RESEARCH ARTICLE

PCR-based clonality assessment in patients with lymphocytic leukaemias: a single-institution experience

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Abstract

PCR-based clonality testing can be performed in all lymphoproliferations by analysing gene rearrangements of antigen receptors, rearrangements that are unique for each kind of lymphocyte. Reactive lymphoproliferations have polyclonally rearranged Ig/TCR genes, whereas malignant proliferations (leukaemias and lymphomas) show clonal rearrangements. The aim of this study was to assess the clinical benefits of clonality testing with previously evaluated consensus primers in leukaemia patients. The study included peripheral blood and bone marrow samples of 67 leukaemia patients (32 B-CLL, 24 B-ALL and 11 T-ALL). Clonality testing was based on PCR amplification of rearranged Ig_H and TCR genes. During diagnosis, monoclonal pattern was found in all analysed B-CLL and T-ALL samples. Testing in B-ALL patients showed positive results in all bone marrow and one peripheral blood samples. Results of clonality testing in B-CLL patients during follow-up were concordant between peripheral blood and bone marrow. Obtained results corresponded to clinical course in all but one patient. In B-ALL group, results of molecular testing in peripheral blood and bone marrow confirmed remission estimated according to clinical criteria in all except one patient. Before any clinical sign of relapse, monoclonal pattern was found in six/seven patients by bone marrow and in three/seven patients by peripheral blood analysis, respectively. Results of molecular monitoring in T-ALL patients did not confirme clinical evaluation in two patients. Obtained results indicate high accuracy of re-evaluated primers for clonality assessment in ALL and CLL patients at the time of diagnosis. Results of clonality testing in B-ALL patients indicate that bone marrow analysis has higher sensitivity compared to analysis of peripheral blood.

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Introduction

According to published data, in 5%–10% of patients with suspect lymphoproliferative disorders, histo/cytomorphology supplemented with immunohistology or flow cytometric immunophenotyping cannot discriminate malignant and reactive processes. In such cases, diagnosis of lymphoid malignancies can be supported by PCR-based clonality testing (Van Dongen *et al*. 2003). The clinical utility of clonality analysis also includes detection of bone marrow infiltration as a part of staging procedure, detection of minimal residual disease following therapy and assessment of ability of purging technique to eradicate residual malignant cells in marrow/blood.

Clonality assesment can be performed in all lymphoproliferations by analysis of antigen-receptor gene rearrangements, which are unique for each kind of lymphocyte. The rearranging process in each immunoglobulin (Ig) or T-cell receptor (TCR) gene during early lymphoid differentiation joins variable (V), diversity (D) and joining (J) gene segments out of the many available segments. Further, nucleotides are deleted and/or randomly inserted at the joining sites, resulting in an enormous diversity of antigen receptors (Abbas and Lichtman 2003; Van Dongen *et al*. 2003). Therefore, reactive lymphoproliferations have polyclonally rearranged Ig/TCR genes, whereas malignant proliferations (leukaemias and lymphomas) show clonal rearrangements.

Previously, we have analysed peripheral blood samples of B-cell and T-cell non-Hodgkin's lymphomas (NHL), re-

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active lymphoproliferations (RL) and healthy persons to evaluate sensitivity and specificity of B-cell clonality testing by polymerase chain reaction (PCR). Using consensus primers, we amplified five different regions of rearranged immunoglobulin heavy chain (Ig_H) genes. All sets included a 3′ consensus primer complementary to J sequence inside framework region IV (FR IV), whereas the 5′ primer was different in each set. The highest sensitivity was obtained with primers amplifying sequences between FR III and FR IV regions i.e., monoclonal B-cell populations were found in 85% of B-NHL samples. Sensitivity of PCR method for B-cell clonality detection was 100% when all five pairs of primers were used at the same time (Cikota *et al*. 2000). Sensitivity of T-cell clonality testing was performed by PCR analysis of mycosis fungoides (MF) samples. Using eight different primers amplifying variable sequences of rearranged TCRγ gene, monoclonal pattern was found in 88% of MF skin samples (Kandolf Sekulović *et al*. 2007). Subsequently, these Ig_H and TCRγ primers were used in routine practice.

Here, we present the results of clonality testing in our institution, and comment on the usefulness and pitfalls of this method in leukaemia diagnostic and follow-up procedures, respectively.

Material and methods

Patients and samples

This study was approved by ethics committee of the Military Medical Academy, Belgrade. Informed consent was obtained from either patients or first degree relatives. A total of 67 leukaemia patients were included in the study: 32 B-cell chronic lymphocytic leukaemia (B-CLL), 24 B-cell acute lymphoblastic leukaemia (B-ALL) and 11 T-cell acute lymphoblastic leukaemia (T-ALL). The age of B-CLL patients ranged from 51 to 80 years (median 63), patients with B-ALL were aged 18–58 years (median 26), while T-ALL patients were 18–35 years (median 25) at diagnosis. All patients were diagnosed according to standard criteria and classification (Harris *et al*. 1999). Patients were treated by standard therapy procedures recommended for their disease and condition. All ALL patients were subjected to high-dose chemotherapy supported with stem-cell transplantation (SCT). Response to therapy was evaluated according to recommendations of National Cancer Institute (Cheson *et al*. 1996; Harris *et al*. 1999).

Peripheral blood (PB) and bone marrow (BM) samples were taken during diagnosis, after the therapy, and at up to 13 points during the follow-up period (2–5 years for B-CLL, 1–5 years for B-ALL, and 2–6 years for T-ALL). The follow-up included serial sampling after three or six month time intervals, depending on the course of disease.

DNA was extracted from PB/BM mononuclear cells by phenol:chlorophorm:isoamil alcohol extraction (Wright and Manos 1990), or by Blood Prep™ Chemistry for ABI PRISM™ 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, USA).

Assessment of B-cell clonality

For PCR amplification of the hypervariable region of Ig_H genes, the following oligonucleotide primers were used: FR III/FR IV: 5′-CTGTCGACACGGCCGTGTATTACTG-3′, 5′-AACTGCAGAGGAGACGGTGACC-3′. Touch down cycle (60°C ↓ 56°C for 1 min) was followed by 35 repeats of basic cycle (annealing step at 55°C for 1 min). PCR products were electrophoresed on 10% polyacrylamide gels (PAGs) after staining with silver nitrate (Sambrook *et al*. 1989). To avoid false-positive results, negative controls containing no template DNA were subjected to the same procedure. PCR products were considered to be monoclonal if only one discrete band within the expected size range of 100–150 bp was observed on the gel after electrophoresis (figure 1).

Assessment of T-cell clonality

To amplify the hypervariable region of rearranged TCRγ genes, the following oligonucleotide primers were used: 5′-CTTCACTCAGATGTCACCTACAACTCCAAGGTTG-3′; 5′-CTTCCTGAAGATGACGCCTCCACCGCAAGGGA-TG-3′; 5′-CTTCCTGGGAATGACTACCACACCTCCAGC-GGTT-3′; 5′-CTTCCTGATGGTAACTCCTACAACTCCAG GGTTG-3′; 5′-GGNACTGCAGGAAGGCAATGGCGCATT CCG-3′; 5′-AAGTGTTGTTCCTACGCCTTT-3′; 5′-AGTT ACTATTCTCCTAGTCCC-3′; 5′-TGTAATGATGGACTTT-GTTCC-3′.

The PCR protocol included 40 repeats of basic cycle (94°C for 40 s, 56°C for 1 min, 72°C for 1 min). The uniformity of rearranged TCRγ genes was analysed on 10% PAG after electrophoresis and silver nitrate staining. In parallel with the samples, negative controls containing no DNA were run. PCR products were considered to be monoclonal if only one discrete band within the expected size range (~200 bp) was observed on the gel after electrophoresis.

Results

Clonality analysis in B-CLL patients

The study included PB and/or BM samples of 32 B-CLL patients. B-cell clonality testing was performed: (i) only at diagnosis in 14/32 patients; (ii) at diagnosis and during the follow-up period in 8/32 patients; and (iii) only during follow-up in 10/32 patients (table 1).

During diagnosis, BM was analysed in 11 patients parallel with PB, while in the other 11 patients only PB was available. In all analysed BM and PB samples, monoclonal Ig_H rearrangements were found (table 2).

Samples taken during follow-up included PB and BM of 18 B-CLL patients. During follow-up period, monoclonal Ig_H rearrangements were detected in 15/18 patients (in one

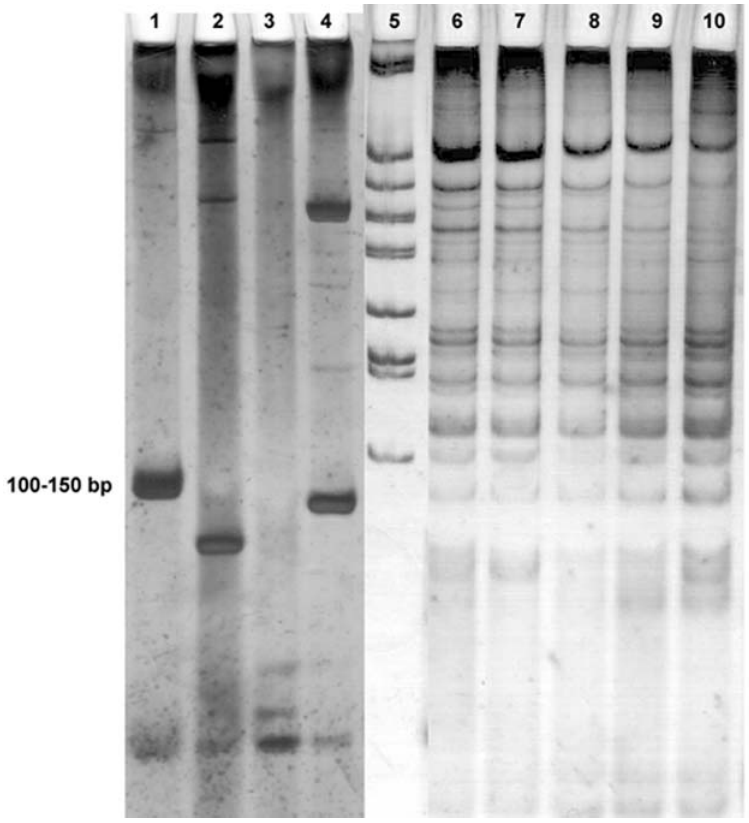

Figure 1. Analysis of B-cell clonality with FR III/FR IV PCR primers. Monoclonal pattern was found in lanes 1, 2 and 4. Polyclonal pattern was found in lanes 5–10.

Table 1. Sampling of peripheral blood and bone marrow in patients with leukaemia.

Disease	Diagnosis	Diagnosis and follow-up	Follow-up
B-CLL	14 (14 PB and 3 BM)	8 (PB and BM)	10 (PB and BM)
B-ALL	2 (PB and BM)	4 (PB and BM)	18 (PB and BM)
T-ALL		5 (PB and BM)	6 (PB and BM)

B-CLL, B-cell chronic lymphocytic leukaemia; B-ALL, B-cell acute lymphoblastic leukaemia; T-ALL, T-cell acute lymphoblastic leukaemia; PB, peripheral blood; BM, bone marrow.

Table 2. Analysis of B-cell and T-cell clonality in leukaemia patients at diagnosis.

	Monoclonal Ig_H rearrangements		Monoclonal TCR_γ rearrangements	
Diagnosis	PB	BM	PB	BM
B-CLL	22/22	11/11	–	–
B-ALL	5/6	6/6	–	–
T-ALL	–	–	5/5	5/5

B-CLL, B-cell chronic lymphocytic leukaemia; B-ALL, B-cell acute lymphoblastic leukaemia; T-ALL, T-cell acute lymphoblastic leukaemia; PB, peripheral blood; BM, bone marrow.

patient with therapy-resistant CLL, 13 patients who achieved partial clinical response and in one patient who achieved complete clinical response). Polyclonal Ig_H rearrangements were found in three patients who achieved complete clinical remission after the therapy. In all analysed patients the same results were obtained in both, PB and BM samples (table 3).

Clonality analysis in B-ALL patients

In the present study 24 B-ALL patients were included. B-cell clonality testing was performed: (i) only at diagnosis in 2/24 patients; (ii) at diagnosis and during the follow-up period in 4/24 patients; and (iii) only during follow-up in 18/24 patients. Both PB and BM samples were analysed at diagnosis and at every follow-up point for each B-ALL patient included in the study (table 1).

At the time of diagnosis, PB and BM samples were available for six patients. All of them had monoclonal Ig_H rearrangements in BM. However, when PB samples were analysed, five patients had monoclonal Ig_H rearrangements and one patient showed a polyclonal pattern (table 2).

Samples taken during follow-up included PB and BM of 22 B-ALL patients. In this group, seven patients relapsed. The presence of monoclonal Ig_H rearrangements were found in six patients: three of them had monoclonal lymphocytes in both BM and PB, while only three patients were positive in BM. In all six patients monoclonal Ig_H rearrangements (molecular relapse) was detected before any clinical sign of relapse. In PB and BM samples of one B-ALL patient polyclonal Ig_H rearrangements were found at relapse.

Another 15/22 patients remained in complete clinical remission after therapy. In one patient from this group monoclonal B-cell population was detected in BM. Despite this finding, the patient remains in complete clinical remission since May, when she was subjected to secondary autologous SCT due to relapse. The remaining 14 patients were in clinical and molecular remission (table 3).

Clonality analysis in T-ALL patients

This study also included 11 patients with T-ALL. T-cell clonality testing was performed in PB and BM samples: (i) at diagnosis and during the follow-up period (5/11 patients); and (ii) only during follow-up (6/11 patients) (table 1).

During diagnosis, in all analysed BM and PB samples monoclonal TCRγ rearrangements were found (table 2).

Analysis of PB and BM during follow-up period showed monoclonal TCRγ rearrangements in three patients (in one patient with therapy-resistant T-ALL, and in two patients who relapsed). In only one patient, monoclonal TCRγ rearrangement was detected before clinical signs of relapse. Further, in one patient with partial clinical response to therapy, polyclonal TCRγ rearrangements were found in PB and BM samples.

In the group of patients who remained in complete clinical remission, polyclonal TCRγ rearrangements were found in PB and BM samples in all follow-up points, excluding one patient with monoclonal T-cell population detected in BM and PB (table 3).

Discussion

One of the major requirements in clinical oncology is early and accurate diagnosis of cancer and monitoring of disease progression/response to therapy. In hematopathology, diagnosis and classification of lymphoproliferative disorders may be problematic for pathologists despite well-established morphological and immunophenotypic criteria. In these situations PCR-based clonality testing can provide valuable additional information.

Published data report many different PCR protocols and primers, each with different sensitivity and applicability. Lack of sufficient primers covering many of V-gene, D-gene and J-gene segments or improper primer annealing (most often as a consequence of somatic hypermutations in Ig genes) may result in false-negative pattern, whereas false-positive results are the most often consequence of contamination with a positive sample. The complexity of rearranging process, variability of Ig and TCR genes, and their susceptibility to

Table 3. Analysis of B-cell and T-cell clonality in leukaemia patients during follow-up period.

		Monoclonal Ig_H rearrangements		Monoclonal TCRγ rearrangements	
Diagnosis	Clinical outcome	PB	BM	PB	BM
B-ALL	CCR	0/15	1/15	–	–
	R++	3/7	6/7	–	–
T-ALL	CCR	–	–	1/7	1/7
	PR	–	–	0/1	0/1
	R+	–	–	2/2	2/2
	Th-resist	–	–	1/1	1/1
B-CLL	CCR	1/4	1/4	–	–
	PR	13/13	13/13	–	–
	Th-resist	1/1	1/1	–	–

B-CLL, B-cell chronic lymphocytic leukaemia; B-ALL, B-cell acute lymphoblastic leukaemia; T-ALL, T-cell acute lymphoblastic leukaemia; PB, peripheral blood; BM, bone marrow; CCR, complete clinical remission; R+, relapse; PR, partial clinical remission; Th-resist, therapy resistant leukaemia.

changes during the evolution of disease make design and evaluation of PCR primers a demanding process. Also, the test results should be interpreted with full knowledge of the immunobiology and hematopathology (Van Dongen *et al*. 2003; Van Krieken *et al*. 2007).

In this article, consensus primers for B-cell and T-cell clonality testing were re-evaluated by analysis of PB and BM samples taken at the time of leukaemia presentation and during the follow-up period.

Clonality testing during diagnosis

Using previously evaluated FR III/FR IV consensus primers for B-cell clonality testing, monoclonal pattern was found in all analysed PB and BM samples of B-CLL patients. Testing in B-ALL patients showed positive results in all BM and in 5/6 PB samples, respectively. Analysis of T-cell clonality by multiplex PCR showed monoclonal TCRγ rearrangements in all analysed PB and BM samples.

Obtained results indicate high accuracy of these primers for B-cell and T-cell clonality assessment in ALL and CLL patients at the time of diagnosis. Results of clonality testing in B-ALL patients indicate that BM analysis has higher sensitivity compared to analysis of PB. Also, leukaemic cells are not always uniformly distributed in the body. However, the possible explanation for PB-negativity in one B-ALL patient may be the presence of some PCR inhibitors in the sample, resulting in a false-negative result. To avoid this problem, some other gene from the genome (for example K-RAS or *β-actin*) should be amplified in parallel with Ig or TCR. In this case, it was not done because diagnosis of B-ALL was confirmed by BM-positivity.

Clonality testing during follow-up

Although many patients with lymphoid malignancies achieve a complete clinical remission (CCR) and even complete pathologic remission by standard morphologic and immunologic criteria, a relatively high proportion of them will ultimately relapse. Clearly, the source of relapse is from persistent malignant cells present at low levels, i.e. minimal residual disease (MRD). These low levels of disease can be detected only by sensitive molecular techniques (Braziel *et al*. 2003). Results of large prospective studies have clearly demonstrated the high prognostic value of MRD monitoring in ALL patients, particularly in children (Knechtli *et al*. 1998; Van Dongen *et al*. 1998; Coustan-Smith *et al*. 2000; Szczepański *et al*. 2001; Marshall *et al*. 2003). However, the treatment with novel polychemiotherapy protocols and SCT can induce CCR and MR in ALL and even B-CLL patients (Provan *et al*. 1996; Balduzzi *et al*. 2001; Piccaluga *et al*. 2004; Dreger *et al*. 2005). Clonality testing with consensus PCR primers after high dose chemotherapy and/or SCT is of limited sensitivity because reactive background lymphocytes obscure the clonal PCR products. PCR sensitivity can be increased up to 1000-fold by use of patient-specific primers, but this methodology is not practical outside the funded clinical trial settings. In this study, we related results of clonality testing by consensus primers to clinical course of ALL and CLL, respectively.

Results of clonality testing in B-CLL patients during follow-up were concordant between PB and BM. Also, obtained results correspond to clinical course in all but one patient who remains CCR despite persistence of MRD. Further monitoring will show whether the patient's immune system will be able to control/eradicate this low level of disease or whether he will relapse.

In B-ALL group, results of molecular testing in PB and BM confirmed remission estimated according to clinical criteria in all except one patient. Further, B-cell clonality testing showed monoclonal Ig_H rearrangements before any clinical sign of relapse in six/seven patients by BM analysis and in three/seven patients by analysis of PB. Despite no significant statistical difference between the sensitivity of PB and BM analyses (Fisher's exact test, $P = 0.363$), qualitative findings suggest that analysis of BM is more informative than analysis of PB for MRD and molecular relapse monitoring. We failed to detect monoclonal Ig_H rearrangements in PB and BM samples of one patient at relapse, probably because changes of Ig_H genes during the evolution of the malignant clone led to improper primer annealing.

Results of molecular monitoring in T-ALL patients confirmed clinical evaluation in all but two patients. Polyclonal TCRγ rearrangements were found in patient with partial clinical response to therapy. On the other hand, residual leukaemia was detected in one patient who remains CCR.

Detection of polyclonal Ig_H or TCRγ rearrangements at relapse and knowledge about accumulation of genetic exchanges during the evolution of malignant disease indicate that the detection of MRD should include some additional genetic markers. This approach requires broader screening at diagnosis, which can be performed by use of microarrays. Further, the presence of monoclonal Ig_H or TCRγ rearrangements can be a marker of a weakened and irrelevant clone that will die by apoptosis or it can be a source of relapse. Positive result of clonality testing indicates monitoring in shorter time intervals and quantification of MRD.

In contrast to lymphoma, diagnosis of leukaemia is not usually problematic. The adjunctive use of special histochemical stains, immunophenotyping and cytogenetic analysis can establish the diagnosis in virtually all leukaemia cases. However, in some patients making the diagnosis is more complicated and clonality testing can be valuable tool. Numerous examples confirmed necessity to integrate clonality testing in routine diagnostic and monitoring procedures for leukaemia patients. Laborious evaluation of PCR primers and methodologies in large collaborative studies resulted in recommendations for clonality testing and quantification of MRD (Gabert *et al*. 2003; Van der Velden *et al*. 2007; Flohr *et al*. 2008). The recommended analyses are reliable, but also very expensive. Considering reliability, duration and costs of

testing, our study indicates that internally evaluated methodology, based on the usage of consensus primers, gives satisfying results in a majority of leukaemia patients.

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