Detection of Clone-Specific Immunoglobulin Heavy Chain Genes in the Bone Marrow of B-cell-Lineage Lymphoma after Treatment

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Division of Molecular Diagnostics, ¹Rheumatology and Hematology, Department of Clinical Medicine, Tohoku University, School of Medicine, ²Department of Pathology, Tohoku University, School of Dental Medicine, Sendai 980-8574, and ³National Sendai Hospital, Sendai 983-8520

HOSHINO, A., FUNATO, T., MUNAKATA, Y., ISHII, T., ABE, S., ISHIZAWA, K., ICHINOHASAMA, R., KAMEOKA, J., MEGURO, K. and SASAKI, T. Detection of Clone-Specific Immunoglobulin Heavy Chain Genes in the Bone Marrow of B-cell-Lineage Lymphoma after Treatment. Tohoku J. Exp. Med., 2004, 203 (3), 155-164 — In order to determine the appropriate treatment of malignant lymphoma, it is important to know the degree to which extra-nodal invasion of lymphoma cells has occurred. We amplified complementarity-determining region (CDR) III genes in 64% of lymph node samples at the onset or relapse of B-cell-lineage non-Hodgkin’s lymphoma (NHL) in 22 patients. By using a clone-specific CDR III probe in each patient, we were able to detect minimal residual disease (MRD) of lymphoma cells in the bone marrow and/or blood in 9 out of 14 cases (64.2%) at the onset of the disease or relapse, whereas abnormal cells in the bone marrow and/or blood were identified by routine morphological analysis in only 4 out of 22 cases (18.2%). This indicates that extranodal invasion of malignant cells may be common in patients with NHL. In some cases, the clone-specific CDR III gene was still expressed in the samples of bone marrow and/or peripheral blood even after chemotherapy, when other markers associated with NHL were no longer expressed. Five out of six cases in this group had a worse outcome associated with NHL. On the other hand, most of the cases whose clone-specific CDR III gene was no longer expressed in the bone marrow and/or in circulation after treatment had a relatively fair prognosis. These results indicate that the detection at molecular level of MRD in extranodal organs may prove useful as a predictor of prognosis for NHL. ——— non-Hodgkin’s lymphoma
Although the treatment currently available for non-Hodgkin’s lymphoma (NHL) can induce clinical remission in the vast majority of patients, most will suffer a relapse (Haioun et al. 1998; Winter 1999) because of the persistence of residual lymphoma cells, which are undetectable using conventional diagnostic procedures (Corradini et al. 1999). An important initiative in the diagnosis and treatment of NHL has been the early detection of minimal residual disease (MRD) (Drexler et al. 1995). If the existence of MRD, which carries with it an increased risk of relapse, is detected reliably during apparent clinical remission, alternative strategies could be applied early while the malignant cell burden is still minimal. In this respect, it is important to determine the involvement of the bone marrow and peripheral blood in NHL. This has often been hampered because of the poor sensitivity of detection by morphological or Southern blot analysis (Okabe et al. 1987), which represents the gold standard methods for clonality assessment. Polymerase chain reaction (PCR) methods are clearly more sensitive than other methods, allowing immunophenotyping and cytogenetic analysis (Bagg and Kallakury 1999). In particular, clonality detected by PCR-based methods may be helpful for establishing the diagnosis of lymphomas (Medeiros and Hernandez 1999), as shown by the detection of the translocation gene at follicular lymphomas (Lopez-Gillermo et al. 1999).

The rearrangement of the variable (\( V_{H} \)), diversity (\( D_{H} \)), and joining (\( J_{H} \)) segments of the IgH gene generates unique DNA sequences that are specific to each B-cell clone (Provan et al. 1996), and, therefore the third complementarity-determining region (CDR III) genes, which are composed of \( V_{H} \text{-N-} D_{H} \text{-N-} J_{H} \) segments, have been applied as clone-specific probes. Accordingly, the PCR amplification of CDR III sequences of IgH genes may be applied widely to detect B-cell-type malignancies, especially NHL (Wu et al. 1997; Donovan et al. 2000; Verhagen et al. 2000; Uchiyama et al. 2003).

In the present study, we investigated the invasion of small numbers of lymphoma cells in extranodal organs to assess MRD in lymphoma. The IgH of specific CDR III region sequences in each patient was examined by PCR amplification as a marker of MRD in NHL. We then used PCR-based methods to determine the degree of involvement of the bone marrow and peripheral blood in B-cell-lineage NHL at diagnosis and after treatment.

**Materials and Methods**

**Patients**

Twenty-two patients (14 males, 8 females) with B-cell-lineage NHL were studied. Of these, 18 were at the primary diagnosis stage, and 4 were at relapse (patients 2, 14, 15, and 22). The median age of the subjects was 58 years (range 33-78) and the median follow-up duration was 18.4 months (range 1-42). Diagnosis of malignant lymphoma was achieved by morphological analysis according to the World Health Organization Classification (Jaffe et al. 2001). Immunophenotypic analysis of B-cell lineage was assessed by flow cytometry (Coulter, UK). All patients were clinically and pathologically staged according to the Ann-Arbor system (Carbone et al. 1986). Bone marrow and peripheral smears were reviewed to confirm the diagnosis and the achievement of remission, which was confirmed by the presence of <5% lymphoma cells in the postinduction marrow. Combination chemotherapy regimens for patients contained doxorubicin. Study protocols were approved by the Committee for the Ethical Issues on Human Genome and Gene Analysis at Tohoku University. All patients
gave their written informed consent for the analysis of their clone-specific immunoglobulin genes.

**DNA extraction**

Genomic DNA from the tissues samples was extracted using conventional methods based on phenol-chloroform-ethanol precipitation (Sambrook et al. 1989) from primary lesions (i.e., the lymph nodes or skin). Mononuclear cells were separated by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation of the bone marrow and peripheral blood was analyzed for detection of lymphoma cells. Genomic DNA from the blood was extracted using a DNA extractor WB kit (Wako, Osaka). In some cases, genomic DNA from the fixed and mounted bone marrow smears was extracted as described previously (Brisco et al. 1993).

**PCR amplification**

We used four sets of primer sequences (comprising V<sub>H</sub> consensus, V<sub>H</sub> family specific, and J<sub>H</sub> consensus) for the PCR amplification of monoclonal rearranged IgH genes. For this all samples from primary lesions were analyzed using the first primer set (V<sub>H</sub>con/J<sub>H</sub>con2). Samples negative for monoclonality were analyzed further until a positive result was observed with the second primer set (V<sub>H</sub>con/J<sub>H</sub>con1), the third primer set (V<sub>H</sub> family specific/J<sub>H</sub>con2), and the fourth semi-nested primer set (V<sub>H</sub>con/J<sub>H</sub>con2-V<sub>H</sub>con/J<sub>H</sub>con1). DNA amplification was performed in an automated thermocycler (GeneAmp PCR system; Perkin-Elmer, CT, USA). One microgram of genomic DNA from a primary lesion was amplified in a 100-μl reaction volume containing 1x PCR buffer (10 mmol/liter Tris-HCl, pH 8.3, 50 mmol/liter KCl, 1.5 mmol/liter MgCl<sub>2</sub>), 200 μmol/liter each deoxynucleotide triphosphate, 0.3 μmol/liter of each primer, and 2.5 U of Taq polymerase (Takara, Tokyo). Reactions with the first primer set (V<sub>H</sub>con/J<sub>H</sub>con2) consisted of 30 cycles of denaturation at 94°C for 90 seconds, annealing at 58°C for 90 seconds, and extension at 72°C for 90 seconds. Reactions with the second (V<sub>H</sub>con/J<sub>H</sub>con1) and the third (V<sub>H</sub> family specific/J<sub>H</sub>con2) primer sets consisted of 30 cycles at 94°C for 90 seconds, 60°C for 90 seconds, and 72°C for 90 seconds. Reactions with the fourth semi-nested primer set (V<sub>H</sub>con/J<sub>H</sub>con2-V<sub>H</sub>con/J<sub>H</sub>con1) consisted of two rounds of PCR cycles. The first one for 1 μg genomic DNA comprised 30 cycles at 94°C for 90 seconds, 58°C for 90 seconds, and 72°C for 90 seconds, and the second one with 2 μl of the first-round product as a template was 20 cycles at 94°C for 90 seconds, 60°C for 90 seconds, and 72°C for 90 seconds. Forty microliters of each PCR product was subjected to electrophoresis on 5% polyacrylamide gels in Tris-borate/EDTA buffer (89 mmol/liter Tris-borate, 2 mmol/liter EDTA), and visualized subsequently with the aid of ethidium bromide staining and ultraviolet illumination.

**Determination of monoclonality**

A PCR product was defined as monoclonal when one or two discrete bands were observed within the size range appropriate for each primer set and there were at least two identical sequences in the sequencing of each PCR product. The size range of the first, second, and fourth primer sets was 70-150 base pairs (bp), as described previously (Yamada et al. 1989; Wan et al. 1992; Lombardo et al. 1996; Zwicky et al. 1996). That of the third primer set was 300-500 bp as described previously (Berman et al. 1988; Deane and Nortan 1990). The PCR product was isolated from polyacrylamide gels by the “crush and soak” method (Sambrook et al. 1989) when it was considered monoclonal.

**Sequencing of PCR products**

Isolated PCR products were cloned into the pGEM-T vector (Promega, WI, USA). Mini-preparation of plasmid DNA was obtained with the alkaline lysis method (Sambrook et al. 1989). Five to ten micrograms of double-stranded plasmid was alkali-denatured, reannealed with 2-4 pmol of fluorescent M13 forward or M13 reverse primers, and used for dideoxy sequencing with T7
DNA polymerase (AutoRead Sequencing Kit; Pharmacia). For each PCR product, both strands of at least 10 clones were sequenced with an automatic laser fluorescence detection DNA sequencer (ALF DNA sequencer, Pharmacia).

**Determination of lymphoma-specific PCR primers and detection probes**

Sequencing was analyzed using Genetyx software (Software Development, Tokyo, Japan), and the V\textsubscript{H}, D\textsubscript{H}, and J\textsubscript{H} regions were identified using data that have been described previously (Siebenlist et al. 1981; Matsuda et al. 1990; Provan et al. 1996) and Genome Net (Kyoto database, Kyoto). A lymphoma-specific PCR primer was determined from CDR III or the V\textsubscript{H}-N-D\textsubscript{H} region of each patient’s monoclonal IgH genes. A detection probe was determined from the D\textsubscript{H}-N-J\textsubscript{H} region. Both were designed to be about 20 bp. The detection probe was 5′ end-labeled with biotin.

**Amplification of lymphoma-specific CDR III regions**

A lymphoma-specific CDR III region was amplified by PCR of the extracted DNA of each patient’s bone marrow or peripheral blood samples (Fig. 1). One microgram of extracted DNA was amplified in a 100-μl reaction volume containing the same buffer as described above. The sense primer was a lymphoma-specific PCR primer synthesized as described above and on J\textsubscript{H} con1 or J\textsubscript{H} con2 and was used for the amplification of each monoclonal IgH gene. PCR for the amplification of a lymphoma-specific CDR III region consisted of 30 cycles of denaturation at 94°C for 90 seconds, annealing at an appropriate temperature for each specific primer for 90 seconds, and extension at 72°C for 90 seconds. In two patients (patients 2 and 21), semi-nested PCR amplification was used with each primer set (a specific primer/J\textsubscript{H} con2-a specific primer/J\textsubscript{H} con1) because the fourth semi-nested PCR primer set was used for the detection of monoclonality. A

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**Fig. 1.** The regime used for molecular analysis.
diluted series of genomic DNA from a primary lesion was amplified as a positive control. DNA from normal mononuclear cells and a sample without template DNA were amplified simultaneously as negative controls.

**Detection of a lymphoma-specific CDR III region**

Fifty microliters of PCR products with lymphoma-specific primers were electrophoresed in 1.5% agarose gels in Tris-acetate/EDTA buffer (40 mmol/liter Tris-acetate, 2 mmol/liter EDTA). Gels were denatured in 0.5 mol/liter NaOH, 1.5 mol/liter NaCl for 60 min and neutralized in 1.0 mol/liter Tris-HCl, pH7.5, 1.5 mol/liter NaCl for 60 minutes. The PCR products were transferred to nylon membrane Immobilon-S (Millipore, Tokyo) using transfer buffer (1.5 mol/liter NaCl, 0.15 mol/liter sodium citrate). DNA was fixed to the membrane by ultraviolet irradiation. Hybridization was carried out in hybridization buffer (0.9 mol/l NaCl, 0.09 mol/l sodium citrate, 0.1% sodium dodecylsulfate, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 100 mg/liter denatured fish sperm DNA) and 10 mmol/liter biotin-labeled probe at an appropriate temperature for the probe. Signals were detected using the NEBlot Phototope Kit (New England Biolabs, Beverly, MA, USA) according to a chemiluminescent reaction under conditions recommended by the manufacturer.

**Statistical analysis**

Comparisons between groups of patients were performed using the Mann Whitney’s U-test. The level of statistical significance was set at \( p \leq 0.05 \).

**RESULTS**

The results at diagnosis in patients with B-cell-lineage NHL are summarized in Table 1. Diffuse large B-cell lymphoma was the main pathological subtype (17/22, 77%) encountered in this study. There were four cases at stage I, five at stage II, five at stage III, four at stage IV, and four at relapse. Amplification of the monoclonal IgH gene from lymph nodes was possible in 14 out of the 22 patients with lymphoma (64%).

The nucleotide sequences of PCR products reactive with clone-specific CDR III probe were identical to those from lymph nodes (data not shown). Invasion of lymphoma cells to the bone marrow and/or circulation was found morphologically in 4 out of 22 patients (18.2%), but PCR analysis revealed amplification of the clone-specific CDR III gene in bone marrow and/or peripheral blood samples from 13 out of 14 patients (92.9%), irrespective of their histological features and stage. These results were reproducible.

In order to establish whether detection of the clone-specific CDR III gene can be used as a predictor of the clinical course of lymphoma, we studied the prevalence of the gene in bone marrow or in peripheral blood following treatment. However, in this study, the prognosis of patients at either diagnosis or relapse did not correlate with molecular detection of lymphoma cells in the bone marrow or peripheral blood samples (Table 1). We examined the bone marrow for MRD in 13 samples from 11 patients soon after the treatment, and analyzed the relationship between the presence of clone-specific CDR III gene and their final prognosis. The results could be divided into two groups in terms of the persistence of clone-specific CDR III gene expression after treatment. Only one patient in group A, where the gene was still amplified in lymphoma cells in the bone marrow and/or peripheral blood even after treatment (Table 1), showed complete remission, as determined by computed tomography, galium scintigraphy or other tests, but the remainder were in partial remission, irrespective of the stage. Five patients (83%) in this group suffered an episode of relapse within 9 months after treatment, and three had a fatal outcome because of malignant lymphoma. On the other hand, patients in group B (Table 1), where molecular detection of lymphoma cells was negative after treatment, had relatively a fair outcome. All were in complete remission soon after the treatment. One (patient 4)
### Table 1. Detection at initial evaluation

<table>
<thead>
<tr>
<th>No.</th>
<th>Histology</th>
<th>Age</th>
<th>Sex</th>
<th>Stage</th>
<th>LN</th>
<th>BM</th>
<th>PB</th>
<th>Histological exam.</th>
<th>Primers for amplification of monoclonal lgH genes</th>
<th>PCR analysis</th>
<th>Treatment</th>
<th>Response</th>
<th>Outcome</th>
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<tr>
<td>1</td>
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<td>70</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>VHcon/JHcon2</td>
<td>+     +</td>
<td>CHOP</td>
<td>CR</td>
<td>CR 3M</td>
</tr>
<tr>
<td>2</td>
<td>follicular</td>
<td>34</td>
<td>M</td>
<td>relapse</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
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<td>+     +</td>
<td>HD AraC</td>
<td>PR</td>
<td>dead 42M</td>
</tr>
<tr>
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<td>43</td>
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<td>II</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
<td>(V/J)2</td>
<td>NA    NA</td>
<td>CHOP</td>
<td>CR</td>
<td>2nd CR 34M (relapse 17M)</td>
</tr>
<tr>
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<td>III</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
<td>VH1/JHcon2</td>
<td>+     +</td>
<td>CHOP</td>
<td>CR</td>
<td>dead 4M (hepatitis)</td>
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<td>5</td>
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<td>-</td>
<td>-</td>
<td></td>
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<td>Tumorectomy</td>
<td>CR</td>
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<td>III</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
<td>VH1/JHcon2</td>
<td>+     +</td>
<td>CHOP</td>
<td>PR</td>
<td>dead 12M</td>
</tr>
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<td>75</td>
<td>M</td>
<td>II</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
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<td>+     -</td>
<td>RT</td>
<td>CR</td>
<td>CR 3M</td>
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<td>33</td>
<td>M</td>
<td>I</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
<td>VHcon/JHcon2</td>
<td>+     +</td>
<td>RT+M-VEPA</td>
<td>CR</td>
<td>2nd CR 40M (relapse 21M)</td>
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<tr>
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<td>63</td>
<td>M</td>
<td>IV</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>VH3/JHcon2</td>
<td>+     +</td>
<td>CAMBO-VIP</td>
<td>PR</td>
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</tr>
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<td>+</td>
<td>+</td>
<td></td>
<td>VH3/JHcon2</td>
<td>+     +</td>
<td>THP-COPE</td>
<td>CR</td>
<td>2nd CR 33M (relapse 22M)</td>
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<tr>
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<td>63</td>
<td>F</td>
<td>IV</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
<td>VH3/JHcon2</td>
<td>+     +</td>
<td>RT-CHOP</td>
<td>PR</td>
<td>dead 17M</td>
</tr>
<tr>
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<td>F</td>
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<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
<td>VHcon/JHcon2</td>
<td>+     +</td>
<td>CHOP</td>
<td>CR</td>
<td>dead 3M (relapse 2M)</td>
</tr>
<tr>
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<td>-</td>
<td>-</td>
<td></td>
<td>VH3/JHcon2</td>
<td>+     +</td>
<td>RT-CHOP</td>
<td>CR</td>
<td>2nd CR 21M (relapse 12M)</td>
</tr>
<tr>
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<td>relapse</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>VHcon/JHcon2</td>
<td>+     +</td>
<td>ESHAP</td>
<td>PR</td>
<td>dead 3M (relapse 2M)</td>
</tr>
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<td>-</td>
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<td>(V/J)2</td>
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<td>RT+THP-COPE</td>
<td>CR</td>
<td>dead 22M (relapse 19M)</td>
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<td>-</td>
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<td>CHOP</td>
<td>PR</td>
<td>dead 12M</td>
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<td>-</td>
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<td>CHOP</td>
<td>CR</td>
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<td>CR</td>
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<td>-</td>
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<td>THP-COPE</td>
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<td>-</td>
<td>-</td>
<td></td>
<td>VHcon/JHcon2</td>
<td>+     +</td>
<td>CHOP</td>
<td>CR</td>
<td>dead 15M (relapse 9M)</td>
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</table>

did not have any signs associated with malignant lymphoma, but died 4 months after treatment due to fulminant hepatitis, probably as a result of infection. Relapse occurred in two patients (patients 8 and 10) at 20 months after treatment. The remaining four patients have been in a complete remission for at least 14-36 months.

**DISCUSSION**

We amplified lymphoma-specific CDR III DNA in 64% of the samples from patients with B-cell-lineage NHL. The amplification was lymphoma-specific, as established by the presence of a single band at electrophoresis using polyacrylamide gel, and, where studied, the nucleotide sequences were identical for all clones. The frequencies for IgH amplification are similar to those reported in the literature (Straka et al. 1994; Wu et al. 1997; Corradine et al. 1999). The reasons why one-third of the samples were negative for IgH amplification may be as follows: (1) primer mismatches within the V and J target regions, (2) partial deletion of 5’-primer target nucleotides in the V and J regions due to junctional diversity, (3) chromosomal translocations involving the IgH gene segments, and (4) incomplete rearrangement (V-D or D-J), which can be detected by Southern blotting but not by PCR. In fact, patients 3 and 18, in whom we failed to amplify specific CDR III genes, had t (14;18), and patient 16 had complicated chromosomal changes involving chromosome 14.

Molecular analysis has been used to detect MRD in acute lymphoblastic leukaemia (Felix et al. 1994) or chronic lymphocytic leukaemia (Dreger et al. 1998). PCR amplification of the breakpoint of bcl-2 was useful for establishing the involvement of bone marrow in patients with follicular lymphoma (Lopez-Gillermo et al. 1999). The amplification of the clone-specific IgH region has also been applied as a molecular marker not only for the diagnosis, but also for the detection of MRD in B-cell-lineage lymphomas (Wu et al. 1997). None of patients with follicular and mantle cell NHL who received PCR-negative stem cell fractions relapsed during the study. On the other hand, persistence of the clone-specific IgH gene was associated with relapse in NHL after autologous transplantation of peripheral blood stem cells (Hardingham et al. 1995; Zwicky et al. 1996).

The clone-specific CDR III PCR used in the present study is sensitive enough to detect MRD in B-cell lymphoma, as shown by positive amplification in one cell among $10^5$ cells (data not shown). We found that lymphoma cells had invaded the bone marrow and/or blood circulation in 9 out of 14 cases (64.2%) with malignant lymphoma at diagnosis or relapse of the disease, whereas abnormal cells were found pathologically in only 4 cases (28.6%) from the same population. A MRD using a CDR III amplification was found in various categories of B-cell lymphoma irrespective of their histological findings and clinical stages. This indicates that an invasion of lymphoma cells into extranodal organs may be common in B-cell lymphoma at their onset or relapse, in contrast with previous reports of microscope observations (Foucar et al. 1982; Bennet et al. 1986; Mckenna and Hernandez 1988; Conlan et al. 1990; Schwozen et al. 1992). It was difficult, however, to determine the correlation between the prevalence of abnormal cells in bone marrows at diagnosis or the relapse, and the prognosis, because most cases were positive for CDRIII-specific amplification in extranodal organs. Recently, by using fluorogenically labeled probes in real-time quantitative PCR assay, a clonal $V_{H}$ has been detected 70.8% of B-NHL cases, and the most frequent $V_{H}$ gene usage being $V_{H}3$ and $V_{H}4$ (88.2%) (Uchiyama et al. 2003). An important finding of present study is that CDR III amplification in the samples of bone marrow persisted even after treatment, when the patients were clinically assessed as being in remission (i.e., according to their hematological and x-ray findings). This also prompted us to evaluate the value of molecular detection of lymphoma cells in extranodal organs with regard to prognosis. The results revealed that the persistence of clone-specific CDR-III
genes in extranodal organs soon after treatment may indicate that the therapy had not been completely successful, because the patients appeared to be in the early stages of lymphoma relapse, and had a poor prognosis associated. This is supported by the observation that all patients with negative amplification in the bone marrow and peripheral blood samples entered complete remission after treatment, and half of them remained in a clinically well state at least during the study period. The remaining patients, however, relapsed 14-25 months later. This indicates the necessity of frequent follow-up evaluation of molecular involvement in the extra-nodal organs, even if patients appear to be in a clinically good condition.

Although the number of cases studied here was small, the data indicate that assessment of persistent molecular involvement in the extra-nodal organs may be useful for evaluating the efficacy of therapy. Further studies are needed to establish the true value of such molecular analysis as a predictor of treatment efficacy in individuals with malignant lymphoma.

References


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