cases no changes in clonal rearrangements were observed: in fact, cerebral spinal fluid analysis at the time of CNS relapse showed MRD positivity, although this was not observed in bone marrow samples. These observations suggest that molecular relapse might not always be evident in the marrow at the time of extramedullary disease.

In conclusion, kinetic studies relying on accurate, reliable blast quantification might help to identify patients with a high risk of relapse.

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Malignant Lymphomas

Combined seminested polymerase chain reaction and heteroduplex analysis for detecting monoclonality of *IgH* rearrangement in patients with follicular lymphoma

A new, sensitive method combining seminested polymerase chain reaction (PCR) and heteroduplex analysis was used to detect follicular lymphoma (FL) cells in peripheral blood. Based on the detection of *IgH* rearrangement in DNA from peripheral blood leukocytes, the method demonstrated the presence of monoclonal B cells in FL patients with high frequency.

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Monoclonality of *IgH* gene rearrangements has been used to distinguish malignant B cells from normal ones. The complementarity-determining region (CDR) in the variable region of the *IgH* gene is unique to each B-cell clone and the CDR III is the useful clonal signature of an individual B cell.[1,2] The main difficulty of PCR-based clonality studies of B-cell malignancies is discrimination between monoclonal and polyclonal products, especially when there is a high background of polyclonal B cells in tumor samples. 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, Department of Internal Medicine, 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 lowstream primer LJH that bound to all published JH gene segments. For reamplification, the lower strand primer (LJH) was replaced by a nested consensus JH primer (VLJH) and an aliquot (1%) of the first PCR was used as template.[5] The seminested PCR conditions have been described previously, but were used 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 products (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 judged to indicate positive 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 ratios 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 stained with ethidium bromide are shown in Figure 1A. TK cells (diffuse large B cell lymphoma) as the positive control (lane 2) and

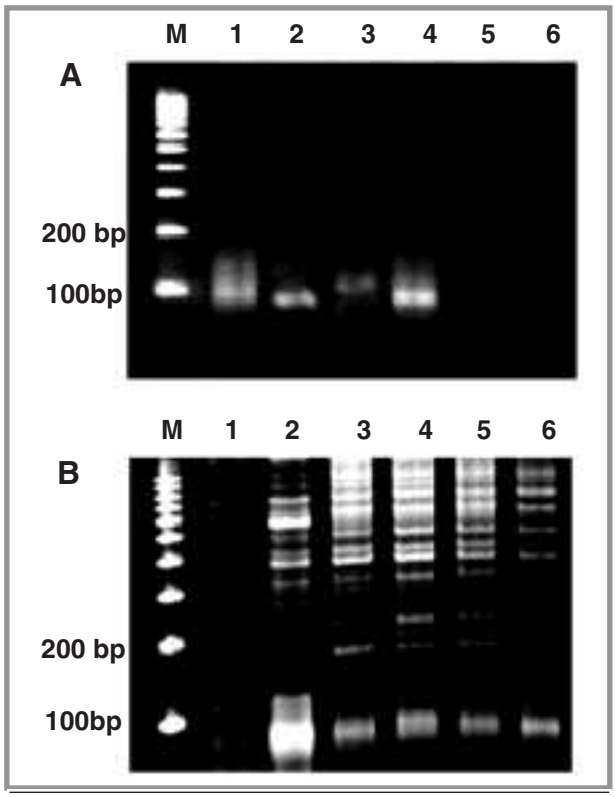

Figure 1. Comparison of the same peripheral blood leukocyte (PBL) DNA samples from FL patients examined by (A) seminested PCR without heteroduplex analysis with agarose gel and ethidium bromide staining, and (B) seminested PCR with heteroduplex analysis with PAGE and SYBR Gold staining. M, DNA size marker; lane 1, PBMC as negative control; lane 2, TK cells as 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 lanes 2-6 in B.

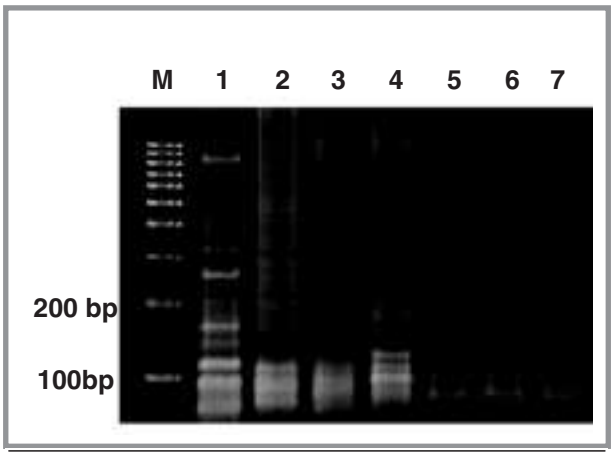

Figure 2. The sensitivity of seminested PCR followed by heteroduplex analysis with PAGE and SYBR Gold staining. DHL-4 cells were diluted in normal PBMC at various concentrations ranging from 10^{-2} to 10^{-6} and the DNA was extracted. M, DNA size marker; lane 1, DHL-4, cells; lane 2, 10^{-2} dilution; 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.

cells from a patient with FL patient (lane 4) both showed a clear monoclonal band. On the other hand, normal PBMC as negative control (lane 1), showed a broad band and FL patients (lane 3) had a very faint band. The seminested PCR alone revealed 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, the heteroduplex analysis with the same samples used in Figure 1A was performed by PAGE and visualized by SYBR Gold dye (Figure1 B). The monoclonal band was much easier to detect than with seminested PCR alone. The monoclonal band was seen in 19 of 24 patients (approximately 79%). In the 24 FL patients, monoclonality in *IgH* rearrangements was found in 2 of 3 patients in stage I, in both of the 2 patients in stage II, in 8 of 12 in stage III and in all 7 patients in stage IV. 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). This combined method detected a monoclonal band in the DNA sample from the mixture containing 1 malignant cell in 10,000 normal PBMC (Figure 2).

In conclusion, we have established a new sensitive method consisting of seminested PCR, heteroduplex analysis with PAGE and SYBR Gold staining. The method detected the monoclonality of *IgH* rearrangement in 19 of 24 peripheral blood leukocyte DNA samples from FL patients and 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 to detect malignant B cells in minimal residual disease or discriminate malignant cells from polyclonal expansions of reactive B cells.

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Key words: seminested PCR, heteroduplex analysis, monoclonality, follicular lymphoma

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Malignant Lymphomas

Sequential chemotherapy regimens followed by high-dose therapy with stem cell transplantation in mantle cell lymphoma: an update of a prospective study

This study is a long-term follow-up analysis evaluating clinical outcome of patients with mantle-cell lymphoma treated by the sequential CHOP and DHAP chemotherapy followed by autografting. The median overall survival of 81 months (95% CI, 66-not reached) and the median event free survival of 51 months (95% CI, 43-not reached) confirm the improvement in outcome obtained by such protocol.

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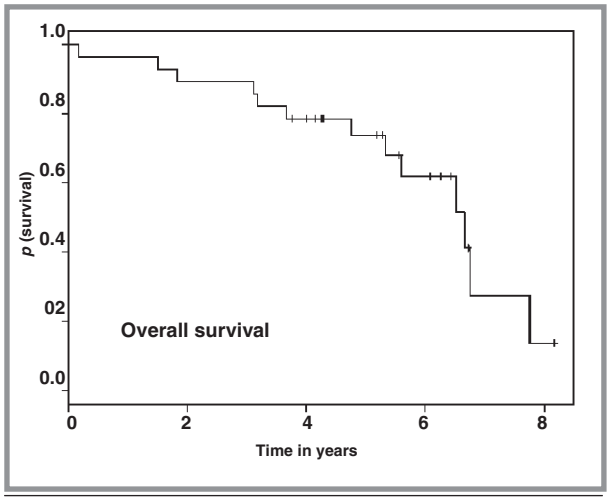

Figure 1. Overall survival (Kaplan-Meier).

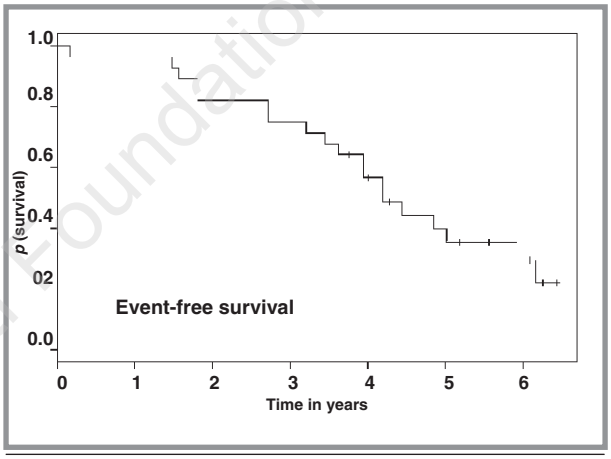

Figure 2. Event-free survival (Kaplan-Meier).

Despite an initial relatively indolent clinical presentation, the prognosis of mantle cell lymphoma (MCL) remains poor with a median survival of less than 3 years.[1-4] We previously reported a prospective study of 28 patients treated between 1995 and 1999 based on the response to initial CHOP therapy with the aims of increasing response rate before autologous peripheral blood stem cell transplantation (APBSCT) and increasing event free and overall survival.[5] Patients who achieved a complete remission (CR) after four cycles of CHOP received total body irradiation (TBI) plus high dose cyclophosphamide and etoposide as a myeloablative regimen before APBSCT. Patients who did not achieve a CR after CHOP were treated with salvage therapy consisting of a high dose cytarabine regimen (DHAP) followed by TBI plus high dose cytarabine plus melphalan (TAM8) and APBSCT. Only two CR and 14 partial responses (PR) were obtained after CHOP. The two patients in CR after CHOP underwent intensification with TBI, high-dose cyclophosphamide-etoposide and unpurged APBSCT. The other twenty-five patients received DHAP and in this group a response rate of 92% (21 CR (84%), two PR (8%)) was observed. Two patients had progressive disease. The twenty-three responding patients received high-dose therapy (TAM8 regimen) followed by APBSCT. There was no conditioning or transplant-related mortality. Here we report the outcome of these patients after a longer median follow-up.

Sixteen (58%) of 28 patients remain alive at the time of data analysis (January, 2004) The median overall survival from diagnosis is 81 months (95% confidence interval, 66-not reached) from the time of diagnosis (Figure 1). In addition, 9 (32%) patients remain alive and progression free (5 patients remaining alive and in remission 6 or more years after treatment). These data result in a median event-free survival of 51 months (95% confidence interval, 43-not reached) (Figure 2).

Our data are similar to those of a published study on the hyper-CVAD/mitoxantrone-cytarabine regimen that contains high dose cytarabine,[6] in which the authors reported a high response rate of 93.5% (CR 38%, PR, 55%) for patients with MCL previously treated (or not). However, the response rate for the subgroup of previously untreated patients was not given and some patients (including some previously treated with chemotherapy) failed to mobilize enough PBSC to harvest for autografting. It is noteworthy that in our study, two courses of DHAP after four cycles of CHOP did not compromise stem cell harvesting, since no patient failed to have adequate peripheral stem cell collection. In a second study, previously untreated 65 years or older patients with MCL were enrolled in sequential phase II trials using hyper-CVAD, alternating with high doses of