Technical Advance

Establishment of a Novel B Cell Clonality Analysis Using Single-Strand Conformation Polymorphism of Immunoglobulin Light Chain Messenger Signals

Satoshi Shiokawa,* Junji Nishimura,[†]
Kouichi Ohshima,[‡] Naokuni Uike,[§] and
Kazuhiko Yamamoto*

From the Department of Clinical Immunology, Medical Institute of Bioregulation, Kyushu University, Beppu, Oita, the Third Department of Internal Medicine,[†] Faculty of Medicine, Kyushu University, the Department of Pathology,[‡] School of Medicine, Fukuoka University, and the Department of Hematology,[§] National Kyushu Cancer Center Hospital, Fukuoka, Fukuoka, Japan*

The remarkable diversity of the complementarity determining region (CDR) 3 of the immunoglobulin (Ig) heavy (H) chain gene rearrangements has been exploited to identify the clonal populations of B cells in B cell malignancies. However, when B cell malignancies of different categories were examined, the overall detection rate was found to be approximately 70%. The development of a simple clonality analysis using Ig light (L) chain CDR3 diversity has been hampered due to the sparseness of knowledge regarding the sequence of V κ and V λ gene segments and the restriction of L chain CDR3 length. Based on the recently reported V κ and V λ gene sequences, we designed V κ and V λ framework 3 consensus primers. We combined the reverse transcriptase polymerase chain reaction (RT-PCR) of IgL chain transcripts with a single-strand conformation polymorphism (SSCP) analysis and then analyzed samples from patients with B cell malignancies. Clonal B cell populations were detected as discrete bands, and identical clones showed a similar mobility in a RT-PCR SSCP analysis. This method was thus found to be a useful supplement to the previously described approach of VH gene amplification for detecting clonal B cell populations. By using SSCP, we were able to determine the clonal identities of B cell expansion in different samples. (*Am J Pathol* 1998, 153:1393-1400)

joining of a DH to a JH gene segment, followed by a rearrangement between the VH gene segment and the intermediate DH-JH join.^{1,2} The productive rearrangement of a κ or λ light (L) chain leads to the surface expression of naive IgM, which thus subjects the B cells to positive and negative selection based on the specificity of its mature antigen receptor.³ Although such rearrangement stops after the loss of recombinase activity in the bone marrow, additional changes in the variable domain gene occur during the immune response.⁴

Antibodies consist of two H chains and two L chains (either κ or λ). Each Ig variable domain contains three intervals of sequence hypervariability termed complementarity determining regions (CDRs) that are separated from each other by four intervals of a relatively constant sequence termed frameworks (FRs).⁵ The juxtaposed CDR domains create an antigen-binding surface. CDR1 and -2 form the outside border of the binding site and are completely encoded by their respective V gene segments. The CDR3 domains are generated by V-(DH)-J joining and are found in the center of the antigen-binding site. The central location of CDR3 in the antigen-binding site attests to the critical role that the sequence and structure of this region plays in defining antigen specificity.⁶

Over the course of the past 15 years, the human VH segments,⁷ DH segments,⁸ JH segments,⁹ V κ segments,¹⁰ J κ segments,¹¹ and J λ segments¹² have been completely mapped and sequenced. However, the map of the V λ locus and the sequences of the germline V λ segments have only recently been determined.^{13,14}

The human κ locus contains 76 V κ genes, of which ~40 are potentially functional based on the sequence of their coding and regulatory regions.¹⁰ Random combinations of these germline V κ gene segments with one of the five J κ gene segments can produce a large array of different V κ L chain rearrangements. The N region nucle-

Supported in part by a grant from Uehara Memorial Foundation.

Accepted for publication August 6, 1998.

Address reprint requests to Dr. Satoshi Shiokawa, Department of Clinical Immunology, Medical Institute of Bioregulation, Kyushu University, Tsurumihara 4546, Beppu, Oita 874-0838, Japan.

In progenitor B cells, the assembly of the immunoglobulin (Ig) heavy (H) chain variable domain begins with the

otides are added to the exposed termini of the rearranging gene segments through the activity of terminal deoxynucleotidyl transferase (TdT).¹⁵ The expression of TdT is thought to be restricted to the pro-B cell stage, when the H chain rearrangement occurs. The TdT protein is not detectable in the cytoplasmic $C\mu^+$ pre-B cells, the stage at which the IgL chain gene segments typically undergo rearrangement. However, there is substantial evidence that N region addition occurs in $V\kappa$ - $J\kappa$ joins derived from B cells of normal individuals.¹⁶ Despite the presence of N region addition, a strict CDR3 length, usually either 9 or 10 amino acids, is maintained in productive rearrangements.

There are 37 $V\lambda$ segments with open reading frames, of which ~30 are expressed, depending on the haplotype.¹⁴ The λ locus contains seven $C\lambda$ gene segments, each preceded by a $J\lambda$ gene segment. The $J\lambda 1$ - $C\lambda 1$, $J\lambda 2$ - $C\lambda 2$, $J\lambda 3$ - $C\lambda 3$, and $J\lambda 7$ - $C\lambda 7$ regions are functional and code for the four distinct Ig λ isotypes, whereas $J\lambda 4$ - $C\lambda 4$, $J\lambda 5$ - $C\lambda 5$, and $J\lambda 6$ - $C\lambda 6$ regions are not functional due to deletions and/or insertions in the $C\lambda$ gene segments.¹² N nucleotide addition and a strict restriction in CDR3 length are also observed in λ L chain rearrangements.¹⁷

The remarkable diversity of the H chain CDR3 has been exploited to identify the clonal populations of B cells.¹⁸⁻²¹ The polymerase chain reaction (PCR) is advantageous for this purpose because it amplifies the CDR3 sequence in question thousands of times.²² The PCR product from a polyclonal B cell population shows a broad band when analyzed on agarose or polyacrylamide gels. DNA from B cell lines and B lymphoproliferative disorders shows a discrete band either on native gel or as an increase in one particular band on denaturing gel. The sensitivity of such methods is limited by the background of the identically sized polyclonal IgH chain gene rearrangement. When agarose gels are used for the electrophoretic analysis, it is often difficult to prove that an ethidium bromide band represents the specific clone and not a mixture of bands from the polyclonal amplification of normal B cells.²³ Even if polyacrylamide gels are used, it may be difficult to distinguish different clones by the band size alone.^{20,24}

A single-strand conformation polymorphism (SSCP) analysis makes it possible to differentiate between the PCR products of equal size but with a different sequence by just one base.²⁵ Davis et al used SSCP analysis to detect clonal IgH chain gene rearrangements after PCR amplification of DNA from patients with B cell malignancies using the consensus sequence primers of the VH and JH gene segments.²⁶ As a result, 1 malignant cell in 500 normal mononuclear cells (0.2%) was thus detected.

In most studies, a VH FR1, FR2, or FR3 consensus primer, or family-specific primers, are used to detect clonal IgH chain gene rearrangements. The detection rate of B cell malignancies varies with the choice of VH primer and also with the disease category. The overall detection rate of each primer was not satisfactory (~70%). The lowest detection rate in all systems was observed in follicular lymphoma.²⁷

If it is possible to detect the clonal L chain gene rearrangements using consensus primers, it is thus a useful supplement to the previously described approach of VH gene amplification to detect clonal B cell populations. This has been difficult because of the sparseness of knowledge regarding the sequence of $V\kappa$ and $V\lambda$ gene segments. In addition, the restriction of the L chain CDR3 length makes it difficult to differentiate clonal from polyclonal proliferation using either agarose or polyacrylamide gels.

Based on the database of human antibody genes (VBASE; MRC Centre for Protein Engineering, Cambridge, UK), we designed $V\kappa$ and $V\lambda$ FR3 consensus primers. We performed a reverse transcriptase (RT)-PCR of the CDR3 region of the IgL chain transcripts with the $V\kappa$ or $V\lambda$ FR3 consensus primer and $C\kappa$ or $C\lambda$ primer, respectively. Due to the restriction of the CDR3 length of the IgL chain rearrangements, the methods depending on the length difference of the PCR products are not appropriate for detecting the difference in the L chain CDR3. We thus combined the RT-PCR of the IgL chain transcripts with SSCP, which detects the difference in the CDR3 sequence, and then analyzed the samples from B cell malignancies to develop a novel technique to analyze the clonal expansion of B cells. By using SSCP, we were also able to determine the clonal identities of B cell expansion in different samples.

Materials and Methods

Patients

Samples from 10 healthy subjects (N1 through N10), 2 rheumatoid arthritis (RA) patients (RA1 and RA2), 4 B-chronic lymphocytic leukemia (B-CLL) patients (CLL1 through CLL4), 6 multiple myeloma (MM) patients (MM1 through MM6), 3 Waldenström's macroglobulinemia (WM) patients (WM1 through WM3), and 16 B cell non-Hodgkin's lymphoma (B-NHL) patients (ML1 through ML16) were studied. A histological examination of the lymph nodes from RA1 and RA2 showed follicular hyperplasia. The diagnosis of B-CLL was made according to the criteria of the International Workshop on Chronic Lymphocytic Leukemia.²⁸ The tumor cells from all of the B-CLL patients, except for those from CLL1, expressed CD5 antigen. Monoclonal gammopathy and Bence-Jones proteinuria were absent in all B-CLL patients. The patients were diagnosed to have MM based on the criteria of Durie and Salmon.²⁹ All of the WM patients were diagnosed according to the criteria of Mackenzie and Fudenberg.³⁰ The diagnosis of B-NHL was made using a proposal from the International Lymphoma Study Group.³¹ Mononuclear cells (MNCs) were isolated from the heparinized peripheral blood (PB) and bone marrow (BM) using the standard Ficoll/Hypaque gradient method.

Reverse Transcriptase Polymerase Chain Reaction

Total RNA from the MNCs and frozen tissue sections were isolated by the previously described acid guan-

Table 1. Nucleotide Sequences of the Primers and Probes

Specificity	Sequence (5' to 3')
V κ FR3 primer	AAGATG/TTTGCAGTTTATTACTG
V λ FR3 primer	AGGAT/CGAGGCTGAT/CTATTACTG
VH FR3 primer	ACACGGCC/TG/CTGTATTACTG
C κ primer	CCAGATTTCAACTGCTCATCA
C λ primer	AGTGTGGCCTTGTTGGCTTG
JH primer	CTGAGGAGACGGTGACC
C κ probe	ACAGATGGTGACGCCACAGT
C λ probe	AGGAGGGT/CGGGAACAGAGTGA
JH probe	ACCA/GG/TG/TGTC/TCCC/TC/TGGCCCCAG

dinium thiocyanate-phenol-chloroform extraction method.³² Total RNA (3 μ g) was converted to first-strand cDNA by using reverse transcriptase (Superscript, BRL, Gaithersburg, MD). The PCR was performed as described by Saiki et al.²² The 50- μ l reaction mixture contained 1 μ l of the RT reaction product, 40 pmol of each primer, and 2.0 U of *Taq* polymerase (Cetus, Norwalk, CT). A total of 35 cycles of amplification were performed (1.5 minutes at 94°C, 2 minutes at 54°C, and 3 minutes at 72°C). To control for possible contamination, mock PCR reaction mixtures lacking template or containing products of the first-strand cDNA reaction without reverse transcriptase were prepared. None of the controls contained amplified products visible on ethidium-stained agarose gels or product detected by SSCP analysis.

Single-Strand Conformation Polymorphism Analysis

The amplified DNA was diluted (1:20) in a denaturing solution (95% formamide, 10 mmol/L EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol) and held at 90°C for 2 minutes. Two microliters of the diluted sample was electrophoresed in nondenaturing 5% polyacrylamide gels containing 10% glycerol. The gel was run at a 35-W constant power for ~2 hours. After electrophoresis, the gel containing DNA was stuck to prewet 3MM paper support and placed on a glass plate. A prewet nylon membrane (Immobilon-S, Millipore Intertech, Bedford, MA) was placed over the gel. After covering the nylon membrane with a second piece of 3MM paper (Whatman International, Maidstone, UK) and paper towels, the DNA

was transferred by putting a second glass plate on top of the stack and weighing it down with a 1-kg weight for 20 minutes. The nylon membrane was thereafter incubated with either a biotinylated internal constant region or JH gene probe. The DNA was then visualized by subsequent incubations with streptavidin, biotinylated alkaline phosphatase, and a chemiluminescent substrate system (Phosphorimager detection kit, New England Biolabs, Beverly, MA).

Primers and Probes

The primers and probes used are described in Tables 1 and 2. The C λ constant region primer hybridizes all functional λ constant region sequences.

DNA Cloning and Sequencing

The slices containing the specific bands were excised from the polyacrylamide gel. The eluted DNA was then reamplified for 35 cycles with the same FR3 and constant region primers used in RT-PCR, except that they had a *Bam*HI and an *Eco*RI cutting site, respectively, and was purified from 1.1% agarose gel. The recovered DNA fragments were ligated in the *Bam*HI/*Eco*RI site of pBluescript SK- (Stratagene, La Jolla, CA) and used to transform the *Escherichia coli* strain XL-1Blue (Stratagene). The clones were picked randomly, and a double-strand DNA template was prepared and sequenced using the DyeDeoxy Terminator Cycle Sequencing kit and the Applied Biosystems automatic DNA sequencer (Applied Biosystems, Riossy, France).

Serial Dilution Experiments

To determine the minimum fraction of the clonal cells that could be detected by SSCP analysis, the MNCs of PB from patients CLL1, CLL2, and CLL4 were serially diluted into the MNCs from healthy individuals, and the samples were assessed by the RT-PCR SSCP analysis.

Southern Blot Analysis

Ten micrograms of DNA was digested with *Eco*RI and *Hind*III. The digests were electrophoresed on a 0.8%

Table 2. Nucleotide Usage at the 3' End Region of FR3 of Human V κ and V λ Genes

		Nucleotide usage																			
V κ (n = 40)																					
G		0	10	40	0	0	15	0	1	40	9	7	17	0	4	0	0	0	0	0	40
A		40	29	0	40	0	2	0	0	0	0	33	22	1	3	0	40	0	0	39	0
T		0	1	0	0	40	23	36	38	0	0	0	1	36	33	40	0	35	40	1	40
C		0	0	0	0	0	0	4	1	0	31	0	0	3	0	0	0	5	0	38	0
Consensus	5'	A	A	G	A	T	G/T	T	T	G	C	A	G	T	T	T	A	T	T	A	C
V λ (n = 31)																					
G		3	25	31	0	0	31	0	29	29	1	0	31	0	3	0	0	0	0	0	31
A		27	4	0	31	0	0	31	2	1	0	0	0	31	0	0	31	0	0	31	0
T		1	1	0	0	22	0	0	0	1	0	29	0	0	12	31	0	28	29	0	31
C		0	1	0	0	9	0	0	0	0	30	2	0	0	16	0	0	3	2	0	31
Consensus	5'	A	G	G	A	T/C	G	A	G	G	C	T	G	A	T/C	T	A	T	T	A	C

Number of V κ or V λ genes analyzed is indicated by n.

agarose gel, transferred to a nylon membrane (Hybond-N+, Amersham Life Science, Little Chalfont, UK) and hybridized overnight with a 32 P-labeled 361-bp probe containing C λ 2. After appropriate washing, the membrane was autoradiographed for 1 day.

Results

To determine whether consensus PCR primers could be designed for the V κ and V λ genes, we examined the nucleotide usage of both genes using the V BASE database. The nucleotide sequence of the 3' end of the FR3 region of V κ and V λ genes was conserved irrespective of the families they belonged to (Table 2). We thus designed 22-bp consensus PCR primers for the V κ and V λ genes in this region.

To obtain information about the mRNA encoding the IgL chain (V κ or V λ) expressed in the selected population of human lymphocytes, the RT-PCR technique was used with a VL FR3 consensus sequence primer and an IgL chain constant region primer. Ethidium bromide staining of conventional agarose gels showed the amplified DNA to have migrated as a single band of $\sim$ 150 bp (data not shown). Because a large degree of diversity should be present within these bands, an attempt was made to discriminate these diversities by employing the SSCP technique, which has been reported to detect even a single nucleotide mutation. The PB and BM MNCs from healthy individuals (N1 through N10) and lymph nodes from RA patients (RA1 and RA2) yielded a smear pattern after both RT-PCR and SSCP analyses (Figure 1). This probably indicates a large degree of heterogeneity in the IgL chain CDR3 repertoire. To confirm the specificity of primers, the PCR product of the κ isotype was hybridized with a C λ probe. In this experiment, no visible signals were observed (data not shown). The same result was obtained when the λ PCR product was hybridized with a C κ probe.

A particular single-stranded DNA can take more than one different molecular shape, depending on the conditions of SSCP electrophoresis.²⁵ When a few discrete dominant bands were detected over the background smear of polyclonal rearrangements, we considered the clonal population to be present in the sample. Total RNA was extracted from the PB MNCs of four patients with B-CLL, six patients with MM, and three patients with WM. The clonal pattern was detected in either a κ or λ analysis in all samples (13/13). Figure 2 shows the results of the RT-PCR SSCP analysis of samples from patients with B-CLL. Total RNA was also extracted from frozen sections of 16 patients with B-NHL. A clonal pattern was observed in 12 patients (75%). In contrast, the lymph nodes from RA1 and RA2, a histological examination of which showed follicular hyperplasia, yielded a diffuse pattern with no discrete bands (Figure 1). The results of an analysis of B cell malignancies are summarized in Table 3. The detection rate of the VL RT-PCR SSCP analysis was 86% (25/29). The lowest detection rate was observed in B-NHL, especially in follicular lymphoma. In CLL, MM, and WM, the L chain isotype of tumor cells was

Figure 1. A clonal analysis of the κ and λ L chain messages of the PB (N1 to N8) and BM (N9 and N10) from healthy subjects and the lymph node from patients with RA (RA1 and RA2). A typical smear pattern was observed in all healthy and RA subjects.

identical to that determined by the RT-PCR SSCP analysis (Table 3). However, the L chain expression pattern of B-NHL determined immunohistochemically was not always identical to that determined by RT-PCR SSCP. In patients ML4, -5, -7, -10, and -13, the monoclonal L chain expression was detected only by RT-PCR SSCP. In patients ML11 and -12, on the other hand, an L chain expression pattern could be determined only by immu-

Figure 2. A clonal analysis of the κ and λ L chain messages of the PB from CLL patients. Patients CLL1 and CLL2 showed clonal bands in a κ isotype analysis. Dominant bands were observed in the λ isotype in patients CLL3 and CLL4.

Table 3. Clinical Features and Results of the RT-PCR-SSCP Analysis

Patient	RT-PCR-SSCP			Surface immunoglobulin	M-protein	Light chain expression		Subtype
	V κ	V λ	VH					
CLL1	+	—	+	$\mu\kappa$ $\mu\kappa$ $\mu\lambda$ $\mu\lambda$	λ $\delta\lambda$ $\delta\lambda$ $\gamma\lambda$ $\gamma\kappa$ $\alpha\lambda$ $\mu\lambda$ $\mu\kappa$ ND			
CLL2	+	—	+					
CLL3	—	+	+					
CLL4	—	+	+					
MM1	—	+	—					
MM2	—	+	—					
MM3	—	+	+					
MM4	—	+	+					
MM5	+	—	+					
MM6	—	+	+					
WM1	—	+	+					
WM2	+	—	+					
WM3	+	—	+					
B-NHL1	—	—	+					
B-NHL2	—	+	—					
B-NHL3	—	+	—					
B-NHL4	—	+	+					
B-NHL5	+	—	+					
B-NHL6	—	+	+					
B-NHL7	+	—	+					
B-NHL8	—	—	+					
B-NHL9	+	—	—					
B-NHL10	—	+	—					
B-NHL11	—	—	—					
B-NHL12	—	—	—					
B-NHL13	—	+	—					
B-NHL14	+	—	+					
B-NHL15	—	+	—					
B-NHL16	—	+	+					

ND, not done.

nohistochemical method. We also performed RT-PCR SSCP analysis using the VH FR3 consensus primer and JH consensus primer. The PB and BM MNCs from healthy individuals (N1 through N10) and lymph nodes from RA patients (RA1 and RA2) showed the same smear pattern as that observed in the κ or λ analysis (data not shown). The results of the VH analysis of B cell malignancies are also shown in Table 3. The detection rate was 66% (19/29). In 17 patients, a clonal pattern was detected in both VH and VL analyses. In eight patients, a clonal pattern was observed only in the VL analysis. The overall detection rate using the VL and VH analyses was 93% (27/29).

To determine the minimum fraction of clonal cells that could be detected by SSCP analysis, serial dilution experiments were performed. The MNCs from the patients CLL1, CLL2, and CLL4 were serially diluted into the MNCs from healthy individuals, and the samples were assessed by the RT-PCR SSCP analysis. Figure 3A shows the clonal SSCP bands to be visible at dilutions of up to 1:100 (1%) in CLL1 and CLL2 and 1:1000 (0.1%) in CLL4 (Figure 3A). Using BM MNCs from a Sjögren's syndrome patient with $\mu\lambda$ paraprotein, we performed both RT-PCR SSCP analysis and a Southern blot analysis. Although no C λ rearrangement was observed in the Southern blot analysis, the RT-PCR SSCP analysis was able to detect clear bands (Figure 3B).

In a high proportion of MM patients, the clonal B cell population can often be detected in PB.³³ We analyzed six patients with MM whose PB lymphocytes were free

from plasma cells by light microscopy. In all MM patients, both BM and PB contained dominant bands of the κ or λ isotype. The dominant bands were present at an identical position in BM and PB (Figure 4). Regarding patients MM1 and MM4, we performed a sequence analysis of the dominant bands in BM and PB and found them to have an identical CDR3 sequence (data not shown).

Discussion

Most of the PCR-based B cell clonality analyses use DNA as the template. However, we could not design consensus primers and probes for the J κ and J λ gene segments. In addition, as the PCR of DNA amplifies the nonfunctional rearrangements as well as the functional rearrangements, the SSCP analysis of the PCR product should therefore produce a complicated pattern. For these two reasons, we chose RNA as the template.

As expected, due to the diversity of the IgL CDR3 region, an analysis of B cells in the PB and BM from healthy subjects exhibited a smear-like pattern that appeared to be a summation of multiple bands with heterogeneous mobilities. The specific immunity in vertebrates depends on the individual's ability to generate a heterogeneous repertoire of an antigen-binding structure. The Ig CDR3 region is important in that it forms the center of the antigen-binding site⁶ and contributes to the generation of diversity in the preimmune Ig repertoire. Our method analyzes the sequence difference in the IgL

Figure 3. A: A RT-PCR SSCP analysis of κ (CLL1 and CLL2) and λ (CLL4) L chain messages. MNCs from PB of CLL1, CLL2, and CLL4 were serially diluted into the PB MNCs of a healthy donor. Lane 1, from CLL1, CLL2, or CLL4; lanes 2 to 6, RT-PCR products from the serial dilutions; lane 7, from a healthy subject. Clonal bands are visible with dilutions of up to 1:100 (1%) in CLL1 and CLL2 and 1:1000 (0.1%) in CLL4. B: A Southern blot analysis and RT-PCR SSCP analysis of BM MNCs from a Sjögren's syndrome patient with $\mu\lambda$ paraprotein. A Southern blot analysis showed no CA rearrangement, whereas clear bands were present in the RT-PCR SSCP analysis. E, *EcoRI*; H, *HindIII*.

chain CDR3 region. If the smear-like pattern observed in healthy individuals reflects the heterogeneous repertoire that is essential to specific immunity, then our method may be useful for evaluating the immune status. The pattern of the RT-PCR SSCP analysis in immunodeficient cases such as malignancy, prolonged treatment with immunosuppressive agents, and bone marrow transplantation is now under study.

Figure 4. A RT-PCR SSCP analysis of the samples from the MM patients (MM1 to MM6). Messages from the BM (left) and PB (right) of six MM patients were used. In MM4, dominant bands were observed in the κ isotype analysis. In MM1–3, MM5, and MM6, clonal bands were present in the λ isotype. The PB from each patient showed dominant signals with a mobility that was identical to that from the BM.

Using the PCR technique, several methods have been established to detect clonal B cell populations in B cell malignancies.^{18–21,23} These methods can be useful as a supplement to a routine morphological and immunohistochemical diagnosis of B cell leukemias and lymphomas. In most studies, a VH FR3 consensus primer hybridizing to the 3' end of most VH genes is used to detect clonal IgH chain gene rearrangements.^{19–21,23} In the T-cell-dependent immune response, B cells undergo a somatic mutation of their Ig variable domain genes, thus resulting in a decrease in the rate of hybridization of the VH FR3 primer to somatically mutated VH genes. Several other mechanisms also exist that explain the failure to detect a clonal population by PCR amplification with VH FR3 consensus primer, including the partial DH-JH rearrangement in immature malignancies,^{34,35} excessive exonucleolytic activity leading to the removal of nucleotides homologous to the 3' end of the FR3 primer, or chromosomal translocation into the IgH locus, such as the t(14;18) in follicular lymphoma. The hybridization rate varies between ~10 and 100% depending on the kinds of B cell malignancies.²⁷ The RT-PCR SSCP approach of VL rearrangements could detect monoclonal B cell popula-

tions in 25 of 29 patients (86%) with B cell malignancies. The detection rate of the VH RT-PCR SSCP analysis was 66% (19/29). If we combined the VL and VH analyses, the overall detection rate increased to 93% (27/29). Our method is thus considered to be a useful supplement to the PCR method using VH FR3 consensus primer. In 17 patients, a clonal pattern was detected in both the VL and VH RT-PCR SSCP analyses. The detection of gene rearrangements at multiple loci is important to minimize false negative results when B cell malignancies show clonal evolution.^{36,37}

Küppers et al used family-specific κ L chain primers to detect the clonal B cell populations.³⁸ In a clonal population, only one or two V κ genes were rearranged and thus produced a PCR product in the reaction with the V κ primer of the respective V κ gene family, whereas in a polyclonal B cell population, the rearrangements of the V κ genes of all families were observed, which resulted in PCR products in all reactions. They also developed a similar method using family-specific λ light chain primers.³⁹ Their methods are simple and provide additional information such as the use of different V gene families in different B cell malignancies. They analyzed B cell leukemias and B-NHL. Their method appears to work only when most of the cells analyzed are clonal. If the clonal B cell is a minor population of the cells analyzed (eg, PB MNCs of MM or WM), their method would produce a polyclonal pattern. In addition, the method gave no information regarding the clonal identities.

In five B-NHL patients, monoclonal L chain expression was detected only by RT-PCR SSCP. The expression level of the L chain in these tumor cells may not be sufficient for the detection by immunohistochemical method. Otherwise, as reported in the H chain expression in CLL, L chain mRNA may not be translated into protein in some patients with B-NHL.⁴⁰

Serial dilution experiments, in which the MNCs from CLL were serially diluted into the MNCs from a healthy subject, revealed that RT-PCR SSCP analysis detected 1 malignant cell in 100 to 1000 MNCs (Figure 3A). Although the sensitivity of the RT-PCR SSCP analysis is considered to be better than that of the Southern analysis (Figure 3B), it may not be sufficient to detect minimal residual disease. In addition, the efficiency with which the consensus VL primers anneal and amplify some IgL gene rearrangements may vary significantly. As a result, the precise sensitivity of this method may vary with different tumors. For disease-monitoring purposes, it is therefore necessary to design tumor-specific primers or probes for either PCR or a Southern blot analysis, respectively.

MM is a malignant tumor involving plasma cells that accumulate in the BM. The tumor cells generally secrete a monoclonal Ig paraprotein that can be identified in the serum, and free IgL chains (Bence-Jones protein) may also be present in urine. It has been shown by morphological, immunofluorescence microscopy, flow cytometric, and molecular techniques that malignant cells circulate in the blood of most patients with active MM.^{33,41-44} The RT-PCR SSCP method was able to detect the presence of common clonal B cell expansion in PB and BM (Figure 4). To determine the presence of clonal B cells in

PB is important for understanding their relationship to the clinical course of MM. The circulating clonal B cells may represent clones that can repopulate tissue sites and contribute to disease dissemination. The RT-PCR SSCP analysis detected clonal B cells in all of the MM PB samples analyzed. The unique features of SSCP also enabled us to easily demonstrate the clonal identities of expanded B cell populations in PB and BM.

MM1 is a patient whose tumor cells secrete free IgL chains (Bence-Jones protein) but not intact Ig molecules. A monoclonal pattern in λ isotype analysis was observed in this patient. In 17% of patients with MM, only the L chain was detected as monoclonal paraprotein.⁴⁵ Although among the IgH chain gene rearrangements in the L chain only MM has not been fully studied, it is clear that our method is applicable in the analysis of this type of MM.

The detection of a clonal population by our method does not prove that malignant cells are present. B cell monoclonal expansion has been detected by a DNA analysis of nonmalignant tissue.⁴⁶ The oligoclonal expansion of B cells was detected in the synovium of rheumatoid arthritis (unpublished data). To verify the relationship between the clonal band and a malignant B cell clone, a careful comparison of the RT-PCR SSCP analysis results and the findings of other diagnostic methods, including morphological, immunofluorescence microscopy, and flow cytometric techniques, is thus necessary in the early phase of the clinical course of the disease.

In summary, we developed a novel clonality analysis of B cell malignancies that used sequence diversity of IgL chain CDR3. A V κ or V λ FR3 consensus primer was used for the amplification of IgL chain CDR3, and SSCP was used for the detection of B cell clonal expansion. Our method together with the VH approach is expected to increase the detection rate of the clonal B cell expansion in B cell malignancies.

Acknowledgments

We thank E. Kohno and M. Eto for their excellent technical assistance.

References

1. Tonegawa S: Somatic generation of antibody diversity. *Nature* 1983, 302:575-581
2. Alt FW, Oltz EM, Young F, Gorman J, Taccioli G, Chen J: VDJ recombination. *Immunol Today* 1992, 13:306-314
3. Goodnow CC, Crosbie J, Adelstein S, Lavoie TB, Smith-Gill SJ, Brink RA, Pritchard-Briscoe H, Wotherspoon JS, Loblay RH, Raphael K, Trent RJ, Basten A: Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. *Nature* 1988, 334:676-682
4. French DL, Laskov R, Scharff MD: The role of somatic hypermutation in the generation of antibody diversity. *Science* 1989, 244:1152-1157
5. Kabat EA, Wu TT, Perry HM, Gottesman KS, Foeller C: Sequences of Proteins of Immunological Interest, eds. Bethesda, MD, US Department of Health and Human Services, 1991
6. Padlan EA: Anatomy of the antibody molecule. *Mol Immunol* 1994, 31:169-217
7. Tomlinson IM, Walter G, Marks JD, Llewellyn MB, Winter G: The

- repertoire of human germline VH sequences reveals about fifty groups of VH segments with different hypervariable loops. *J Mol Biol* 1992, 227:776-798
8. Corbett SJ, Tomlinson IM, Sonnhammer ELL, Buck D, Winter G: Sequence of the human immunoglobulin diversity (D) segment locus: a systematic analysis provides no evidence for the use of DIR segments, inverted D segments, "minor" D segments or D-D recombination. *J Mol Biol* 1997, 270:587-597
9. Ravetch JV, Siebenlist U, Korsmeyer S, Waldmann T, Leder P: Structure of the human immunoglobulin μ locus: characterization of embryonic and rearranged J and D genes. *Cell* 1981, 27:583-591
10. Schable KF, Zachau HG: The variable genes of the human immunoglobulin κ locus. *Biol Chem Hoppe-Seyler* 1993, 374:1001-1022
11. Hieter PA, Maizel JV, Leder P: Evolution of human immunoglobulin κ J region genes. *J Biol Chem* 1982, 257:1516-1522
12. Vasicek TJ, Leder P: Structure and expression of the human immunoglobulin λ genes. *J Exp Med* 1990, 172:609-620
13. Fripiat J-P, Williams SC, Tomlinson IM, Cook GP, Cherif D, Le Paslier D, Collins JE, Dunham I, Winter G, Lefranc M-P: Organization of the human immunoglobulin lambda light-chain locus on chromosome 22q11.2. *Hum Mol Genet* 1995, 4:983-991
14. Williams SC, Fripiat J-P, Tomlinson IM, Ignatovich O, Lefranc M-P, Winter G: Sequence and evolution of the human germline V λ repertoire. *J Mol Biol* 1996, 264:220-232
15. Desiderio SV, Yancopoulos GD, Paskind M, Thomas E, Boss MA, Landau N, Alt FW, Baltimore D: Insertion of N regions into heavy-chain genes is correlated with expression of terminal deoxynucleotidyl transferase in B cells. *Nature* 1984, 311:752-755
16. Foster SJ, Brezinschek H-P, Brezinschek RI, Lipsky PE: Molecular mechanisms and selective influences that shape the kappa gene repertoire of IgM⁺ B cells. *J Clin Invest* 1997, 99:1614-1627
17. Byer S, Bridges SL Jr: Immunoglobulin lambda light chain expression in patients with rheumatoid arthritis and in normal individuals. *Arthritis Rheum* 1997, 40:S246
18. Deane M, Norton JD: Detection of immunoglobulin gene rearrangement in B lymphoid malignancies by polymerase chain reaction gene amplification. *Br J Haematol* 1990, 74:251-256
19. Trainor KJ, Brisco MJ, Wan JH, Neoh S, Morley AA: Gene rearrangement in B- and T-lymphoproliferative disease detected by the polymerase chain reaction. *Blood* 1991, 78:192-196
20. McCarthy KP, Sloane JP, Wiedemann LM: Rapid method for distinguishing clonal from polyclonal B cell populations in surgical biopsy specimens. *J Clin Pathol* 1990, 43:429-432
21. Deane M, McCarthy KP, Wiedemann LM, Norton JD: An improved method for detection of B-lymphoid clonality by polymerase chain reaction. *Leukemia* 1991, 5:726-730
22. Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA: Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 1988, 239:487-491
23. Ramasamy I, Brisco M, Morley A: Improved PCR method for detecting monoclonal immunoglobulin heavy chain rearrangement in B-cell neoplasms. *J Clin Pathol* 1992, 45:770-775
24. Pollard P, Owen G, Worwood M: PCR-based immunogenotyping at the Ig heavy chain CDR3 locus: improvements in resolution. *Br J Haematol* 1993, 84:169-171
25. Orita M, Iwahana H, Kanazawa H, Hayashi K, Sekiya T: Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proc Natl Acad Sci USA* 1989, 86:2766-2770
26. Davis TH, Yockey CE, Balk SP: Detection of clonal immunoglobulin gene rearrangements by polymerase chain reaction amplification and single-strand conformational polymorphism analysis. *Am J Pathol* 1993, 142:1841-1847
27. Aubin J, Davi F, Nguyen-Salomon F, Leboeuf D, Debert C, Taher M, Valensi F, Canioni D, Brousse N, Varet B, Flandrin G, Macintyre EA: Description of a novel FR1 IgH PCR strategy and its comparison with three other strategies for the detection of clonality in B cell malignancies. *Leukemia* 1995, 9:471-479
28. International Workshop on Chronic Lymphocytic Leukemia: Chronic lymphocytic leukemia: recommendations for diagnosis, staging, and response criteria. *Ann Intern Med* 1989, 110:236-238
29. Durie BG, Salmon SE: A clinical staging system for multiple myeloma: correlation of measured myeloma cell mass with presenting clinical features, response to treatment, and survival. *Cancer* 1975, 36:842-854
30. Mackenzie MR, Fudenberg HH: Macroglobulinemia: an analysis for forty patients. *Blood* 1972, 39:874-889
31. Harris NL, Jaffe ES, Stein H, Banks PM, Chan JKC, Cleary ML, Delsol G, Wolf-Peeters CD, Falini B, Gatter KC, Grogan TM, Isaacson PG, Knowles DM, Mason DY, Muller-Hermelink H-K, Pileri SA, Piris MA, Ralfkiaer E, Warnke RA: A revised European-American classification of lymphoid neoplasms: a proposal from the International Lymphoma Study Group. *Blood* 1994, 84:1361-1392
32. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987, 162:156-159
33. Billadeau D, Ness BV, Kimlinger T, Kyle RA, Therneau TM, Greipp PR, Witzig TE: Clonal circulating cells are common in plasma cell proliferative disorders: a comparison of monoclonal gammopathy of undetermined significance, smoldering multiple myeloma, and active myeloma. *Blood* 1996, 88:289-296
34. Steenbergen EJ, Verhagen OJHM, van Leeuwen EF, von dem Borne AEG, van der Schoot CE: Distinct ongoing Ig heavy chain rearrangement processes in childhood B-precursor acute lymphoblastic leukemia. *Blood* 1993, 82:581-589
35. Wasserman R, Galili N, Ito Y, Reichard BA, Shane S, Rovera G: Predominance of fetal type DJH joining in young children with B precursor lymphoblastic leukemia as evidence for an in utero transforming event. *J Exp Med* 1992, 176:1577-1581
36. Cleary ML, Galili N, Trela M, Levy R, Sklar J: Single cell origin of bigenotypic and biphenotypic B cell proliferations in human follicular lymphomas. *J Exp Med* 1988, 167:582-597
37. Zelenetz AD, Chen TT, Levy R: Clonal expansion in follicular lymphoma occurs subsequent to antigenic selection. *J Exp Med* 1992, 176:1137-1148
38. Kuppers R, Zhao M, Rajewsky K, Hansmann M-L: Detection of clonal B cell populations in paraffin-embedded tissues by polymerase chain reaction. *Am J Pathol* 1993, 143:230-239
39. Kuppers R, Willenbrock K, Rajewsky K, Hansmann M-L: Detection of clonal λ light chain gene rearrangements in frozen and paraffin-embedded tissues by polymerase chain reaction. *Am J Pathol* 1995, 147:806-814
40. Malisan F, Fluckiger A-C, Ho S, Guret C, Banchereau J, Martinez-Valdez H: B-chronic lymphocytic leukemias can undergo isotype switching in vivo and can be induced to differentiate and switch in vitro. *Blood* 1996, 87:717-724
41. Berenson J, Wong R, Kim K, Brown N, Lichtenstein A: Evidence for peripheral blood B lymphocyte but not T lymphocyte involvement in multiple myeloma. *Blood* 1987, 70:1550-1553
42. Billadeau D, Quam L, Thomas W, Kay N, Greipp P, Kyle R, Oken MM, Van Ness B: Detection and quantitation of malignant cells in the peripheral blood of multiple myeloma patients. *Blood* 1992, 80:1818-1824
43. Boccadoro M, Omede P, Massaia M, Dianzani U, Pionpo P, Battaglio S, Meregalli M, Pileri A: Human myeloma: several subsets of circulating lymphocytes express plasma cell-associated antigens. *Eur J Haematol* 1988, 40:299-304
44. Pilarski LM, Belch AJ: Circulating monoclonal B cells expressing p-glycoprotein may be a reservoir of multidrug resistant disease in multiple myeloma. *Blood* 1994, 83:724-736
45. Ameis A, Ko HS, Pruzanski W: M-components: a review of 1242 cases. *Can Med Assoc J* 1976, 114:889-892
46. Fishleder A, Tubbs R, Hesse B, Levine H: Uniform detection of immunoglobulin-gene rearrangement in benign lymphoepithelial lesions. *N Engl J Med* 1987, 316:1118-1121