescent PCR; CQ-PCR

Introduction

Acute leukemia is the most common malignancy in children accounting for approximately one-third of all

childhood cancers [1]. Treatment of acute lymphoblastic leukemia (ALL) comprises induction, remission and maintenance chemotherapy. This leads to recovery in 95-98% of children and 50% of affected adults. At

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diagnosis, patients may have a total of approximately 10^{12} malignant cells [2]. The disease is considered to be in complete remission when fewer than 5% of the cells in bone marrow samples are morphologically identifiable as blasts [3]. Evidences show that leukemic cells persist for at least sometime after the onset of remission. Despite the risk of adapted therapy, 25-30% of the patients with childhood ALL relapse while there are no concrete individual predictive factors have yet been found to identify this subgroup. These relapses are thought to stem from regrowth of the original leukemic cells remained undetectable during or after treatment. Therefore assessment of minimal residual disease (MRD) during remission is of vital importance for individualization of treatment and relapse prediction [4]. MRD is defined as the lowest level of disease detectable in patients in complete remission by the available methods [5]. Various techniques would produce different levels of sensitivity allowing detection of MRD between 10^{-2} - 10^{-6} . The highest sensitivity (10^{-6}) achieved by PCR based clone-specific immunoglobulin heavy chain (IgH) and T-cell receptor (TCR) genes rearrangements detected by radio labeled allele specific oligonucleotide (ASO) probing [6]. However, inspite of its desirable level of sensitivity, this approach has several practical limitations including being labor intensive, time taking, costly and potential dangers of working with radioactive isotopes. These difficulties encouraged searching for more suitable alternatives. Fluorescent PCR found to be safe, rapid and comparatively easy to perform method with an acceptable level of sensitivity (10^{-2} to 10^{-4} leukemic cells).

Materials and Methods

Preparation of Competitor

Bone marrow samples were obtained from 40 patients with precursor-B ALL after induction, consolidation, reconsolidation and intensification therapy. Analysis of clonally rearranged immunoglobulin heavy chain (IgH) gene (Fig. 1) carried out by complementarily determining region 3 (CDR3) amplification in the presence of an internal competitor with known copy number. PCR products were analyzed by automated laser fluorescent express (ALF Express) DNA sequencer (Pharmacia Biotech, Sweden) both for detection and quantitation of MRD.

pBR322 plasmid was amplified using specific forward and reverse primers (Table 1). To the 5' end of the pBR322 forward primer the sequence for the FRIII consensus primer and to the 5' end of reverse primer the JH consensus primer sequence were added (Fig. 1). In

this way FRIII and JH primer sites incorporated into the PCR products. PCR reaction for competitor fragment production contained the followings: 10 mM Tris-HCl pH 8.2, 50 mM KCl, 0.125 mM each dNTPs, 1.5 mM MgCl₂, 0.1 mg/ml gelatin, 50 pmoles of each of the primers and 1U Taq DNA polymerase. Amplification performed for 25 cycles of 95°C, 67°C and 72°C, 1 min each. The PCR products electrophoresed on 1.5% agarose gel stained with ethidium bromide and visualized using UV transilluminator. The corresponding bands then excised from the gel and DNA fragments eluted using standard protocol. Genequant DNA/RNA spectrophotometer (Eppendorf) was used to estimate the quantity of the purified competitor molecules.

DNA Preparation from Bone Marrow Samples

Two hundred microliters of fresh anticoagulated bone marrow sample that was aspirated for usual diagnostic purposes layered on ficol gradient to separate out lymphocytes. The separated lymphocytes were then transferred into a new tube and disintegrated by lysis buffer and proteinase K. The extracted DNA was then ethanol precipitated, air dried and finally resuspended in 100 µl of sterile water.

Quantitative Competitive PCR

The competitive quantitative PCR method is based on co-amplification of an exogenous template as an internal standard that competes with the target sequence for the same primers. In this way any variable affecting amplification has the same effect on both target and competitor.

Known copy number of competitor was mixed with 1 µg of ALL DNA sample and PCR amplification was performed for 35 cycles. The numbers of leukemic cells were then calculated by comparison of the competitor and target DNA peak areas on ALF express DNA sequencer.

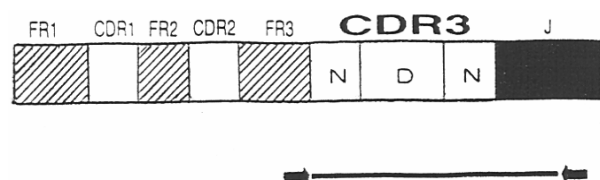


Figure 1. Structure of the rearranged IgH gene with position of primers (arrows) used for the FR3 fingerprinting. A solid black bar indicates the length of the amplified PCR products. FR, frame work region; CDR, complementarily determining region; N, random nucleotides; D, diversity segment; J, joining segment.

Table 1. Oligonucleotide Primers used for generation of competitor molecules and amplification of CDR3 region of IgH gene

1	Forward Primer of competitor	5'-CCGAGGACACGGCCGTGTACTACT-3'
2	Reverse Primer of competitor	5'-ACCTGAGGAGACGGTGACCAGGGTAAGCCT-3'
3	Labeled Consensus JH Primer	5'-ACCTGAGGAGACGGTGACCAGGGT-3'
4	FRIII Consensus Primer	5'-CCGAGGACACGGCCGTGATTACTG-3'

An equivalence point was established at presentation by diluting a clonal sample in to a normal one. To find out the right concentration of competitor and target molecules, series of competitor dilution was added to different target dilutions and amplified for 35 cycles.

Analysis of PCR Products

Three microliters of the fluorescently labeled amplified DNA was mixed with an equal volume of loading dye (0.5% dextran blue in 100% formamide) and denatured for 5 min at 90°C prior to loading on a 6% denaturing polyacrylamide gel (Amersham Biosciences). Electrophoresis was carried out on Alfexpress DNA sequencer. Fragment analysis was carried out using Allele Links software and the size; peak area, amplitude and height of the amplified fragments were calculated for subsequent interpretation.

Results

Due to the clonal rearrangements of IgH gene, in most of the cases a distinct peak of 470 bp was observed in the presentation B-cell precursor ALL bone marrow DNA samples. In some cases two or more clonal rearrangements were observed in the presentation bone marrow DNA samples. Equivalence points for samples were established by amplification of various known concentrations of competitor molecules along with 1 μ of DNA extracted from bone marrow samples. It was assumed that 1 μ of DNA was equivalent to about 2×10^5 cells and that each leukemic cell contains one copy of the clone. In this way number of the molecules per μ g DNA calculated between 2.4×10^5 to 5.3×10^5 cells (Fig. 2). Amplified products from normal DNA samples and bone marrow DNA samples obtained from patients who had entered in complete remission phase represent a smear instead of a distinct peak. This is due to the return of polyclonal nature of IgH gene (Fig. 3).

After induction therapy 53% of the patients who were considered to be in clinical remission phase still had detectable MRD. The level of MRD was less than 10^{-3} in 11 patients and more than 10^{-3} in another 10

patients. After consolidation therapy 17 patients (43%) had detectable MRD. Out of them 59% had $<10^{-3}$ and 43% with MRD level of $>10^{-3}$. Subsequent to maintenance-2 or intensification therapy still in 12 patients (30%) MRD was observed, among them 34% had MRD level of more than 10^{-3} . Relapse occurred in 11 out of 40 patients. All of them were MRD positive with mostly 10^{-3} or more leukemic cells. These results are summarized in Table 2.

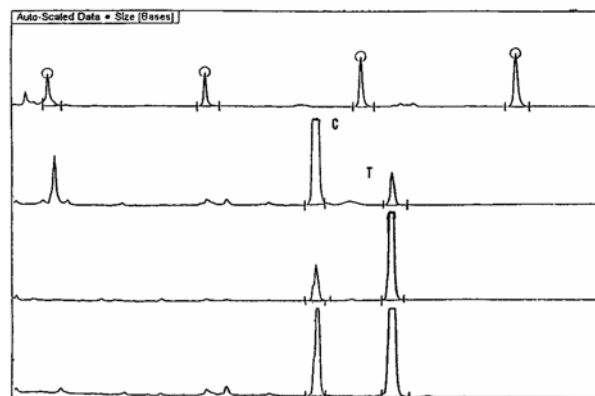


Figure 2. Determination of equivalence points between competitor molecules (C) and target DNA molecules (T) in a competitive quantitative PCR reaction.

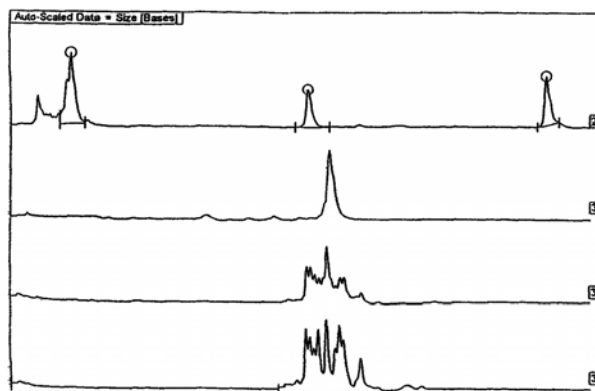


Figure 3. A single clonal rearrangement is seen in the presentation bone marrow (lane 33) and its absence from remission bone marrow (lanes 34 and 35).

Table 2. MRD status (presence and quantity) in different stages of treatment

MRD determination stage	MRD status with percentage of patients (No. of patients with relapse)			
	MRD Negative	MRD Positive	Residual blast or quantity of MRD	
			<10 ⁻³	>10 ⁻³
After induction therapy	47	53	52	48
After consolidation therapy	57	43	59	41
After interval therapy	63	37 (4)	66 (2)	34 (2)
After intensification therapy	70	30 (6)	66 (2)	34 (4)

Discussion

At present ALL patients are classified as standard or high-risk based on clinical and laboratory findings such as age, sex, WBC count, cytogenetic, biochemical and so on [7]. Interestingly, the majority of relapses occur in children classified as standard risk at presentation [8]. Lack of well defined criteria to predict clinical response to treatment and relapse in the standard risk group emphasizes the necessity of a reliable prognostic marker with a high degree of specificity and sensitivity. Criterion, which helps towards identification of patients who may destined to relapse within the first 6 months from presentation, considered as valuable tools in disease management. This stage of treatment is still early enough to allow a change in treatment strategy hence excluding a significant number of patients being unnecessarily over treated under normal circumstances. On the other hand, there are patients who despite entering remission based on morphological examination, need more intensive treatment because they still have a substantial tumor burden [9]. It has been clearly demonstrated before that, higher the level of disease the greater the chance of relapse [10].

It is shown that the detection limit of cytogenetic techniques in MRD assessment is 10⁻² neoplastic cells and double-color immunofluorescence analysis goes up to 10⁻⁴ neoplastic cells [11], whereas sensitivity of PCR-based strategies reach the limit of 10⁻⁴-10⁻⁶ leukemic cells [12].

Yokota S. *et al.* [11] performed PCR analysis on bone marrow and peripheral blood samples during complete remission assessed according to conventional clinical and laboratory criteria. They showed that MRD was detectable in the bone marrow of all patients investigated 2 to 6 months after remission and in more than 50% of patients on maintenance therapy 7 to 19 months after diagnosis. Roberts *et al.* [12] used quantitative PCR assay in children with B-precursor ALL and found evidence of MRD in 15 out of 17 patients who remained in prolonged remission after the

completion of treatment. Cave H. *et al.* [13] performed a multi-center study to evaluate the clinical significance of MRD detection in childhood ALL. They concluded that the level of residual leukemia significantly related to the risk of early relapse. They also stated that in remission, detection of MRD by PCR acts as a powerful prognostic factor in childhood ALL. Goulden N.J. *et al.* [14] performed a retrospective analysis of MRD on 66 ALL children categorized under standard risk group. In this study PCR of IgH, TCR δ and TCR γ gene rearrangements and allele-specific oligoprobeing were employed. Those 33 patients who relapsed had significantly higher numbers of residual cells compared to those who remained in continuous clinical remission.

MRD detection also helps in monitoring patients with childhood ALL for evaluation of effectiveness of treatment specially if measured during the first 3 months of treatment. In this way patients with good prognosis could be differentiated from those with poor prognosis hence helps to decide whether and how to modify treatment [15].

Our obtained results through application of quantitative fluorescent IgH PCR showed that after induction therapy, although based on morphological criteria patients were entered into clinical remission, but still 53% had detectable MRD. Among MRD positive patients in clinical remission more than half of them had more than 10⁻³ residual blasts.

During the course of chemotherapy there was a steady fall in the number of patients with positive MRD but about 30% remained positive until the end of therapy. Relapse occurred in 11 patients which were among those who remained MRD positive in their third and fourth stages of treatment. Out of the 11 patients with relapse 63% had level of MRD higher than 10⁻³ leukemic cells.

Based on our results that majority of relapses occurred in patients with positive MRD at the 6th month of treatments, this time point could be selected to evaluate MRD for prediction of relapse. In this way not only the repeated bone marrow sampling which is

difficult, time consuming and painful to patients, is not necessary to perform but also the predictive value for relapse assessment and treatment outcome could be precisely performed.

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