Bcl-2 GENE REARRANGEMENT DETERMINED BY PCR AS A MEAN TO DETECT MINIMAL RESIDUAL DISEASE IN MALIGNANT LYMPHOMAS

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Abstract

Objective: To develop a sensitive method to detect minimal residual disease and to elucidate the significance of bcl-2 gene rearrangement in diagnosis and treatment of malignant lymphoma. Methods: Using polymerase chain reaction (PCR) to detect bcl-2 gene rearrangement and using serial dilution method to define the sensitivity of PCR. Results: In 9 different malignant lymphoma cell lines, Su-DHL-4 and Su-DHL-6 were shown bcl-2(MBR)/JH rearrangement, the sensitivity of PCR was $1:10^5$. In 16 patients with follicular lymphoma, the peripheral blood and bone marrow were PCR positive in 4 cases both at initial diagnosis and after complete remission. Conclusion: Detection of bcl-2 gene rearrangement by PCR provides a sensitive and specific assay of minimal residual disease. It is helpful to improve staging of disease, prognosis and evaluation of the treatment results.

Key words: Lymphoma, Polymerase chain reaction, Bcl-2 gene, Gene rearrangement.

Minimal residual disease (MRD) plays a causative role in tumor recurrence and establishment of effective and sensitive assessment of MRD could be very useful for staging and evaluation of treatment of disease. In this study, we assessed the usefulness of bcl-2/JH PCR analysis on occult lymphoma cells in the peripheral and bone marrow of follicular lymphoma patients.

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MATERIALS AND METHODS

Patients and Cell Lines

Sixteen patients with follicular non-Hodgkin's lymphoma (F-NHL) including 11 males and 5 females were diagnosed by pathological and immunologic procedures. Age range of these patients was 23 to 65 years old. 9 kinds of malignant lymphoma cell lines[1] (Su-DHL-1, Su-DHL-4, Su-DHL-6, Su-DHL-8, Su-DHL-9, Su-DHL-10, Daudi, SB, 8392) all derived from B-cell lymphomas, and provided by ATCC.

Sample Preparation

Peripheral blood 5 ml and bone marrow samples 2 ml to be analyzed were collected from patients at diagnosis and after complete remission. The mononuclear cells were separated by Ficoll-Hypaque centrifugation and rinsed twice with PBS.

DNA Preparation

The genomic DNA was extracted by standard phenol/chloroform methods, precipitated in ethanol and resuspended in sterile TE buffer for storage at 4°C.

PCR Amplification

Primer Sequences[2]

Bcl-2 gene major breakpoint region primer (MBR):5'-TTAGA GAGTT GCTTT ACGTG-3'; bcl-2 gene minor cluster region primer (MCR): 5'-GACTC CTTTA CGTGC TGCTA CC-3'; Immunoglobulin heavy chain gene primer (JH): 5'-ACCTG AGGAG ACGGT GACCA GGGT-3'; The primers were synthesized by Cybersyn Company.

PCR Reaction

The PCR mixture contained 1 μg genomic DNA, 0.2 mmol/L of each of four deoxynucleotides, 25 pmol/L of each primer, 1.5 mmol/L of $MgCL_2$, 10 mmol/L Tris·HCL (pH 8.0), 50 mmol/L KCL, 0.1% Triton X-100 (v/v), 2U of Taq polymerase. The mixture were subjected to 45 cycles PCR amplification using Eppendorff Masterthermal cycler. Cycle conditions included a 94°C denaturation (45 seconds, first cycle 5 min), a 55°C annealing (45 seconds), a 72°C extension (60 seconds), after the final cycle, tubes were placed at the extension temperature for 5 min. As a control for preventing contamination, PCR reactions without genomic DNA were routinely included in each run.

Assay of PCR Production

A volume of 6 μl PCR products was added in 1.5% argrose gel containing 0.5 μg/ml EB, after electrophoresis at 5 V/cm for 30 min, the gel was placed under ultraviolet ray to analyze the results. The molecular marker was pBR322/Hinf I.

RESULTS

Bcl-2/JH Gene Rearrangement in Cell Lines

Two patterns of bcl-2/JH gene rearrangement, MBR/JH and MCR/JH, were respectively detected in 9 kinds of lymphoma cell lines, bcl-2 (MBR)/JH fusion gene is a predicted 230 bp fragment, bcl-2 (MCR)/JH fusion gene predicts 500 bp. The results showed that Su-DHL-4 and Su-DHL-6 presented 230 bp fragment and suggested that bcl-2 gene breakpoint occurred at MBR. Other cell lines had no MBR/JH rearrangement. None of cell lines demonstrated MCR/JH rearrangement.

The Sensitivity of PCR Amplification

The actual conditions of the PCR assay were first optimized, and the results suggested that the greatest amplification was obtained by 1.5 mmol/L Mg concentration of PCR buffer, and annealing temperature of 55°C, a 72°C extension temperature and 45 PCR cycles. In order to assess the sensitivity of PCR amplification, 1×10^7 cells from the Daudi cell line without bcl-2/JH rearrangement were admixed with serial 1:10 dilutions of cells from Su-DHL-6 cell line with bcl-2/JH rearrangement, DNA was then extracted and amplified by PCR. Figure 1 demonstrated that the PCR product can be seen at a dilution of $1:10^5$ (lane 6), and suggested that the sensitivity of this assay was $1:10^5$, that is to say, one tumor cell with bcl-2/JH rearrangement was detectable from 10^5 normal cells by PCR amplification.

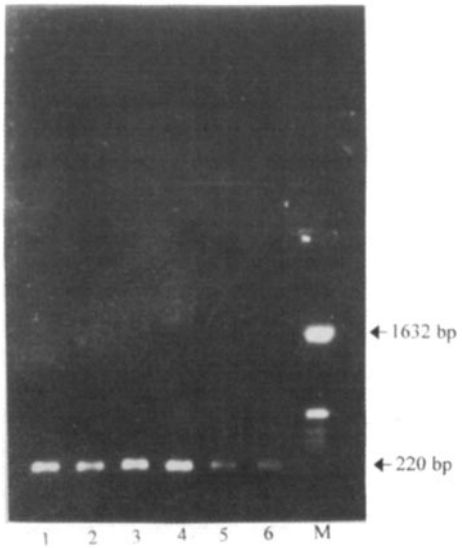

Fig. 1. The sensitivity of PCR amplification. M: Marker, lane 1 to lane 6 represent Su-DHL-6 in Daudi at dilutions of 1:1, 1:10, $1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$ respectively.

Detection of Occult Lymphoma Cells

The clinical features of 16 patients with follicular non-Hodgkin's lymphoma were summarized in Table 1. In 16 cases of untreated patients, 4 cases demonstrated PCR positive cells both in bone marrow and peripheral blood specimens including one patient with stage II disease, two with stage III, one with stage IV (Figure 2). Only one of 4 positive specimens was felt to be positive morphology. All of bcl-2 gene breakpoints occurred in MBR. When complete remission was obtained after chemotherapy with CHOP or CHOP-Bleo regimens in 4 PCR positive patients, occult lymphoma cells were still detectable both in bone marrow and peripheral blood specimens from all of these patients.

DISCUSSIONS

Patients with MRD run a substantial risk of tumor recurrence, and many different options can be chosen to monitor MRD.[3] However, usefulness of conventional morphological and DNA analysis assay (e.g. cytogenetics, Southern blotting, flow cytometry) is limited by bad sensitivity and technique constraint. On the other hand, due to high sensitivity and specificity, PCR technique has become an important approach to detect MRD.

The most frequent translocation in human lymphoma is the t(14;18) ($q^{32};q^{21}$), which juxtaposes a putative oncogene, bcl-2, from 18q21 with one of the six J segments of the immunoglobulin heavy chain

locus on chromosome 14.[4] On chromosome 18, approximately 60% bcl-2 gene breakpoints occur within the 150 bp major breakpoint region (MBR) at the untranslated region in the third exon of bcl-2 gene. In comparison, 30% of breakpoints occur at the minor cluster region (MCR), a second site located approximately 30 kb downstream from bcl-2 gene. The clustering of breakpoints at MBR and MCR has made it possible to use PCR amplification to identify cells containing bcl-2/JH gene rearrangement, and this tumor-specific molecular marker can be used to follow the natural history of lymphoma and search for MRD. In present study, the sensitivity of detecting occult cells with bcl-2/JH rearrangement by PCR technique was $1:10^5$ determined by serial dilution and showed more than 1000 times better than that of light microscopy (1:100), cytogenetics (5:100), and Southern blotting (1:100). The result showed that detection of bcl-2/JH gene rearrangement by PCR was a sensitive approach and could be used in clinical detection of MRD in lymphoma patient

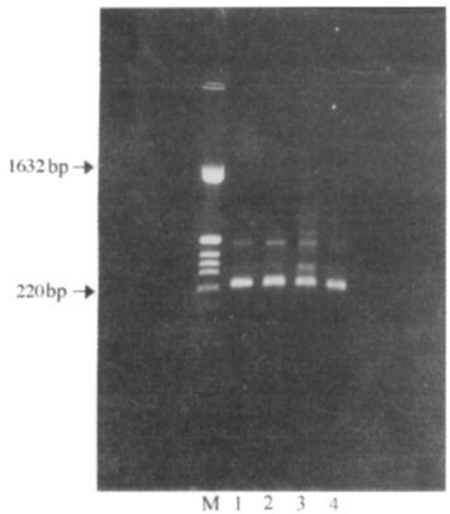

Fig. 2. Bcl-2 rearrangement in BM and PB samples of F-NHL. M: Marker, lane 1 to lane 4 demonstrated MBR/JH rearrangement.

Table 1. The clinical features and the PCR results of 16 patients of F-NHL

No.	Sex	Age	Stage	Pathological subtype	Bone marrow involvement	PCR results	
						MBR	MCR
1	F	23	I	SC	–	–	–
2	M	38	II	SC	–	+	–
3	M	46	II	M	–	–	–
4	M	52	IV	LL	+	–	–
5	F	39	III	SC	–	+	–
6	M	56	II	M	–	–	–
7	M	65	I	M	–	–	–
8	M	63	II	SL	–	–	–
9	F	48	IV	SC	+	–	–
10	F	50	IV	LC	–	–	–
11	M	64	III	LC	–	+	–
12	M	61	IV	SC	+	–	–
13	M	56	II	M	–	–	–
14	F	42	IV	SC	+	+	–
15	M	51	IV	M	–	–	–
16	M	64	III	SC	–	–	–

F: female; M: male; SC: small cleaved cell subtype; M: mixed cell subtype; LC: large cleaved cell subtype.

Bone marrow is the most common site of lymphoma involvement. The evidences of previous and present studies found that the bone marrow, even in stage I and II patients, frequently contained lymphoma cells when tested by PCR, and suggested that the bone marrow involvement in NHL might occur at onset or during the course of the disease.[5,6] These can result in upstaging many patients. Some oncologist thought that those patients with PCR positive tumor cells should be in stage IV.[6] Our study also showed that occult cells with bcl-2/JH rearrangement were still detectable by PCR in bone marrow and peripheral blood after complete remission, and suggested that aggressive chemotherapy often fails to eradicate bcl-2/JH positive cells from bone marrow even when this become morphologically negative. This results is in agreement with observations by Gribben et al.[7] and Yuan et al.[8] Although PCR-detectable lymphoma cells could persist in some patients with long CR duration, persistence of MRD is

a harbinger of eventual relapse. Gribben et al. analyzed a total of 542 bone marrow samples obtained from NHL patients in CR, and found that disease-free survival was markedly increased in patients with no PCR-detectable lymphoma cells in the marrow compared with those in whom residual cells were detected ($P<0.00001$), and the presence of detectable lymphoma cells was associated with a 48 fold increase in the risk of relapse after BMT. Therefore, prolonged follow-up is essential for PCR positive patients.

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