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# Malignant Cell Detection in Burkitt's Lymphoma Using Third-complementarity-determining Region (CDRIII), Clone-specific Probe Developed by Sequencing DNA from Stored Slides

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**The DNA sequence of the third-complementarity-determining region (CDRIII) of the immunoglobulin heavy chain (IgH) gene in a case of Burkitt's lymphoma was determined by polymerase chain reaction (PCR) using template DNA extracted from a smear stored at room temperature for more than one year. The DNA sequence obtained from the stored slide was compared with that of DNA from a frozen lymph node biopsied at the initial presentation. The sequences were shown to be identical, implying that DNA from a smear on a stored slide can be used as a source of DNA for PCR amplification, sequencing, and development of a clone-specific probe. Using oligonucleotides generated from one of the CDRIII sequences of the IgH gene as molecular probes, a retrospective study for the malignant clone on the smears was carried out. Malignant cells were detectable in the peripheral blood at an early stage of bone marrow relapse but not in the peripheral blood or bone marrow at the initial presentation. No malignant clone was detected in the bone marrow when testicular infiltration was diagnosed by examination of a pathological specimen. Thus, the technique permits molecular analysis of hematologic malignancies of B-cell lineage in cases where fresh or frozen specimens are not available.**

Key words: Third-complementarity-determining region — B cell leukemia/lymphoma

Malignant cells of lymphoma/leukemia have been detected in peripheral blood and bone marrow by routine morphological examination of blood smears. Their presence in B-cell lymphoma/leukemia has also been implied by conventional Southern analysis of immunoglobulin (Ig) genes,[1,2] which offers a sensitivity of 5–10% in the detection of clonal rearrangements.[3,4] A hypervariable sequence known as the complementarity determining region III (CDRIII) of Ig heavy (IgH) chain is generated by the rearrangement of Ig heavy chain gene during the differentiation of B lymphocytes. Since acute lymphoblastic leukemia (ALL) and lymphoma of B cell lineage result from the clonal expansion of a malignant B lymphocyte, the CDRIII sequence can be exploited as a specific marker for the malignant clone. Using CDRIII-PCR methods, it has become possible to detect malignant clones with up to 0.01% sensitivity.[5,6]

A common practical problem, however, is that many patients with lymphoma or leukemia are treated before collection of samples suitable for DNA analysis is considered. Therefore, we wondered whether DNA from stored smears could be used as a source of template DNA for PCR amplification of the CDRIII sequence of IgH gene in order to conduct a retrospective study for the presence of malignant cells. This approach may enable clinicians to detect the malignant cells in those patients already on treatment and also patients who were diagnosed before present-day techniques became available.

## MATERIALS AND METHODS

**Case report** A boy was presented because of a right cervical lymph-node swelling on 17th October, 1988. A biopsy of the swollen node revealed a malignant lymphoma of diffuse small non-cleaved type with surface immunoglobulin kappa chain but no heavy chain. A work-up for staging showed involvement of abdominal lymph nodes without extranodal dissemination. Treatment was started with multiagent chemotherapy. Response was prompt; all the pathological nodes had disappeared after one course of therapy and he was thought to have entered remission. No recurrence occurred until 4th September, 1989 when bilateral testicular swelling was noted. A biopsy of both swollen testes revealed diffuse proliferation of the blasts with an appearance similar to that at initial diagnosis. Testicular relapse was diagnosed but the bone marrow remained free of blasts at this time. Local irradiation to both testes and chemotherapy with a new combination of multiagents were started. In spite of this treatment, bone marrow examina-

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tion performed on 17th October, 1989 revealed FAB L3 lymphoblasts of 59.2% and at this time similar lymphoblasts were noted in a peripheral blood smear (up to 2% leukocytes). He died on 11th December, 1989.

**Clinical samples** Materials used for DNA analysis were as follows: 1. Frozen lymph node obtained at the initial diagnosis. 2. Bone marrow and peripheral blood smears made on 22nd October, 1989 and stored at room temperature for more than one year. 3. Several other bone marrow or peripheral blood smears obtained at the time when testicular involvement was uncovered (12th September, 1989), before the leukemic transformation (25th, 30th September, and 2nd October, 1989) and after the leukemic transformation (17th October and 13th November, 1989). All the smears were stored at room temperature for more than one year.

**DNA extraction** DNA from fresh lymph node was extracted as previously described.[7] Air-dried unstained slides stored at room temperature were taken from the archives of the Department of Padiatrics at Tokyo University Hospital. DNA was extracted from smears according to the method described by Fey *et al.*[8] Smears were scraped off the glass slides with a scalpel blade and the resulting powdered material was transferred to Eppendorf tubes. The material was first lysed in 0.9 ml of distilled water and in 300 $\mu$l of a nonionic detergent (Nonidet P40, 0.1% solution, lysis time 10 min) and the nuclear pellets were incubated in 200 $\mu$l of lysis buffer (10 m*M* Tris-HCl at pH 8.0, 10 m*M* NaCl, 10 m*M* EDTA), with sodium dodecyl sulfate (SDS: 2%) and proteinase K (2 $\mu$g/$\mu$l) at 37°C for 6–12 h. DNA extraction with phenol and chloroform was performed as previously described.[2] DNA was precipitated from the aqueous phase with sodium chloride and ethanol. The precipitates were centrifuged at 12,000 rpm for 20 min in a high-speed micro centrifuge (Tomy, Tokyo), vacuum-dried and redissolved in 20 $\mu$l of TE buffer. Ten $\mu$l of the extracted DNA was subjected to PCR amplification.

**Southern hybridization analysis** High-molecular-weight DNA extracted from the frozen lymph node was subjected to analysis for the rearrangement of IgH chain gene. Eight $\mu$g of genomic DNA was digested to completion with 3–5 U/$\mu$g of the restriction enzyme *Bam*HI. The digests were size-fractionated on 0.7% agarose gels and transferred to nylon membranes (GeneScreen Plus, Dupont). Genomic blots were hybridized with $^{32}$P-labeled DNA probe, washed at 65°C in 2×SSC (0.3 *M* NaCl, 0.03 *M* Na citrate) and 0.1% sodium dodecyl sulfate (SDS) and autoradiographed. A human genomic 5.8 kb *Bam*HI-*Hin*dIII fragment of $IgJ_H$ gene ($\lambda$ CH28-6[2]) was used as a probe. The probe was $^{32}$P-labeled by the random primer method.

**Oligonucleotide probes** Oligonucleotides were synthesized by the solid-phase phosphoramidite method on an Applied Biosystems model 391 DNA synthesizer and purified by HPLC. The primers used for amplification of the CDRIII region are 5′-GGCCGCGGACACGGCCGTGTATTACTG-3′ (VSac) and 5′-GGGTCGACCTGAGGAGACGGTGACCAG-3′ (JSal). These primers were ligated with linkers: *Sac*II for $V_H$ and *Sal*I for $J_H$ (underlined). The oligonucleotide probe used for hybridization is 5′-TCGACTTCTGGGGCCAAGGG-3′. Sequences determined by PCR and used as diagnostic probes are shown in Fig. 2. As a positive control to indicate the quality and quantity of extracted DNA, phosphoglycerate kinase (PGK) gene was amplified using another aliquot of DNA. The primers for the amplification of the PGK gene are 5′-CAGGGGTCCTAGGCTTGGA-3′ for the 5′ primer and 5′-GGATCCATCCCCGAAGGTGA-3′ for the 3′ primer. The sequence of the hybridization probe is 5′-TTTCTAGCCGCATTTTCCC-3′. The diagnostic probe was end-labeled using [$\gamma$-$^{32}$P]ATP (3000 Ci/mmol, Amersham Japan; 1 Ci=37 GBq), 50 m*M* Tris-HCl (pH 7.6), 70 m*M* $MgCl_2$, 10 m*M* dithiothreitol, 1 m*M* spermidine hydrochloride and 10 units of T4 polynucleotide kinase (Takara Shuzou, Kyoto). Radiolabeled oligomers were further purified before hybridization by Biogel P60 gel filtration.

**Polymerase chain reaction and analysis of amplification products** PCR was carried out as described by Saiki *et al.*[9] with the precautions recommended by Kwok and Higuchi[10] in order to avoid false-positives. A negative control reaction with no template DNA was run in parallel with the DNA amplifications and PCR analyses. DNA (10 $\mu$l) was amplified in a reaction mixture of 2.5 $\mu$l of 10 × Taq polymerase buffer (500 m*M* KCl, 100 m*M* Tris-HCl pH 8.3, 150 m*M* $MgCl_2$, 0.1% gelatin), 2 $\mu$l of 2.5 m*M* each dNTPs, 100 pmol of each primer, DDW to 25 $\mu$l and mineral oil to prevent evaporation. The sample was initially denatured at 97°C for 7 min, followed by the addition of 2.5 units of *Thermus aquaticus* (Taq) DNA polymerase (Perkin-Elmer/Cetus, Norwalk, CA). The initial five cycles each consisted of an annealing step at 49°C for 30 s, an elongation step at 72°C for 1 min and a denaturation step at 94°C for 30 s followed by 25 cycles under the same conditions except for the annealing and extension steps, which were done at 72°C for 1 min. A total of 30 cycles were carried out in a Thermal Cycler (Perkin-Elmer/Cetus). PGK gene was amplified as a positive control to analyze the quality and quantity of extracted template DNA in the samples in which no amplification of CDRIII gene was observed. Four $\mu$l of each reaction product was analyzed by gel electrophoresis in 3% NuSieve/1% Seakem agarose gels (FMC) in 0.089 *M* Tris borate/0.002 *M* EDTA. For Southern blotting the electrophoresed DNA was transferred onto nylon membranes (Gene Screen Plus,

DuPont) using 0.4 *N* NaOH and hybridized at 55°C with $^{32}$P-labeled diagnostic oligonucleotide probe. After hybridization, the filters were washed in 5×SSPE (1× SSPE=0.15 *M* NaCl/0.01 *M* $NaH_2PO_4$, pH 7.0/1 m*M* EDTA) and 0.1% SDS at 5°C below the calculated melting point of the oligonucleotide probe. Filters were exposed to X-ray film at −70°C overnight with an intensifying screen (DuPont Lightning Screen Plus).

**DNA cloning, sequencing and computer analysis** An aliquot of the material amplified by PCR was digested with *Sal*I and *Sac*II restriction enzymes (Takara Shuzou), purified by gel electrophoresis and recovered from the gel by using a Gene Clean (BIO 101, USA). The recovered DNA was ligated to the *Sal*I/*Sac*II site of Bluescript KS vector (Stratagene, La Jolla, CA) and trnasfected into *Escherichia coli* (strain DH5$\alpha$). Transformants containing CDRIII DNA were detected by hybridization with $^{32}$P-labeled $J_H$ oligonucleotide probe (5′-TCGACTTCTGGGGCCAAGGG-3′). The cloned fragment was subjected to nucleotide sequencing by the dideoxy method[11] using Sequenase version 2.0 (United States Biochemical Corp, Cleveland, OH) and M13 M3 and M13 RV (Takara Shuzou) as primers. Computer analysis of DNA-sequencing data was performed with the sequence analysis software DNASIS (Hitachi, Japan).

## RESULTS

**Determination of the DNA sequence of CDRIII region from stored bone marrow smears** Genomic DNA extracted from a bone marrow smear at the stage of leukemic transformation (17th October, 1989) was amplified by PCR and the CDRIII region was sequenced. A nuiversal $V_H$ gene oligonucleotide primer (Vsac), which displays sequence homology with all members of the $V_H$ family thus far identified, was used as a 5′ primer. The $J_H$ antisense oligonucleotide primer (Jsal) which matches the 3′ ends of the six $J_H$ segments was used as a 3′ primer. Two faint bands of 133 bp and 118 bp species were detected by ethidium bromide staining of the electrophoresed amplified DNA. Cloning and sequencing of the bands generated two different kinds of clones (three clones termed KI′ and five clones termed KII′ in Fig. 2).

Southern hybridization analysis revealed two rearranged alleles of IgH gene in the DNA from the lymph node at the initial presentation (20th October, 1988) (Fig. 1). In order to compare the DNA sequence obtained from the bone marrow smear with that of the lymph node, the CDRIII region of genomic DNA extracted from the lymph node (20th October, 1988) was amplified. Two distinct bands of the same size as those of the DNA from the stored slide were detected by ethidium bromide staining. Cloning and sequencing of these two bands generated 4 clones with the sequence termed KI and 2 clones with the sequence termed KII in Fig. 2.

**Single point mutation in the sequence of one of the clones (KII′) at the stage of leukemic transformation** When the KI′ and KII′ sequences were compared with the se-

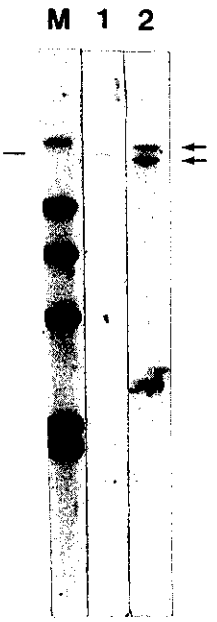


Fig. 1. Southern blot analysis of IgH gene in the lymph node biopsied at the initial presentation. —: germ line band. →: rearranged allele. M: λ *Hin*dIII marker. lane 1: germ line control. lane 2: lymph node on 20th October 1988.

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                    VH ↓
KI   5'-ACTGTGCAAGAGAGTTAGTGCGATTGTGGTGGTGAACGGACCA
KI'  5'-ACTGTGCAAGAGAGTTAGTGCGATTGTGGTGGTGAACGGACCA
                          ProKI
                      ↓ JH
     GCATGAACGTGGCTGGTTCGACCCCTGGGGCCAGGGAACCCTGG-3'
     GCATGAACGTGGCTGGTTCGACCCCTGGGGCCAGGGAACCCTGG-3'
      CAATGTGACAACTGGTTCGACTCCTGGGGCCAAGGAACC
                          JH5
                    VH ↓
KII  5'-ACTGTGCGACAGAAGCCGGAGTAGTACCAGGCCGCT
KII' 5'-ACTGTGCGACAGAAGCCGGAGTAATACCAGGCCGCT
                              *
                ↓ JH
     CTTCGAACTTCCAGCACTGGGGCCAGGGCACCCTGG-3'
     CTTCGAACTTCCAGCACTGGGGCCAGGGCACCCTGG-3'
     CTGAATACTTCCAGCACTGGGGCCAGGGCACC
                      JH1
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Fig. 2. DNA sequence comparison of CDRIII of Burkitt's lymphoma. KI, KII: DNA sequences obtained by the analysis of DNA from fresh lymph node. KI′, KII′: DNA sequences determined using stored slide. Arrows indicate the end of $V_H$ and $J_H$ sequences. Asterisk denotes single point mutation (G to A) in KII′ sequence. ProKI: Sequence used for clone-specific probe.

quences of KI and KII, one single point mutation of KII′ was identified (from G to A at the 20th nucleotide from the 5′ end of each of the cloned fragments). As all five of the KII′ sequences showed an identical G to A change, it is very unlikely that this single nucleotide change was due to Taq polymerase error. KI (KI′) and KII (KII′) were revealed to involve the rearrangement of $J_5$ and $J_1$, respectively.

**Detection of malignant clones using a clone-specific oligonucleotide probe in DNA extracted from stored peripheral blood and bone marrow smears** An oligonucleotide diagnostic probe corresponding to ProKI in Fig. 2, homologous to segments of the CDRIII DNA sequence within KI and KI′, was synthesized. Its specificity in recognizing the CDRIII DNA of lymphoma cells was tested against the RCR products of DNA from the leukemic cells of a common ALL (cALL) patient, fetal liver and normal adult peripheral blood. No positive signal was detected against the PCR amplified material from these samples. ProKI specifically detected the PCR amplified products of this Burkitt's lymphoma cell DNA (data not shown).

RCR studies using template DNA extracted from bone marrow or peripheral blood smears on slides serially obtained and kept at room temperature were carried out retrospectively using ProKI sequence as a molecular probe (Fig. 2). DNA from the bone marrow and peripheral blood slides prepared at the initial presentation gave no positive signal, indicating very little possibility of infiltration by lymphoma cells into the bone marrow or peripheral blood. The bone marrow smear obtained when testicular relapse was diagnosed ( 12th September, 1989) gave no positive signal after DNA amplification. No positive signal was present in the peripheral blood until 2nd October, 1989. A positive signal was detected in the peripheral blood on 17th October, 1989, when bone marrow infiltration by tumor cells was detected. A strong positive signal was observed at 133 bp in the bone marrow smear taken on 13th November, 1989, corresponding to the predominant blast cell infiltration (97.4%) in the bone marrow (Fig. 3).

In order to rule out the possibility of a false negative reaction, amplification of the PGK gene by PCR was performed. As the results show, all the samples with no amplification of CDRIII gene gave a positive signal for PGK after 30 cycles of amplification, indicating that the genomic DNA recovered from the smears was sufficient for PCR analysis (Fig. 3B, lanes 2–7).

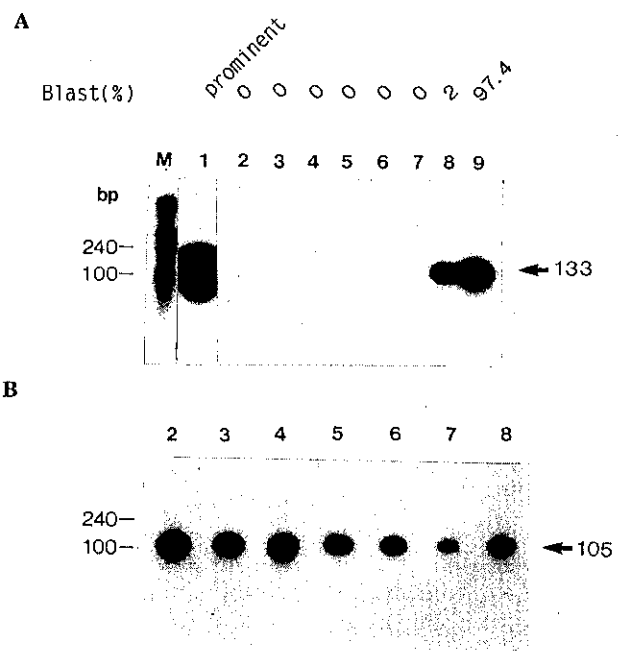


Fig. 3. Detection of malignant clones by PCR analysis of DNA from serially stored slides. A) PCR study was carried out as described in "Materials and Methods." Blast percent was determined by morphological examination under the microscope. B) PCR study of PGK gene as an internal positive control shows that the samples contain sufficient template DNA to analyze. PGK gene was amplified using the template DNA with no amplification (lanes 2–7) and amplification (lane 8) of CDRIII gene. As CDRIII region was amplified using the template DNA from lymph node (20th October, 1988, lane 1) and bone marrow (BM) smear (13th November, 1989, lane 9), PGK gene was not studied. lane 1: frozen lymph node biopsied on 20th October, 1988. lane 2: BM smear on 20th October, 1988. lane 3: peripheral blood (PB) smear on 20th October, 1988. lane 4: BM smear when testicular involvement was uncovered (12th September, 1989). lane 5: PB smear on 25th September, 1989. lane 6: PB smear on 30th September, 1989. lane 7: PB smear on 2nd October, 1989. lane 8: PB smear when bone marrow infiltration of malignant cells was identified (17th October, 1989). lane 9: BM smear on 13th November, 1989.

## DISCUSSION

Yamada *et al.* have developed a highly sensitive method for the detection of minimal disease in B lineage leukemias/lymphomas by the use of third complementarity-determining region (CDRIII)-specific probes.[5,6] This technique exploits the N nucleotide sequences of $V_H$-D-$J_H$ joining that occur physiologically in cells of B-cell lineage to identify DNA sequences specific to malignant clones in patients with B-cell malignancies. When compared with conventional Southern hybridization which enables a diagnosis of relapse only when the leukemic blast cell population in the marrow exceeds 1:20, this approach enables the detection of leukemia-specific sequences with a sensitivity of 1:10,000.[5] Since the therapeutic strategy for acute leukemias and lymphomas requires the accurate detection of residual malignant cells

during the course, this approach offers one of the most useful tools for improving the clinical therapeutic strategy.

A common practical problem associated with this approach, however, is that fresh or frozen malignant cells are not available from all the patients. If the original malignant cells cannot be characterized by molecular analysis, subsequent studies may be severely compromised. Therefore it is of clinical importance to widen the scope of this approach to those patients from whom no fresh material is available. As bone marrow and peripheral blood smears from patients with any hematological disease are stored for years, it might be clinically useful if DNA from stored smears could be used as a source of DNA for determining the clone-specific DNA sequence. Using DNA from a bone marrow smear stored at room temperature for more than one year as a PCR template, we have determined the clone-specific DNA sequence in a case of Burkitt's lymphoma. The sequence was compared with that obtained from DNA extracted from the lymph node at the initial presentation (20th October, 1988). As the results show, three KI′ clones obtained from the stored smear were identical to the sequence of KI, which was determined from the DNA of the lymph node (20th October, 1988). Five KII′ clones, obtained from the stored smear, showed a single point mutation from G to A. This is probably because of the extensive somatic hypermutation of Ig gene hypervariable region, as has been demonstrated in some B cell neoplasms.[12] In order to avoid possible false-negative results when the sequence which covers the region of this mutation is used as a probe, a sequence corresponding to ProKI sequence (Fig. 2) was used as a clone-specific oligonucleotide probe. Our results show that when fresh or frozen tumor cells are not available as a source of DNA for the development of a clone-specific probe, stored smears can be used as an alternative material. Furthermore, even DNA from May-Gruenwald Giemsa stained smears was useful for DNA amplification and sequence determination (data not shown). We could not find any D segment reported homologous to the suspected D segment within KI (KI′) and KII (KII′) in either of the EMBL or Gen Bank data bases. This is probably because of either the extensive deletion of 5′ and 3′ D segments or the involvement of novel D segments.

Using this highly sensitive technique, DNA from bone marrow and peripheral blood smears serially obtained and stored were examined retrospectively for the presence of malignant cells. As the results show, malignant cells were not detected in bone marrow or peripheral blood at the time of initial diagnosis. DNA extracted from the bone marrow smear taken when testicular infiltration was diagnosed (12th September, 1989) showed no positive signal, again ruling out the possibility of bone marrow infiltration by malignant cells. The peripheral blood smear taken on 17th October, 1989, when leukemic transformation was diagnosed on the bone marrow smear, however, showed a positive signal. A careful examination of the peripheral blood smear on the microscope suggested that about 2% of the mononuclear cells were blast cells, corresponding to the result obtained by PCR analysis. Until 17th October no peripheral blood smear showed a positive signal. Since the PGK gene was amplified from all the DNA samples from bone marrow and peripheral blood, in which no amplification of CDRIII gene was demonstrated, the negative result was shown not to be due to either DNA degradation or failure to obtain sufficient DNA for the analysis. Accordingly, we showed this approach to be useful in a retrospective study of the clinical stages of a Burkitt's lymphoma patient.

Recent studies in the application of molecular techniques to clinical studies have shown that the variable region gene of the T cell recepter (TCR)$\delta$ as well as the TCR$\gamma$ gene can also be used as clone-specific DNA markers for T cell leukemias.[13, 14] As they are based on the amplification of N nucleotide sequences of VDJ (TCR$\delta$) or VJ (TCR$\gamma$) rearrangements, our approach for development of a clone-specific probe by PCR and sequence analysis of DNA from stored smears can also be applied to T cell leukemias. Thus our approach will widen the scope of molecular analysis of hematologic malignancies of both B- and T-cell lineages to include the investigation of cases where the original malignant cells are not available except for smears on the stored slides. This method can be particularly useful when the amount of bone marrow material is barely adequate to make a few smears for morphological observation, including cytochemical and immunocytochemical studies, because of a dry tap in bone marrow aspiration. In such cases, the smears can be employed for DNA analysis after the completion of morphological observation.

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