**FOLIA HISTOCHEMICA ET CYTOBIOLOGICA**
Vol. 43, No. 1, 2005
pp. 19-23

# Modification of immunocytochemical ZAP-70 assay for potential clinical application in B-cell chronic lymphocytic leukemia

**Agnieszka Bojarska-Junak[1], Jacek Roliński[1] and Jerzy Kawiak[2]**

[1]Department of Clinical Immunology, University School of Medicine, Lublin;
[2]Department of Clinical Cytology, Medical Center of Postgraduate Education, Warsaw, Poland

**Abstract:** The ZAP-70 protein is a member of the Syk/ZAP protein tyrosine kinase family, normally expressed in T cells and NK cells but not found in normal, mature B cells. The protein plays a critical role in the initiation of T-cell signaling. Leukemic cells from patients with B-cell chronic lymphocytic leukemia (B-CLL) that expressed nonmutated immunoglobulin V genes were found to express levels of ZAP-70 protein that were comparable to those detected in T cells of healthy adults. The ZAP-70 protein expression can be evaluated by flow cytometry and may be used as a prognostic marker in B-CLL patients. We modified the method of immunocytochemical assessment of ZAP-70 expression. The traditional two-step method with monoclonal anti-ZAP-70 antibody in the first step followed by FITC-conjugated goat anti-mouse IgG was changed for one-step method with monoclonal anti-ZAP-70 antibody labeled by Zenon Alexa Fluor 488. The method is simple and fast. The major advantage of Zenon labeling technique is its compatibility with simultaneous staining of surface antigens. The cells may be earlier immunostained for CD3, CD19 and/or CD5 to compare of the ZAP-70 kinase expression in B and T cells.

**Key words**: B-CLL, ZAP-70, Flow cytometry

## Introduction

The B-cell chronic lymphocytic leukemia (B-CLL) has variable clinical course. Although the median survival time of patients with this form of leukemia is around 10 years, in individual patients the prognosis is extremely variable. Thus, some patients have a long survival without major progression while others have more aggressive disease with progressive clinical features and require earlier therapy [2, 12]. The prognostic factors and categories are well defined, starting from the clinical staging systems [1, 13] which are the classical guidelines. Other prognostic factors include the performance status, age, pattern of bone marrow involvement, serum levels of $\beta_2$-microglobulin and soluble CD23 molecule, P53 expression, somatic mutation of $V_H$ immunoglobulin genes and different genomic aberrations [8, 10, 15].

There is an intense search for the prognostic markers that might facilitate the treatment of patients according to individual prognosis. It appeared in multivariate analyses that $IgV_H$ gene mutational status is a good predictor of clinical outcome. Patients having B-CLL cells with nonmutated $IgV_H$ genes often have progressive disease and have poorer prognosis, whereas patients whose leukemic cells express mutated $IgV_H$ regions more often have a slowly progressing disease [6, 7]. The prognostic value of the $IgV_H$ mutational status is independent of the clinical stages [2]. However, most clinical diagnostic laboratories are not prepared to perform routinely sequencing of $IgV_H$ genes, the analysis is time consuming and expensive.

It has been found that the expression of the gene coding for ZAP-70 protein (zeta-associated protein 70) is high in B-CLL cells of patients with nonmutated $IgV_H$ genes and low or absent in B-cells of patients with mutated genes [2, 14, 14]. ZAP-70 is a tyrosine kinase protein [3] essential for T cell signaling but not found in normal peripheral blood B cells. Hence, this marker appears to be a good discriminator between the two groups of B-CLL patients [2, 4, 14].

This study presents a modification of previously described immunocytochemical method [5] for flow cytometric examination of ZAP-70 protein expression

**Correspondence:** A. Bojarska-Junak, Dept. Clinical Immunology, University School of Medicine, Jaczewskiego 8, 20-950 Lublin, Poland; e-mail: agnieszkajunak@poczta.onet.pl

in B cells from patients with B-CLL. The modified procedure is a one-step immunocytochemical method using monoclonal anti-ZAP-70 antibody conjugated with labeled Fab fragments of Fc-specific anti-mouse IgG2a antibody.

## Materials and methods

**Reagents.** Anti-ZAP-70 antibody, clone 2F3.2 (mouse monoclonal $IgG_{2a}$) (lot. 24920) was purchased from Biomol Research Laboratories and clone 29 mouse $IgG_{2a}$ (cat. 610239) from Pharmingen. Goat anti-mouse FITC-conjugated IgG (lot. 41868) was provided by Becton Dickinson. Zenon™ Alexa Fluor® 488 Mouse $IgG_{2a}$ Labeling Kit (lot 75C4-1 and 75C3-1) was obtained from Molecular Probes. Monoclonal antibodies: anti-CD19 PE, anti-CD3 PE, anti-CD5 CyChrome and the negative controls were obtained from Caltag Laboratories. Monoclonal antibody anti-CD19 CyChrome was purchased from Becton-Dickinson. Monoclonal antibody anti-CD3 conjugated with magnetic beads was provided by Miltenyi Biotec.

**Patients and samples.** Peripheral blood samples were obtained from 22 newly diagnosed, untreated patients with B-CLL. The diagnosis of B-CLL was made on the basis of clinical examination, as well as morphological and immunological criteria. All peripheral blood samples were collected in heparinized tubes and immediately processed. Mononuclear cells were isolated by density gradient centrifugation on Gradisol L (Aqua Medica, Poland) for 25 min at 400 × g, at room temperature. The interphase cells were removed and washed twice in phosphate-buffered saline (PBS) and then resuspended at $1\times10^6$ cells per tube for future assays. Peripheral blood from 11 healthy persons was also analyzed. The written consent was obtained from the patients and healthy volunteers.

**Two-step ZAP-70 immunocytochemical reaction using FITC-conjugated goat anti-mouse IgG.** The CD19+ B cells used in this experiment were purified by negative selection using the magnetic-activated cell-sorting (MACS) system. They were purified from patients' blood mononuclear cells by treatment with the specific monoclonal antibody (mAb) against T lymphocytes (anti-CD3) conjugated with magnetic beads according to the manufacturer's instruction. The purification was verified by flow cytometry: B-CLL cells contained more than 97% of CD19+ lymphocytes. B lymphocytes from 10 healthy adults were also separated in the same way.

Expression of ZAP-70 in the purified CD19+ cells was measured by an indirect labeling procedure using FITC-conjugated goat anti-mouse IgG. Briefly, purified B lymphocytes were fixed with 1% paraformaldehyde in PBS for 15 min at room temperature. Next, 70% ethanol was added to permeabilize the cell membranes (1 h at -20˚C). After fixation and permeabilization, cells were incubated with 0.5 µg, 1 µg or 2 µg of anti-ZAP-70 primary antibody per $10^6$ cells for 30 min at room temperature. Next, the cells were washed with PBS and then treated with FITC-conjugated goat anti-mouse IgG as a secondary antibody. Following an additional 30-min incubation at room temperature, cells were washed with PBS and analyzed by flow cytometry.

**One-step ZAP-70 immunocytochemical reaction using Zenon™ Alexa Fluor® 488 Mouse $IgG_{2a}$ Labeling Kit.** For ZAP-70 immunocytochemical reaction, concentrations of 0.5 µg, 1 µg or 2 µg of anti-ZAP-70 antibody per $10^6$ cells were tested. The best results were obtained with 1µg mAb/$10^6$ cells, and it was used in further experiments. Five µl of Zenon mouse IgG labeling reagent, which contains a fluorophore-labeled Fab fragment, were mixed with 1 µg (5 µl) of the antibody solution. The mixture was incubated for 5 minutes at room temperature. Then 5 µl of the Zenon blocking reagent was added to the reaction mixture. The solution was incubated for additional 5 min and applied to the cell sample.

One hundred µl of peripheral blood mononuclear cells ($1\times10^6$ cells/ml) were added to a flow cytometry tube and immunostained with monoclonal antibodies against the cell-surface markers: CD19 PE, CD19 CyChrome, CD5CyChrome or CD3 PE. The cells were then fixed in 1% paraformaldehyde solution in PBS for 15 min at room temperature, permeabilized with 70% ethanol for at least 1 h at -20˚C and washed with PBS. After centrifugation, the supernatant was discarded. Next, anti-ZAP-70 antibody, labeled using the Zenon mouse IgG labeling reagent, was added to the sample tubes. The samples were incubated for 10 or 30 min at room temperature, washed once with PBS and immediately analyzed by flow cytometry. The best results were obtained following an incubation for 30 min, and it was used in further experiments. The cells identified as CD3-positive cells and CD19-negative lymphocytes (generally T and NK cells) served as internal positive controls (Fig. 1).

**Flow cytometry analysis**. Samples were analyzed by flow cytometry using a Becton Dickinson FACSCalibur instrument equipped with 488-nm argon laser. Five data parameters were acquired and stored: linear forward and side scatter (FSC, SSC), FL-1(FITC), FL-2(PE) and FL-3(CyChrome). An acquisition gate was established based on FSC and SSC that included mononuclear cells and excluded dead cells and debris. For each analysis, 10 000 events were acquired and analyzed using CellQuest software.

**Statistical analysis.** Nonparametric statistics was used. Median values as well as 25th percentile (P25) and 75th percentile (P75) values were calculated. The two-group Mann-Whitney U-test was used for comparison of the results.

## Results

In our first assessment of ZAP-70 protein expression, we followed description of other authors [3], using two-step immunocytochemical procedure. The B CD19+ cells from untreated patients (n=18) with B-CLL were purified by negative selection using specific mAb against T lymphocytes conjugated with magnetic beads. More than 97% of the cells in each case expressed CD19. In these samples assessed with two-step procedure, the proportion of B cells expressing ZAP-70 was 13.6% to 83.8%. In healthy blood donors (n = 10), the proportion of B cells expressing ZAP-70 was in the range of 4.3% to 10.3 %. Median value was 7.1%, P25-P75 = 6.3-8.4%.

Then we tested the modified, one-step immunocytochemical procedure with the peripheral blood mononuclear cells to assess ZAP-70 protein expression. In this technique, fluorochrome-labeled antibodies against cell surface markers were also used. The sequence of analysis for identification of CD19+/CD5+ leukemic cells and CD3 T cells expressing ZAP-70, following three or two-color labeling is presented in Figure 1. The percentage of ZAP-70+/CD19+/CD5+ cells in untreated patients (n = 22) was in the range of 2.4-83.7%. As observed by Crespo *et al.* [5], in patients in whom 20% or more of the leukemic cells are positive for ZAP-70, *IgVH* is nonmutated, whereas $IgV_H$ mutations are found in patients in whom less than 20% of the leukemic cells are positive for ZAP-70. Assuming this cut-off value, 12 patients tested with the modified method had ZAP-70-positive CD19+/CD5+ leukemia cells in the lower range

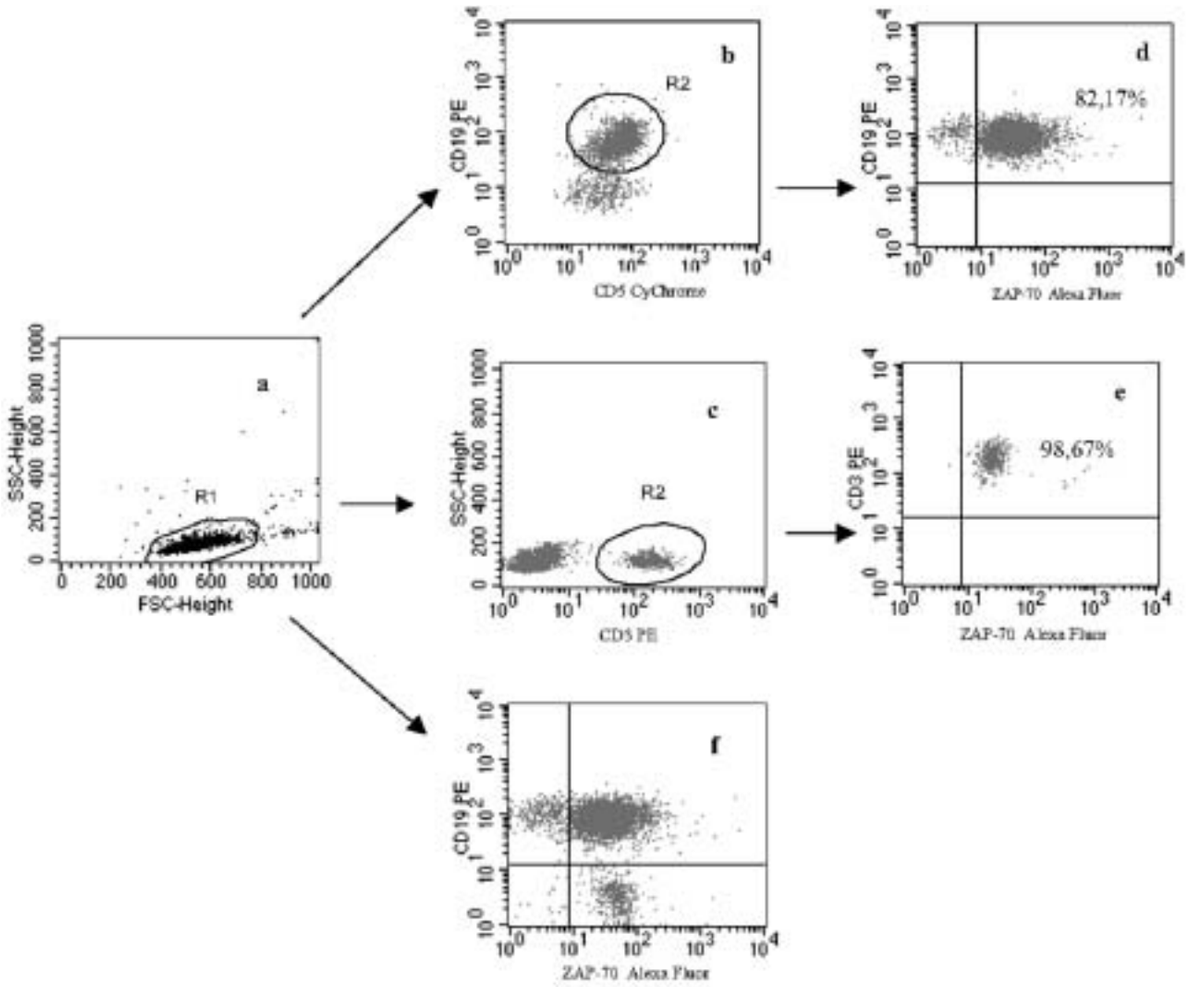


**Fig. 1.** The dot plots show representative data from one B-CLL patient, illustrating the method for identification of CD19+/CD5+ leukemic cells and CD3+ T-cells expressing ZAP-70 following three- or two-color staining. ZAP-70 antibody was labeled using Zenon technique. An acquisition gate was established based on FSC and SSC that included mononuclear cells. Region R1 was drawn around the lymphocytes **(a)**. Next, the R1 gated events were analyzed for CD19+PE/CD5+CyChrome or CD3 PE staining and positive cells (CD19+/CD5+ or CD3+) were selected (region R2) **(b, c)**. We used dot plots of CD19 PE versus CD5 CyChrome **(b)** or SSC versus CD3 PE **(c)**. The final dot plots: CD19 PE vs ZAP-70 **(d)** and CD3 PE vs ZAP-70 **(e)** were established by combined gating of events using R1 and R2. The numbers in the upper right quadrant on the dot plots **d, e** represent the percentage of CD19+/CD5+/ZAP-70+ and CD3+/ZAP-70+ cells. The dot plot **f** shows ZAP-70 expression after all lymphocyte gating (R1). Lower right quadrant shows ZAP-70 expression by CD19- lymphocytes (generally T and NK cells), which provided a useful positive control for antibody activity.

(2.4-19.1%, median value 10.7%, P25-P75 = 7.1-14.8%). The other ten patients had ZAP-70-positive leukemia cells in the higher range (20.5-83.7%, median value 47.4%, P25-P75 = 27.3-49.0%). In all samples, ZAP-70 was positive in CD3+ T cells, which were considered as the internal standard.

In samples from healthy volunteers, the proportion of B cells (CD19+) expressing ZAP-70 was 0.3% to 5.6%, median value 3.7%, P25-P75 = 1.0-2.0%. These results are in accordance with findings of other authors [5, 11]. The percentage of ZAP-70-positive B lymphocytes for healthy persons when tested with both described procedures are significantly different ($p=0.0003$), what suggests that the preparation of the cells and the immunocytochemical procedure influence the results. Figure 2 shows ZAP-70 protein expression by B CD19+ and T CD3+ cells from healthy person.

The monoclonal anti-ZAP-70 antibody of clone 29 from Pharmingen labeled with Zenon and tested with the one-step procedure was found to nonspecifically bind to peripheral blood B lymphocytes (CD19+) of healthy persons as well as of patients. Therefore, data presented above included only results of flow cytometric analysis of samples labeled by anti-ZAP-70 antibody purchased

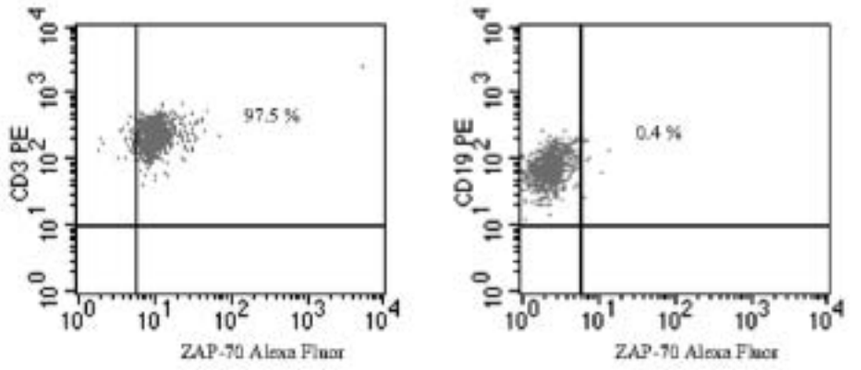


**Fig. 2.** Dot plots from flow cytometry showing ZAP-70 protein expression by normal B (CD19+) and T (CD3+) cells.

from Biomol Research Laboratories. The antibody of the clone 29 was previously used for immunoprecipitation or Western blot analyses only [4].

## Discussion

ZAP-70 expression is an unusual finding in chronic lymphocytic leukemia B-cells, since the protein has not been reported in normal circulating B cells. Recent studies have shown that expression of the protein tyrosine kinase ZAP-70 may serve as a prognostic marker in B-CLL which will eventually predict survival of the patients [2, 5]. The analysis of B-CLL cells suggests that the $IgV_H$ gene mutation status is stably coexpressed with ZAP-70 protein [2], while expression of another marker - CD38 antigen may change in the course of the disease [6]. Moreover, the study of Crespo *et al.* [5], who used the same clone (2F3.2) anti-ZAP-70 antibody as that used in the present study, showed that the leukemic cells without $IgV_H$ mutations express at least 20% ZAP-70-positive cells. In our experiments, we observed expression of ZAP-70 protein in a small subpopulation of peripheral blood B lymphocytes of healthy volunteers. A significantly lower median value of this subpopulation was observed after the modified procedure as compared with the double-step method, possibly due to the nonspecific, higher background in the latter. These differences, however should not influence the clinical prognostic result in most patients, specially since some authors propose 15% ZAP-70-positive cells as the cut-off value [11]. The leukemic cells of patients with $IgV_H$ mutations have barely detectable levels of ZAP-70. On the other hand, in the immunoblot analyses of Chen *et al.* [4], B-CLL cells with nonmutated immunoglobulin V genes were found to express levels of ZAP-70 protein that were comparable to those detected in the peripheral blood T-cells of healthy adults. In our pilot study [9], an increased percentage of ZAP-70-positive leukemic cells was associated with short time to progression, lymphocyte count and disease risk status. Moreover, the majority of ZAP-70-negative leukemia cells were observed in patients with stable disease.

In our assessment of ZAP-70 protein expression, two different approaches were used to determine the best method of detecting purified anti-ZAP-70 monoclonal antibody ($IgG_{2a}$): FITC-conjugated goat anti-mouse IgG and Zenon Mouse IgG labeling procedure. In general, both methods may be used to identify ZAP-70 expression, however each method has its advantages and limitations. In our opinion, Zenon Mouse IgG labeling procedure is simple and gives reproducible results, as required for clinical procedures. This technology is designed for use with a particular mouse monoclonal antibody isotype ($IgG_{2a}$) and may be applied in other immunocytochemical procedures as well. For assessment of cell-surface phenotype, mouse antibodies of other isotype ($IgG_1$) may be used. In the procedure, individual cells can be sequentially analyzed for the expression of surface markers and intracellular ZAP-70 protein.

In summary, the Zenon labeling of ZAP-70 antibody used in the immunocytochemical method produces less nonspecific background than the two-step procedure with FITC-conjugated goat anti-mouse IgG. The modified assay is easy to perform, does not require purification of the B cells, and may be suggested to become an established clinical prognostic test available to all B-CLL patients.

**Acknowledgements:** Supported in part by grant 501-1-1-24-01/03 and Polish Scietific Committee (KBN) 2 P05B 119 27.

## References

[1] Binet JL, Auquier A, Dighiero G, *et al.* (1981) A new prognostic classification of chronic lymphocytic leukemia derived from a multivariate survival analysis. Cancer 48: 198-206
[2] Bosch F, Villamor N (2003) ZAP-70 expression in chronic lymphocytic leukemia: a new parameter for an old disease. Haematologica 88: 724-726
[3] Chan AC, Iwashima M, Turck CW, Weiss A (1992) ZAP-70: a 70 kd protein-tyrosine kinase that associates with the TCR zeta chain. Cell 71: 649-662

[4] Chen L, Widhopf G, Huynh L, Rassenti L, Rai KR, Weiss A, Kipps T (2003) Expression of ZAP-70 is associated with increased B-cell receptor signaling in chronic lymphocytic leukemia. Blood 100:4609-4614
[5] Crespo M, Bosch F, Villamor N, Bellosillo B, Colomer D, Rozman M, Marce S, Lopez-Guillermo A, Campo E, Montserrat E (2003) ZAP-70 expression as a surrogate for immunoglobulin-variable-region mutations in chronic lymphocytic leukemia. N Engl J Med 348: 1764-1775
[6] Damle RN, Wasil T, Fais F, Ghiotto F, Valetto A, Allen SL, Buchbinder A, Budman D, Dittmar K, Kolitz J, Lichtman SM, Schulman P, Vinciguerra VP, Rai KR, Ferraini M, Chiorrazzi N (1999) Ig V gene mutation status and CD38 expression as a novel prognostic indicators in chronic lymphocytic leukemia. Blood 94: 1840-1847
[7] Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK (1999) Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. Blood 94:1848-1854
[8] Klein U, Tu Y, Stolovitzky GA, Mattioli M, Cattoretti G, Husson H, Freedman A, Inghirami G, Cro L, Baldini L, Neri A, Califano A, Dalla-Favera R, Hamblin TJ (2001) Gene expression profiling of B cell chronic lymphocytic leukemia reveals a homogeneus phenotype related to memory B cells. J Exp Med 194: 1625-1638
[9] Kowal M, Dmoszyńska A, Bojarska-Junak A, Roliński J, Wawrzaszek P (2003) Prognostic significance of ZAP-70 expression in B-cell chronic lymphocytic leukemia (CLL). Blood 102: 349b
[10] Montserrat E (2002) Classical and new prognostic factors in chronic lymphocytic leukemia: where to now? Haematol J 3: 7-9
[11] Orchard JA, Ibbotson RE, Davis ZA, Wiestner A, Rosenwald A, Thomas PW, Hamblin TJ, Staudt LM, Oscier DG (2002) ZAP-70 expression and prognosis in chronic lymphocytic leukemia. Lancet 363:105-111
[12] Polliack A (2003) Current therapeutic options for subgroups of chronic lymphocytic leukemia. Planning risk-adapted treatment according to recognized prognosis factors. Haematologica 88: 726-728
[13] Rai KR, Sawitsky A, Cronkite EP, Chanana AD, Levy RN, Pasternack BS (1975) Clinical staging of chronic lymphocytic leukemia. Blood 46: 219-234
[14] Rosenwald A, Alizadeh AA, Widhopf G, Simon R, Davis RE, Yu X, Yang L, Pickeral OK, Rassenti LZ, Powell J, Botstein D, Byrd JC, Grever MR, Cheson BD, Chiorazzi N, Wilson WH, Kipps TJ, Brown PO, Staudt LM (2001) Relation of gene expression phenotype to immunoglobulin mutation genotype in B cell chronic lymphocytic leukemia. J Exp Med 194: 1639-1648
[15] Zwiebel JA, Cheson BD (1998) Chronic lymphocytic leukemia: staging snd prognosis factors. Semin Oncol 25: 42-59

*Received March 1, 2004*
*Accepted after revision October 11, 2004*