Key words: cost, resource utilization, mycosis fungoides, cutaneous T-cell lymphoma, rare disease, Italy.

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Malignant Lymphomas

Seminested polymerase chain reaction and heteroduplex analysis detects the monoclonality of *IgH* rearrangement in follicular lymphoma patients with high sensitivity

A new method, combining seminested polymerase chain reaction (PCR) with heteroduplex analysis, was utilized to detect follicular lymphoma (FL) cells in peripheral blood. The method, based on the detection of *IgH* rearrangements in DNA, detected the presence of monoclonal B cells in FL patients with a high frequency.

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The monoclonality of *IgH* gene rearrangements has been applied to distinguish malignant B cells from normal ones. The complementarity–determining region (CDR) in the variable region of the *IgH* gene is unique for each B cell clone and the CDR III is the clonal signature of an individual B cell.[1,2] The main difficulty of PCR-based clonality studies of B-cell malignancies is to discriminate between monoclonal and polyclonal products, especially when there is a high background of polyclonal B cells in the tumor sample. To discern between them more clearly, we utilized heteroduplex analysis in which PCR products are denaturated at high temperatures and subsequently renatured to induce homoduplex or heteroduplex formation. Genomic DNA was isolated from 24 patients (18 males, 6 females) with histologically verified FL in the Hematology and Medical Oncology Division of Hasan Sadikin Hospital, Bandung, Indonesia. Consensus PCR primers used for amplifying the *IgH* gene were: FR3A (framework 3A), FR1c (framework 1c) for the 3' end of the V region, and LJH (low JH), VLJH (very low JH) for the 3' end of the J region.[3,4] The first round of amplification was performed using an upstream consensus primer FR3A or FR1c and a downstream primer LJH that binds to all published JH gene segments. For reamplification, the lower strand primer (LJH) was replaced by a nested consensus JH primer (VLJH) and a small amount (1%) of the first PCR was used as the template.[5] The seminested PCR conditions were those described previously, with some modifications.[6] For heteroduplex analysis, PCR products were denaturated at 94°C for 10 min and subsequently cooled at 40°C to induce duplex formation. The hetero- and/or homoduplex (10 μL) were characterized by polyacrylamide gel electrophoresis (PAGE) with 10% non-reducing gel in 0.5 ×TBE buffer.[7] The gel was stained with a 1:1000 dilution of SYBR Gold dye in 0.5×TBE buffer for 30 min and visualized under ultraviolet light. One or two thin clear bands within the expected size (75-140 bp for FR3A and 330-350 bp for FR1c) were taken to signify positivity for clonality.[5,7] For sensitivity studies, peripheral blood mononuclear cells (PBMC) from normal subjects and DHL-4 cells (diffuse large B cell lymphoma) were mixed to make a ratio of one DHL-4 cell in 10^2, 10^3, 10^4, 10^5 and 10^6 PBMC and the total cell number was adjusted to 5×10^6. The DNA was extracted from the mixture using a Qiamp DNA Mini Kit (Qiagen).

The PCR products, loaded on 4% agarose gel and stained with ethidium bromide, are shown in Figure 1A. TK cells (diffuse large B cell lymphoma), used as the positive control (lane 2), and a FL patient (lane 4) showed a clear monoclonal band. On the other hand, normal PBMC as the negative control (lane 1) showed a broad band and FL patients (lane 3) had a very faint band. The seminested PCR alone gave a monoclonal band in 10 of 24 samples. However, it was very difficult to judge the presence of a clear monoclonal band in ethidium bromide-stained agarose gel. Therefore, heteroduplex analysis with the same samples shown in Figure 1A was performed by PAGE and visualized by SYBR Gold dye (Figure 1B). The monoclonal band was much easier to detect than with seminested PCR alone. A monoclonal band was seen in 19 of 24 patients (approximately 79%). Among the 24 FL patients, monoclonal *IgH* rearrangement was detected in 2 of 3 patients in stage I, both patients in stage II, 8 of 12 in stage III and all 7 in stage IV. In addition, there was no significant difference in clinical characteristics between patients with and without monoclonality. The method also identified monoclonality in NALM-6 (pre-B acute lymphoblastic lymphoma), RPMI 1788 (B acute lymphoblastic lymphoma) and HD-MY-Z (nodular sclerosis Hodgkin's lymphoma) cell lines. This combined method detected a monoclonal band in the DNA sample from the mixture containing 1 malig-

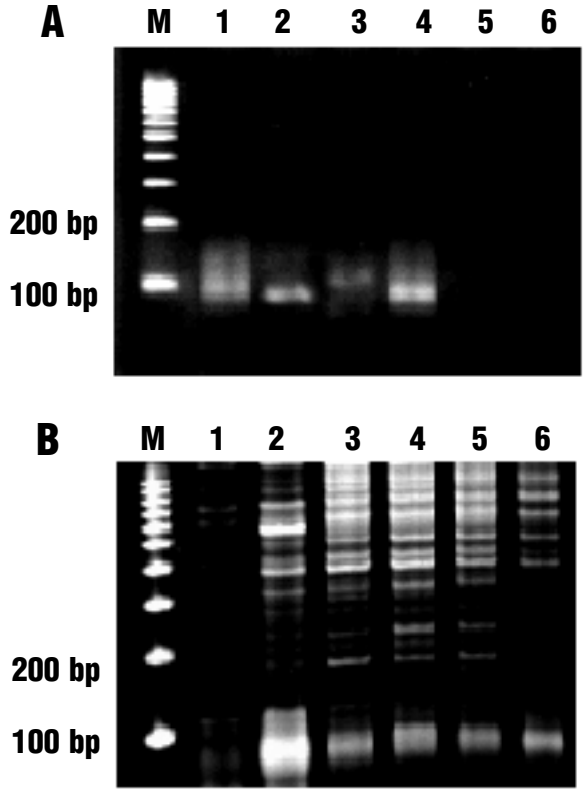

Figure 1. Comparison of the same peripheral blood leukocyte (PBL) DNA samples from FL patients using (A) seminested PCR without heteroduplex analysis with agarose gel and ethidium bromide staining, and (B) seminested PCR with heteroduplex analysis with PAGE and SYBR Gold staining. M, DNA size marker; lane 1, PBMC as the negative control; lane 2, TK cells as the positive control; lanes 3-6, PBL from FL patients. Note that a clear monoclonal band is present between 75 and 140 bp in lanes 2 and 4 in A and in lanes 2-6 in B.

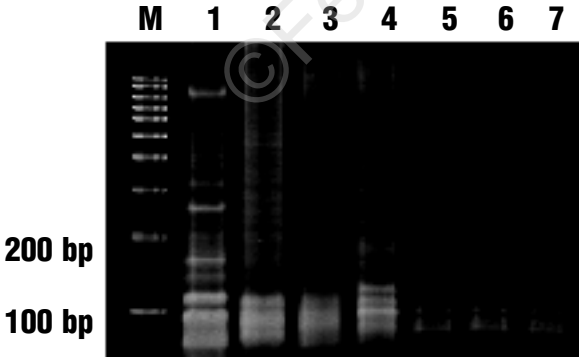

Figure 2. The sensitivity of seminested PCR followed by heteroduplex analysis with PAGE and SYBR Gold staining. DHL-4 cells were diluted in normal PBMC at various concentrations ranging from 10-2 to 10-6 and the DNA was extracted. M, DNA size marker; lane 1, DHL-4, cells; lane 2, 10-2 dilution; lane 3, 10-3 dilution; lane 4, 10-4 dilution; lane 5, 10-5 dilution; lane 6, 10-6 dilution; lane 7, normal PBMC. Note that monoclonal bands could be seen until a 10-4 dilution of DHL-4 cells.

nant cell in 10,000 normal PBMC (Figure 2).

We have established a new sensitive method for detecting monoclonality of *IgH* rearrangements. The method consists of seminested PCR, heteroduplex analysis with PAGE and SYBR Gold staining. This method detected monoclonality of the *IgH* rearrangement in 19 of 24 peripheral blood leukocyte DNA samples from FL patients and it was more sensitive than seminested PCR with PAGE and ethidium bromide.[8,9] The method could detect 1 malignant cell in 10,000 PBMC and was approximately 10 times more sensitive than silver nitrate-stained PAGE after heteroduplex analysis.[7] Our method might be useful for detecting malignant B cells in minimal residual disease or discriminating such cells from a polyclonal expansion of reactive B cells.

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