High expression of cyclin B1 predicts a favorable outcome in patients with follicular lymphoma

Erik Björck, Sara Ek, Ola Landgren, Mats Jerkeman, Mats Ehinger, Magnus Björkholm, Carl A. K. Borrebaeck, Anna Porwit-MacDonald, and Magnus Nordenskjöld

Substantial research has been dedicated to the study of the relationship between genetic mechanisms regulating cell functions in tumors and how those tumors respond to various treatment regimens. Because these mechanisms are still not well understood, we have chosen to study the genetic makeup of 57 tumor samples from patients with follicular lymphoma (FL). Our goal was to develop a prognostic tool, which can be used as an aid in determining FL patients with tumors genetically predisposed to a successful treatment with the CHOP (cyclophosphamide, vincristine, doxorubicin, prednisone) regimen. To select relevant genes, high-density oligonucleotide arrays were used. There were 14 genes highly expressed in FL patients that responded well to CHOP chemotherapy, and 11 of these were involved in G_2/M transition of the cell cycle, in mitosis, or in DNA modulation. A high expression of *CCNB1* (cyclin B1), *CDC2, CDKN3A, CKS1B, ANP32E,* and *KIAA0101,* but not of the proliferation-related antigen Ki-67, was associated with better survival rate in a univariate analysis. *CCNB1* expression had an independent prognostic value when included in a multivariate analysis together with the 5 parameters of the follicular lymphoma international prognostic index. (Blood. 2005;105:2908-2915)

Introduction

Follicular lymphomas (FLs) are the second largest group of non-Hodgkin lymphomas (NHLs) in the Western world. The clinical course of FL is often indolent, with a median survival of 10 years.[1] Some patients can remain untreated with no signs of progression for several years, while others may suffer a rapid progression of the disease.[2] The heterogeneity of the clinical course has led to various options of treatment including watchful waiting, oral alkylating agents, purine nucleoside analogues, combination chemotherapy, interferon, and monoclonal antibodies.[3] However, it is still unclear which patients will benefit from aggressive treatment. The International Prognostic Index, originally developed for aggressive NHL,[4] was not very useful in predicting outcome for FL NHL patients since only few of the FL patients were categorized in the high-risk group.[5] In the recently published Follicular Lymphoma International Prognostic Index (FLIPI),[5] 8 parameters had independent prognostic value: age; Ann Arbor stage; number of nodal sites; hemoglobin level; lactate-dehydrogenase (LDH); sex; bone marrow involvement; and peripheral blood lymphocyte count. Age was the strongest predictor of survival and was selected together with stage, number of nodal sites, hemoglobin level, and LDH to build the FLIPI.

Prediction of treatment outcome is important to avoid overtreatment of some patients and to identify others in whom less intensive treatment is likely to fail. Initial response to chemotherapy has been shown to be associated with prolonged survival in patients with FL.[6] Also, several studies tried to define which of the so-called tumor-related factors have impact on survival; patients with FL grade 3 with a significant diffuse component had a shorter survival compared with those with FL grades 1 and 2.[7] Overexpression of the *BCL-XL* or *BCL-2* mRNA, or mdm2 protein has been reported to correlate with an inferior survival, while patients with tumors demonstrating high expression of bcl-6 had a favorable prognosis.[8-11] Molecular and cytogenetic studies have indicated that a partial deletion of chromosome 6, or deletion of the short arm of chromosome 17, or trisomy 12 have been associated with adverse clinical outcome.[12,13]

The potential link between a good response to chemotherapy and a good clinical outcome prompted us to apply the response to cyclophosphamide, vincristine, doxorubicin, prednisone (CHOP)[14] as criteria to define the groups of FL patients in whom the differential gene expression in tumor samples taken at diagnosis was investigated. A group of differentially expressed genes has been defined, and the impact of these genes' expression on patients' outcome was further investigated in a cohort of 57 patients with FL.

Patients, materials, and methods

Patient characteristics

Lymph node tumor samples taken at diagnosis from 57 FL patients were included in this study. The 44 samples that were diagnosed at Karolinska

From the Department of Molecular Medicine, the Department of Medicine, Division of Hematology, and the Department of Pathology, Karolinska University Hospital and Karolinska Institutet, Stockholm, Sweden; the Department of Immunotechnology, Lund University, Lund, Sweden; and the Department of Oncology, Department of Pathology, Lund University Hospital, Lund, Sweden.

Submitted July 16, 2004; accepted November 25, 2004. Prepublished online as *Blood* First Edition Paper, December 2, 2004; DOI 10.1182/blood-2004-07-2721.

Supported by grants from the Swedish Cancer Society, the Swedish Society of Medicine, the Stockholm County Council, the Swedish Society for Medical Research, the King Gustave V Jubilee Foundation, and BioInvent International AB (S.E., C.A.K.B.).

Reprints: Anna Porwit-MacDonald, Department of Pathology, Radiumhemmet, Karolinska University Hospital, SE-171 76 Stockholm, Sweden; e-mail: anna.mac-donald@karolinska.se.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

Hospital, Stockholm, Sweden, were a part of a consecutive series of 66 diagnostic specimens frozen from 1994 to 1999. Of 66 samples, 22 were excluded due to poor RNA quality (n = 14), insufficient material (n = 5), and insufficient microarray data quality due to low number of genes with a present call (16.2%, 22.3%, 22.4%) (n = 3). To increase the number of investigated tumors, we have also included 13 samples obtained from patients diagnosed at Lund University Hospital, Sweden (1996-2001). Tumor characteristics and clinical data of included patients are given in Table 1. There were 31 women and 26 men. The median age at diagnosis was 60 years (range, 28-85 years). Clinical staging was performed according to the Ann Arbor classification[15]; 21 patients had clinical stages I to II and 36 patients, stages III to IV. According to the FLIPI,[5] 28 patients belonged to the low-risk group, 16 patients to the intermediate-risk group, and 13 patients to the high-risk group. The distribution among risk groups did not differ from that of the patients with FL that were used to design the FLIPI. At last follow-up, 35 patients were alive and 22 patients had died. Median follow-up time for patients still alive was 65 months (range, 20-117 months). The histopathologic slides of all cases were revised and classified according to WHO classification as FL grade 1 (n = 12), grade 2 (n = 32), and grade 3 (n = 13).[1] Approval for this study was obtained from the ethics committee at Karolinska Institutet; informed consent was not required since the analyses were performed on retrospective patient material.

RNA extraction and microarray hybridization

Frozen tissue (30 to 50 mg; −70°C) from tumor samples was homogenized with a Polytron tissue tearer (Biospec Products, Bartlesville, OK) in 1 mL Trizol (Invitrogen, Carlsbad, CA). Prior to RNA isolation, 5 samples (patient nos. 23, 36, 46, 53, 54) were purified to 85% to 95% tumor content with T-cell depletion as previously described.[16] RNA was extracted with Trizol according to the manufacturer's instructions, followed by purification with RNeasy mini kit (Qiagen, Hilden, Germany). To evaluate the RNA purity, the $A_{260/280}$ ratio (1.9-2.1) was determined by spectrophotometry, followed by denaturating gel electrophoresis where the RNA quality was visually estimated by the presence of 2 sharp RNA bands. Furthermore, the RNA quality was confirmed by analysis in an Agilent 2100 Bioanalyser (Agilent Technologies, Palo Alto, CA). All preparation of mRNA, in vitro transcription and hybridization of labeled cRNA, and scanning of the U95Av2 oligonucleotide array (Affymetrix, Santa Clara, CA) were performed as previously described.[17]

Microarray analysis

The expression level for each probe set was given as a signal value and a detection call (present, absent, or marginal) by the Microarray Suite 5.0 software (Affymetrix). To enable interarray comparison, the signal values were scaled in Microarray Suite, against a target value of 100. The scaling factor was 0.68 to 3.43, and 46 of 57 samples had results within a factor of 3 (0.79-2.25). The range of probe sets with a present call was 34.3% to 54.9%. Microarray Suite comparison analysis (batch analysis) was used to compare the arrays. The created comparison files were imported into Genespring 6 (Silicon Genetics, Redwood City, CA) for gene selection and further analysis.

Selection of patients according to response to CHOP chemotherapy

The WHO criteria for evaluation of the response to chemotherapy were applied to define groups of patients with complete remission (CR), partial response (PR), and progressive disease (PD).[18] The algorithms used to define probe sets that were differentially expressed in patients who responded to CHOP therapy with CR or PD are given in Table 2, and details of treatment are shown in Table 1. To define the probe sets with differential expression between CR and PD patients, 2 main comparisons were made. Tumors selected for the first comparison fulfilled all of the following strict criteria: (1) CHOP was given as the first-line treatment; (2) time to treatment was less than 6 months from diagnosis; (3) 4 or more courses of CHOP were given; and (4) treatment response was either CR or PD.

In this comparison, 3 tumors from patients with PD (nos. 8, 11, 14) were compared with 10 tumors from patients who obtained CR (nos. 13, 25, 30, 33, 35, 38, 50, 51, 54, 55). The results of this comparison were used as a main selection of probe sets. To further select relevant probe sets and to narrow the number of probe sets, a second comparison was made using the following criteria: (1) CHOP was given either as the second-line treatment (minimum 2 courses) or as the first-line treatment with 3 courses given (patient no. 45); and (2) treatment response was either CR or PD.

In this second comparison, 5 tumors from patients with PD (nos. 2, 7, 9, 10, 19) were compared with 3 tumors from patients with CR (nos. 45, 48, 56). The average time between the biopsy and CHOP treatment was 1.7 months (range, 0-5 months) in the first comparison and 33 months (range, 1-84 months) in the second comparison. Since they did not fulfill the selection criteria, 6 tumors from patients who received CHOP were not included in the first or second comparison. These patients either responded with PR or received only one course of CHOP.

To compare samples from patients responding with PD with samples from CR patients, comparison files were created with Microarray Suite 5.0. The comparison analysis was based on 2 algorithms: change algorithm and signal log ratio algorithm. Briefly, the change algorithm indicates increase, decrease, or no change, and the signal log ratio algorithm calculates the log ratio (base 2), between the compared arrays. A detailed description of the algorithms has been published.[19]

To consider the expression of a probe set significantly lower in the PD group compared with CR group, it had to be down-regulated by both algorithms in both comparisons. To consider the expression of a probe set significantly higher in the PD group compared with the CR group, it had to be up-regulated by both algorithms in both comparisons. A cross-comparison was also made where the tumors from the CR patients from the first comparison were compared with tumors from PD patients of the second comparison and vice versa (Table 2).

Tumor samples used to identify genes coexpressed with *KIAA0101*

Since *KIAA0101* was one of the genes selected by the previous comparisons and the function of this gene is unknown, we investigated the expression pattern of this gene and its correlation with the global gene expression pattern in other samples. To avoid bias from the 21 FLs of the previous comparisons, this correlation study was done using the results from the remaining 36 FL together with 21 mantle cell lymphomas and 11 samples representing 5 different B-cell populations from reactive tonsils. The mantle cell lymphomas and B-cell populations have previously been described in detail.[16,17]

Immunohistochemistry

The protein expression of Ki-67, cyclin B1, and cyclin A was evaluated by immunohistochemistry (IHC). Briefly, 5-μm paraffin sections were deparaffinized, rehydrated, and cooked in a microwave oven (in citrate buffer, pH 6: for cyclin A, at 750 W until boiling then at 500 W for 10 minutes and for cyclin B1, 10 minutes at 750 W and 10 minutes at 450 W; in citrate buffer, pH 7.3: for Ki-67, at 300 W for 3 × 10 minutes). Incubation was carried out for 1 hour at room temperature with primary antibodies for cyclin B1 (Clone 7A9, dilution 1:40; Novocastra Laboratories, Newcastle upon Tyne, United Kingdom) and cyclin A2 (Clone 6E6, dilution 1:100; Novocastra). Cyclin B1 and cyclin A visualization was carried out using the immunoperoxidase method, with Strept ABComplex/HRP Duet Mouse/Rabbit and diaminobenzidine (DAB) used as substrate (DakoCytomation, Glostrup, Denmark). For Ki-67 (Clone M7240, dilution 1:50; DakoCytomation), the slides were stained using a NexES IHC staining module (Ventana Medical Systems, Tucson, AZ) with enhanced DAB paraffin procedure. Distinct nuclear staining pattern was obtained in all tumors with cyclin A and Ki-67 antibodies. For cyclin B1, only 51 of the 57 tumors were successfully stained, despite repeated staining attempts in the remaining 6 cases. Cyclin B1 showed preponderant cytoplasmic staining (Figure 1).

To evaluate cyclin B1 and cyclin A expression, 2 different counting methods were used. In the first method, 4 representative high-power fields (HPFs) were evaluated and the numbers of positive cells per field were

Table 1. Characteristics of 57 follicular lymphoma patients studied

Patient	Cyclin B1*	Age, y	Sex	Stage	Hemoglobin level, g/L	FLIPI	WHO grade	Ki-67†	Cause of death	Follow-up, mo	Treatment in temporal order‡
1	11.4	81	F	IIA	102	1	3A	3	Colon cancer	15	KNOSPE
2	11.8	68	F	IIIA	123	3	2	1	Lymphoma	56	KNOSPE, CHOPx3, MIME
3	17.7	42	M	IA	160	0	2	1	Alive	82 +	RT, rituximab
4	26.7	49	F	IIIA	124	2	2	2	Alive	65 +	KNOSPE
5	26.8	76	M	IA	172	1	2	1	Alive	87 +	No treatment
6	28.1	67	M	IA	159	1	2	1	Alive	84 +	RT
7	30.6	61	F	IIA	146	1	1	0	Lymphoma	51	KNOSPE, RT, CNOPx5 + MTX, LD-ACOP-B
8	32.7	83	M	IVB	127	2	2	1	Lymphoma	8	CHOPx6, RT
9	34.2	28	F	IIIA	143	2	2	1	Lymphoma	56	KNOSPE, RT, rituximab, fludarabine, CHOPx5, RT
10	34.3	42	M	IVA	125	2	2	0	Lymphoma	89	Flu/Cycl, CHOPx3, MIME, RT, gemcitabine
11	34.5	60	M	IVA	115	4	1	0	Alive	53 +	Rituximab, CHV(O)Px6, RT, KNOSPE, MIME, Flu/Cycl
12	35.9	53	F	IIIA	133	1	1	1	Lymphoma	80	KNOSPE, CHOPx8, Flu/Cycl, MIME
13	36.0	75	M	IIIB	153	3	2	1	Lymphoma	44	CHOPx8, KNOSPE, ENAD
14	36.7	77	M	IVA	111	5	3B	3	Lymphoma	7	CHOPx6
15	40.4	59	F	IA	124	0	2	1	Lymphoma	55	RT, CHOPx8, ENAD, MIME
16	40.6	57	F	IVA	138	1	1	1	Lymphoma	81	KNOSPE, CHOPx6, rituximab, RT, Flu/Cycl
17	43.0	75	M	IIIA	142	4	2	3	Lymphoma	41	KNOSPE
18	43.0	82	F	IA	118	2	2	1	Lymphoma	64	KNOSPE
19	46.6	66	M	IVA	135	2	1	0	Lymphoma	10	Prednimustine, CHOPx2, RT-CEP
20	47.0	79	M	IIIB	109	4	2	1	Lymphoma	8	KNOSPE
21	47.9	73	M	IIIA	172	4	1	1	Lymphoma	95	KNOSPE
22	47.9	60	F	IA	133	0	2	1	Alive	63 +	No treatment
23	48.1	43	F	IIIA	118	2	2	1	Alive	20 +	No treatment
24	50.7	45	M	IIIA	154	1	2	1	Alive	50 +	No treatment
25	51.1	68	F	IVB	145	4	1	0	Alive	67 +	CHOP×8, KNOSPE, MIME
26	51.8	47	F	IVA	136	1	2	2	Alive	72 +	KNOSPE
27	52.6	56	F	IVB	139	1	1	0	Lymphoma	82	CdA, KNOSPE
28	54.1	61	M	IA	159	1	3A	2	Alive	59 +	RT
29	59.0	57	F	IVA	135	1	2	1	Alive	87 +	CHOPx1, RT
30	63.1	28	M	IVB	114	3	3B	3	Alive	65 +	CHOPx7 + SCT
31	64.9	56	M	IA	147	0	1	1	Alive	56 +	No treatment
32	68.3	43	F	IVA	142	3	2	1	Alive	112 +	CdA
33	68.7	61	F	IVA	140	2	3B	2	Lymphoma	54	CHOPx2, CHVPx6
34	74.4	60	F	IVA	134	2	2	1	Alive	72 +	Chlorambucil, rituximab
35	77.4	85	F	IIA	128	2	3B	3	Lymphoma	38	CHOPx5, gemcitabine
36	80.2	56	F	IVA	140	1	1	1	Alive	29 +	No treatment
37	81.5	59	M	IIIA	158	1	1	0	Alive	58 +	No treatment
38	84.4	78	F	IIIA	147	3	2	1	Alive	117 +	CHOPx4
39	85.0	80	M	IVB	84	4	1	1	Lymphoma	1	No treatment
40	86.3	71	F	IA	144	1	2	1	Alive	56 +	RT
41	88.7	44	F	IVA	140	2	2	1	Alive	54 +	No treatment
42	88.8	70	F	IA	127	1	2	1	Alive	70 +	RT
43	90.6	72	M	IVA	133	3	2	1	Lymphoma	43	KNOSPE, CHOPx6
44	90.7	46	M	IVB	145	1	2	2	Alive	63 +	Rituximab + interferon alfa
45	96.0	37	F	IIB	135	0	2	1	Alive	57 +	CHOPx3
46	104.6	56	F	IIIA	156	1	2	1	Alive	28 +	Chlorambucil, rituximab
47	105.1	34	M	IA	160	0	2	1	Alive	65 +	RT
48	105.6	54	F	IVA	137	1	2	1	Alive	53 +	Rituximab, chlorambucil, CHOPx6
49	119.8	54	F	IIIA	135	1	2	1	Alive	63 +	CHOPx6, MIME, rituximab
50	130.8	61	F	IIA	136	2	3B	3	Alive	68 +	CHOPx6
51	153.0	47	M	IIIA	97	2	3B	3	Alive	82 +	EPOCHx8
52	153.5	67	F	IA	137	2	3A	3	Alive	58 +	RT
53	173.2	54	M	IIB	140	0	2	1	Alive	81 +	Prednimustin, chlorambucil, prednisone, fludarabine
54	175.4	56	M	IIA	127	0	3B	3	Alive	31 +	CHOPx5
55	181.8	64	M	IIIB	126	2	3B	3	Alive	70 +	CHOPx7
56	200.6	69	F	IA	132	2	3B	3	Alive	81 +	RT, CHOPx8
57	246.8	77	M	IA	151	1	3B	3	Cardiac	48	RT

*Relative expression of RNA of probe set 34736_at.

†Immunohistochemistry Ki-67: 0 indicates less than 10%; 1, 10% to 30%; 2, 30% to 50%; and 3, more than 50% cells positive.

‡KNOSPE indicates chlorambucil, prednisone; CHOP, cyclophosphamide, doxorubicin, vincristine, prednisone; MIME, mitoguazone, ifosfamide, methotrexate, etoposide; RT, radiotherapy; CNOP, cyclophosphamide, mitoxantrone, vincristine, prednisone; MTX, methotrexate; LD-ACOP-B, low-dose doxorubicin, cyclophosphamide, vincristine, prednisone, bleomycin; Flu/Cycl, fludarabine, cyclophosphamide; CHVP, cyclophosphamide, doxorubicin, teniposide, prednisone; ENAD, etoposide, mitoxantrone, cytarabine, prednisone; RT-CEP, lomustine, etoposide, chlorambucil, prednisone; CdA, cladribine; EPOCH, etoposide, cyclophosphamide, doxorubicin, vincristine, prednisone; and SCT, stem cell transplantation.

Table 2. Algorithm used to find probe sets with differential expression in PD and CR patients

	1st comparison	2nd comparison	Cross-comparison
Compared groups of patients	3 PD* vs 10 CR†	5 PD‡ vs 3 CR§	3 PD* vs 3 CR§ + 10 PD† vs 3 CR‡
Comparison files	3 × 10 = 30	5 × 3 = 15	10 × 5 + 3 × 3 = 59
Comparison files with probe sets altered (%)	21/30 (70)	10/15 (67)	41/59 (69)
Change algorithm: probe sets decreased	51	338	62
Signal log ratio algorithm: probe sets of − 1 or less	217	617	109
Probe sets selected by both algorithms	28	207	35
Probe sets selected by 1st and 2nd comparison	16	16	10‖

*Patient numbers 8, 11, 14.
†Patient numbers 13, 25, 30, 33, 35, 38, 50, 51, 54, 55.
‡Patient numbers 2, 7, 9, 10, 19.
§Patient numbers 45, 48, 56.
‖Probe sets common in 1st, 2nd, and cross-comparison.

calculated. We also applied a "hot-spot" (HS) approach where the percentages of positive cells were counted out of a total of 500 cells from the 3 to 4 most positive areas of the slide. All counting of cells was facilitated with a Weibul grid. Evaluation of the proliferative fraction with Ki-67 was performed as previously described.[20]

FISH analysis of topoisomerase II alpha

Fluorescent in situ hybridization (FISH) was performed with the Vysis LSI topoisomerase II alpha *(TOPO2A)* probe according to the manufacturer's instructions (Vysis, Downers Grove, IL) on tumor tissue imprints from 18 tumors (patient nos. 2, 8, 9, 10, 11, 13, 14, 18, 25, 29, 30, 33, 35, 39, 45, 50, 55, 56). Signals from 200 interphase nuclei of each imprint were analyzed with Smart Capture 2 (Digital Scientific, Cambridge, United Kingdom) software.

Statistical analysis

Survival curves were calculated with the Kaplan-Meier method and compared with log-rank test.[21] In the univariate analysis, the cohort of 57 patients was dichotomized by the median expression of each studied probe set. Cox proportional hazard regression was used for multivariate analysis.[22] Survival analysis was calculated both for cause-specific survival (CSS) and overall survival (OS). CSS was defined as the time from diagnosis to death of lymphoma or death judged to be related to treatment of the disease. OS was defined as the time from diagnosis to death, irrespective of cause. A chi-2 test and a Mann-Whitney test were used to compare the expression of Ki-67 antigen in tumors from patients with CR and patients with PD and between cases with high and low expression of cyclin B1, as well as to evaluate the distribution of patients with regard to FLIPI groups. To evaluate the correlation between expression of RNA (microarray analysis) and protein (IHC), the Pearson correlation coefficient was calculated. The statistical analysis was performed with Minitab 14.1 (Minitab, State College, PA) and Statistica 6.1 (StatSoft, Tulsa, OK). *P* values less than .05 were considered statistically significant.

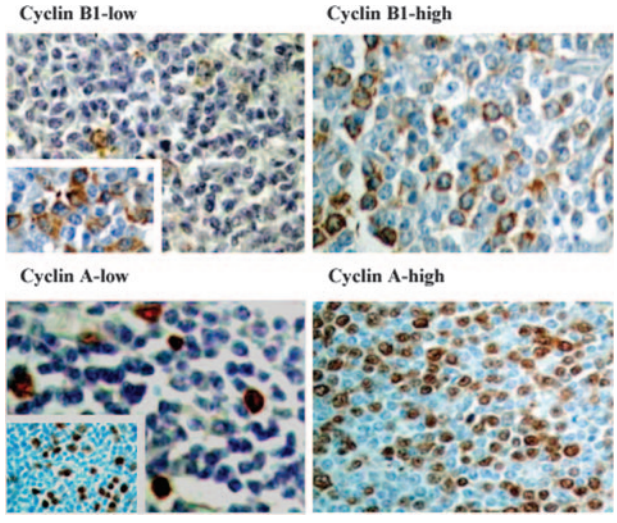

Figure 1. Immunohistochemistry of cyclin B1 and cyclin A. Examples of follicular lymphomas with high and low expression of cyclin A and cyclin B1. (Left) Tumor from patient no. 9 showing low fractions of cyclin B1–positive cells (magnification, × 200) and cyclin A–positive cells (magnification, × 600). Inserts show regions with higher expression, so called hot spots (magnifications, × 600 and × 100). (Right) Tumor from patient no. 55 with high percentages of cyclin B1–positive cells (magnification, × 400) and cyclin A–positive cells (magnification, × 200). Original magnifications, × 200 (top left panel and inset; bottom right panel); × 400 (top right panel; bottom left panel and inset). A Nikon Optishot microscope equipped with 20 ×/0.40 and 40 ×/0.65 objective lenses (Nikon, Tokyo, Japan) was used to visualize images. Pictures were taken with a Nikon CoolPix 990 camera, and Adobe Photoshop 6.0 software was used to zoom images to their present magnitude (Adobe, San Jose, CA).

Results

Differential gene expression in tumors with diverse response to chemotherapy

There were 16 probe sets representing 14 genes that showed high expression in the CR group and low expression in the PD group in both comparisons (Tables 2-3). The cross-comparison analysis identified 10 probe sets in common with the selected 16 probe sets (Table 3). Of the 14 selected genes, 11 were involved in cell cycle, mitosis, or DNA modulation, while 3 had other or unknown functions. No probe set fulfilled the criteria of high expression in the PD group and low expression in the CR group with all algorithms in both the first and the second comparison.

Prognostic significance of the identified gene expression

Levels of expression of 6 of the 14 selected genes showed in univariate analysis a statistically significant impact on both CSS and OS in the whole cohort of 57 FL patients (Table 3). Since the *CCNB1* gene is a key regulator of the G_2/M transition of the cell cycle, and the other genes that have significant impact on survival have a direct or indirect interaction with *CCNB1,*[23-25] we have chosen this gene expression for further analysis. The Kaplan-Meier curves comparing the survival of patients with *CCNB1* high and low tumors (Figure 2) show that a high expression of *CCNB1* is associated with a better survival rate ($P = .010$). When *CCNB1* expression and the FLIPI factors were tested in a multivariate analysis, a high expression of *CCNB1* was independently associated with favorable prognosis (Table 4). Age, stage, hemoglobin, and *CCNB1* still retained their independent prognostic values when the WHO histologic grade was entered in the multivariate analysis. To avoid a potential bias from data that might skew the results, 4 additional survival analyses were made: (1) to see if the importance of *CCNB1* expression was independent of the response to first-line

Table 3. Probe sets up-regulated in tumors from patients with a good response to CHOP

Function/probe set	Gene symbol	Gene name	*P*, log-rank test*
Cell-cycle related			
1945_at	*CCNB1*	Cyclin B1†	.020
34736_at	*CCNB1*	Cyclin B1†	.010
1803_at	*CDC2*	Cell division cycle 2, G_1 to S and G_2 to M	.004
37347_at	*CKS1B*	CDC28 protein kinase regulatory subunit 1B	.005
1599_at	*CDKN3*	Cyclin-dependent kinase inhibitor 3†‡	.005
40348_s_at	*ANP32E*	Acidic (leucine-rich) nuclear phosphoprotein 32 family, member E†	.005
40347_at	*ANP32E*	Acidic (leucine-rich) nuclear phosphoprotein 32 family, member E†	NS
32263_at	*CCNB2*	Cyclin B2	NS
Mitosis			
40041_at	*KNTC2*	Kinetochore associated 2 (highly expressed in cancer, HEC)†	NS
35699_at	*BUB1B*	BUB1 budding uninhibited by benzimidazoles 1 homolog beta (yeast)†	NS
36863_at	*HMMR*	Hyaluronan-mediated motility receptor (RHAMM)	NS
DNA modulation			
40145_at	*TOP2A*	Topoisomerase (DNA) II alpha 170 kDa†	NS
38065_at	*HMGB2*	High-mobility group box 2†	NS
Other/unknown			
38116_at	*KIAA0101*	KIAA0101 gene product†	.004
35071_s_at	*GMDS*	GDP-mannose 4,6-dehydratase	NS
39056_at	*PAICS*	Phosphoribosylaminoimidazole carboxylase§	NS

*Log-rank test for cause-specific survival.
†Probe sets that were selected with cross-comparison; see Table 2 for details.
‡Other name: CDK2-associated dual specificity phosphatase.
§Other name: phosphoribosylaminoimidazole succinocarboxamide synthetase.
NS indicates not significant.

chemotherapy, we excluded the 13 patients of the first comparison; (2) to investigate if the prognostic significance of *CCNB1* was valid in low-grade FL, we excluded the 13 patients with FL grade 3; (3) we excluded the 5 purified samples that might have given a false high value of *CCNB1;* and (4) to determine whether or not the scaling factor influenced the survival analysis, we excluded the 11 samples that were not within the recommended scaling factor of 3.[19]

In each of these 4 analyses, the significant prognostic impact of *CCNB1* expression was retained.

High expression of each of the genes *CDC2, CDKN3, CKS1B, ANP32E,* and *KIAA0101* was also associated with a good outcome in a multivariate analysis independent of the factors of FLIPI (relative risk: *CDC2,* 3.6; *CDKN3,* 3.0; *CKS1B,* 5.0; *ANP32E,* 3.7; and *KIAA0101,* 3.0). Since the expression levels of all these genes were significantly correlated with *CCNB1,* no further multivariate analyses were made including the factors of FLIPI. The results for *ANP32E* should be interpreted with caution since only one probe set (40348_s_at) had prognostic impact on survival.

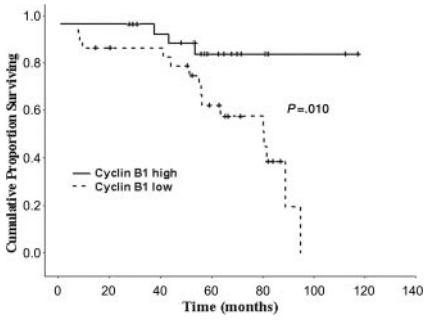

Figure 2. Prognostic significance of *CCNB1* expression in follicular lymphomas. Cause-specific survival depending on expression of probe set 34736_at. Patients were divided in groups (cyclin B–high, solid line; cyclin B–low, dashed line) by the median expression of *CCNB1.* Difference in survival was calculated with log-rank test.

Since both cyclin B1 *(CCNB1)* and cyclin A *(CCNA2)* are essential for G_2/M transition in the cell cycle, the impact of *CCNA2* expression on survival was analyzed. There were 2 probe sets for *CCNA2* present on the u95Av2 array (1943_at and 40697_at). High expression of both was statistically significant for CSS, but only high expression of the 1943_at probe set was significant for OS (data not shown). Cyclin A did not have independent prognostic impact when included in a multivariate analysis combined with the parameters of the FLIPI. No significant difference in survival could be seen when the mRNA expression levels of other cyclins (C, D1, D3, E, F, G2, T1, T2) and of the proliferation marker Ki-67 were analyzed.

Protein expression of cyclin B, cyclin A, and Ki-67

The FL samples showed a large variation in the number of cells showing protein expression of cyclin B1: the HPF method showed a range of 13 to 204 positive cells (median, 61 cells) and the HS method, 4.2% to 47.8% (median, 11.8%) positive cells. The HS method gave the best correlation when comparing RNA and protein expression, with a correlation coefficient of 0.67 ($P = 6 \times 10^{-5}$) (Figure 3). However, we could not define a cut-off level for cyclin B1 protein expression, which could divide the patients into 2 groups with statistically significant differences in survival. This

Table 4. Multivariate analysis of prognostic impact of cyclin B1 and FLIPI parameters on overall survival

	Adverse factor	Relative risk	Range	*P*
Age	≥ 60	5.6	1.7-18.7	.005
Ann Arbor stage	III-IV	4.4	1.3-15.2	.020
Nodal sites	> 4	0.3	0.09-1.02	.054
Hemoglobin	< 12 g/dL	3.0	1.0-8.9	.043
Serum LDH level	> ULN	1.0	0.33-3	.99
Cyclin B1*	≤ 59	3.0	1.1-8.3	.036

ULN indicates upper limit of normal.
*Relative expression of RNA of probe set 34736_at.

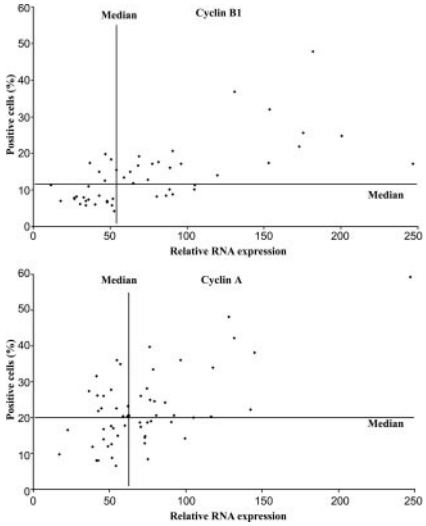

Figure 3. Correlation between RNA and protein expression of cyclin B1 and cyclin A in follicular lymphomas. RNA expression for *CCNB1* was measured by probe set 34736_at and for *CCNA2* by probe set 1943_at. The percentages of cyclin B1–positive cells (top) and cyclin A–positive cells (bottom) with immunohistochemistry were calculated with the hot-spot method (see "Patients, materials, and methods"). Correlation coefficient between RNA and protein for cyclin B1 was 0.67 (*P* value, 1×10^{-7}) and for cyclin A, 0.63 (*P* value, 2×10^{-7}).

was due to discrepant results found in 11 patients; 6 patients (nos. 36, 40, 41 43, 46, 47) had RNA expression above the median but low fractions of cells expressing protein, and 6 patients (nos. 14, 18, 19, 20, 24, 28) had RNA below the median but high fractions of cells expressing protein (Figure 3). Cyclin A expression with the HPF method varied between 31 and 520 positive cells, with a median of 91 cells. The results obtained with the HS method ranged between 6.6% and 59.2%, with a median of 20.4% positive cells. The HS method gave the best correlation when comparing RNA and protein expression, with the correlation coefficient of 0.63 ($P = 2 \times 10^{-7}$) (Figure 3). For cyclin A, a cut-off of 18% positive cells gave a statistically significant difference on the CSS ($P = .033$) but not on the OS. When this cut-off value was used in a multivariate analysis together with the prognostic factor of the FLIPI, cyclin A did not have an independent prognostic value.

The fractions of cells expressing Ki-67 were compared in groups of patients with different responses to CHOP chemotherapy and also in tumors with high and low expression of cyclin B1. A significantly higher expression of Ki-67 was seen both on RNA and protein level in the tumors from CR patients compared with the PD group ($P < .05$). Also, tumors with high expression of the *CCNB1* gene had a significantly higher incidence of high Ki67 expression on RNA levels ($P < .05$) but not on protein levels.

FISH analysis of *TOP2A*

Since the *TOP2A* gene was overexpressed in tumors from patients with favorable response to CHOP, we performed FISH analysis to assess if the overexpression was due to genomic amplification. A normal pattern with 2 signals for chromosome 17 centromere and 2 signals from *TOP2A* was found in 85% to 95% of the cells of the investigated tumors.

Genes showing coexpression with *KIAA0101*

Since the function of *KIAA0101* is unknown, we have attempted to identify the probe sets with expression pattern similar to *KIAA0101* in order to elucidate the role of this gene. In the 68 samples that were used for this analysis, 21 genes had a correlation coefficient above 0.8 with *KIAA0101*. Of these genes, 11 were involved in mitosis *(CENPA, TPX2, KIF11, KNTC2, BUB1R, KPNA2, ZWINT, KIF2C, DLG7, AURKB, PTCC1),* 5 genes were cell-cycle related *(CCNB2, UBE2C, CDC20, CDKN3, EZH2, CDC2),* 2 had DNA modulating function *(HMGB2, TOP2A),* and 3 had other functions *(TYMS, KIF14, EZH2).* The strongest correlation was obtained with *CCNB2,* with a correlation coefficient of 0.89 ($P = 1.3 \times 10^{-22}$). This pattern of expression suggests that *KIAA0101* is involved in mitosis or cell-cycle regulation.

Discussion

In this study we compared gene expression in diagnostic samples from FL patients with diverse response to CHOP chemotherapy and investigated the prognostic impact of such expression pattern.

There were 14 genes that showed a high expression level in the group with a good response to CHOP and low levels in the group with poor response. Of the 14 selected genes, 11 were involved in cell-cycle regulation *(CKS1B, CDKN3, ANP32E, CCNB1, CCNB2, CDC2),* mitosis *(HMMR, BUB1B, KNTC2),* or DNA modulation *(TOP2A, HMGB1).* This may indicate that the tumors responding well to CHOP chemotherapy had more cells in an active proliferating phase than the tumors from nonresponders. In agreement with this observation was the fact that the protein expression of the proliferation marker Ki-67 was higher in the group with good response to chemotherapy compared with the group of nonresponders. The components of the CHOP regimen interact in different modes with the proliferating cells. The active metabolite of cyclophosphamide binds to DNA and inhibits replication and cell division, and vincristine prevents the spindle formation of mitotic cells and causes cell-cycle arrest. Both of these drugs are most likely lethal when the target cells are in the replicative phase. In this context, it is interesting to note that *TOP2A* was among the selected genes. *TOP2A* alters the topologic state of DNA during transcription, and it is also the main target of doxorubicin.[26] It is likely that a high expression of *TOPO2A* facilitated the response to doxorubicin in the studied CR patients. A high expression of *TOPO2A* has been found in Wilms tumor samples from patients with good response to doxorubicin.[27] Järvinen et al showed that genomic amplification and overexpression of *TOPO2A* was correlated with higher sensitivity to doxorubicin in breast cancer cell lines.[28] In our FL patients, the overexpression of *TOPO2A* was most likely posttranscriptionally regulated since no amplification could be seen at a genomic level of investigated tumors. Our observation indicating that more active proliferating cells can be found in FL sensitive to chemotherapy is in agreement with previous studies.[20,29]

Most of the 14 selected genes have their main function during the G_2/M phase of the cell cycle. *CCNB1* and *CDC2* form a complex, which is essential for the G_2/M transition of the cell cycle. This complex has a peak during the G_2/M phase and must be degraded before the completion of the cell division. Other selected genes interacting with *CDC2* were *CKS1B, CDKN3, CCNB2,* and *TOPO2A.*[23,25,30] A link between *ANP32E* and *CDC2* has also been established, since *ANP32E* inhibits the *CDC2* inhibitor *PP2A.*[24] *BUB1B* and *KNTC2* are both attached to the kinetochore and involved in the spindle checkpoint.[31,32] They are involved in 2 different inhibition pathways of the *APC/C-CDC20* complex. An activated *APC/C-CDC20* complex is believed to contribute to the degradation of the *CCNB1/CDC2* complex,[33,34] and thus high

expression of *BUB1B* and *KNTC3* is indirectly related to high expression of *CCNB1/CDC2*. Another gene involved with mitosis is *RHAMM,* which has a role in spindle pole stability. *RHAMM* has interestingly also been linked to the function of *CCNB1*.[35,36] A link to *CCNB1/CDC2* or to the mitotic spindle was not found for *GMDS* and *PAICS*. The function of *KIAA0101* is not known, but interestingly its expression was strongly correlated to that of cyclin B2 and of other genes involved in mitosis or cell-cycle regulation. It is therefore reasonable to speculate that *KIAA0101* might have a similar function.

We also investigated the impact of the mRNA expression levels for the 14 selected genes on prognosis. A high expression of 6 of these genes *(CCNB1, CDC2, CDKN3, CKS1B, KIAA0101,* and *ANP32E)* was associated with better prognosis in the univariate analysis (Table 1; Figure 2). Since all of these have their main function during the G_2/M transition of the cell cycle and *CCNB1* is a key regulator of this transition, *CCNB1* was selected for further analysis. Multivariate Cox regression analysis of *CCNB1* and the clinical parameters of the FLIPI revealed that age, stage, hemoglobin, and *CCNB1* all had independent prognostic value (Table 4).

We could not show any significant impact on survival for levels of the proliferation marker Ki-67. The prognostic impact of proliferation in lymphomas measured by Ki-67 protein expression remains controversial.[20,37-39] However, we could show that a high expression of cyclin A was correlated with a longer survival in the univariate analysis but not in the multivariate analysis. There are only a few clinical studies comparing expression of cyclin A and cyclin B1 in lymphoma. Low expression of cyclin A measured by Western blot has been shown to correlate to a longer survival in NHL.[40] Cyclin B1 evaluated by IHC could not be related to survival in the study by Jin and Park.[41]

Our results showed a good correlation between the results of microarray and IHC for both cyclin B1 and cyclin A. Despite this good correlation, it was difficult to determine the prognostically significant cut-off for percentage of positive cells. A possible reason for this could be the uneven distribution of cyclin B1–positive and cyclin A–positive cells in the tissue. The strongest correlation between RNA levels and immunohistochemistry findings was obtained when the analysis was restricted to cells only from the most positive areas of the slide. This may suggest that the groups of cells with high expression may be of biologic importance for progression of the disease. It is therefore plausible to conclude that the possibilities to reproducibly quantify immunohistochemistry are limited, and that it would be valuable to study the impact on survival of cyclin A and cyclin B1 with other methods that would secure a more accurate measurement of the protein expression. Flow cytometry analysis of *CCNB1*[42] could be an attractive alternative method in a prospective study of follicular lymphomas. An alternative approach in the diagnostic setting is to measure RNA levels of these genes with quantitative polymerase chain reaction (PCR).[43]

In conclusion, we have shown that a high expression of genes associated with the G_2/M transition of the cell cycle, mitosis, and DNA modulation indicates the possibility of a favorable response to CHOP chemotherapy in FL patients. High RNA expression of cyclin B1 was significantly correlated with longer survival. The prognostic value of cyclin B1 was independent when tested in a multivariate analysis including the established prognostic parameters of the FLIPI.

Acknowledgments

The authors would like to thank Margareta Waern, Stefanie Bug, and Anna-Barbara Witte for skillful technical assistance; Ingrid Svensson for advice on statistical concerns; Marie Bohlin for help retrieving information on treatment of the patients; and Göran Björck and Lewis Edgel for linguistic consultation.

References

1. Jaffe ES, Harris NL, Stein H, Vardiman JW. Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues. Lyon, France: IARC Press; 2001.
2. Advani R, Rosenberg SA, Horning SJ. Stage I and II follicular non-Hodgkin's lymphoma: long-term follow-up of no initial therapy. J Clin Oncol. 2004;22:1454-1459.
3. Reiser M, Diehl V. Current treatment of follicular non-Hodgkin's lymphoma. Eur J Cancer. 2002; 38:1167-1172.
4. A predictive model for aggressive non-Hodgkin's lymphoma: The International Non-Hodgkin's Lymphoma Prognostic Factors Project. N Engl J Med. 1993;329:987-994.
5. Solal-Céligny P, Roy P, Colombat P, et al. Follicular Lymphoma International Prognostic Index. Blood. 2004. Prepublished on May 4, 2004, as DOI 10.1182/blood-2003-12-4434 (now available as Blood. 2004;104:1258-1265).
6. Davidge-Pitts M, Dansey R, Bezwoda WR. Prolonged survival in follicular non Hodgkins lymphoma is predicted by achievement of complete remission with initial treatment: results of a long-term study with multivariate analysis of prognostic factors. Leuk Lymphoma. 1996;24:131-140.
7. Hans CP, Weisenburger DD, Vose JM, et al. A significant diffuse component predicts for inferior survival in grade 3 follicular lymphoma, but cytologic subtypes do not predict survival. Blood. 2003;101:2363-2367.
8. Zhao WL, Daneshpouy ME, Mounier N, et al. Prognostic significance of bcl-xL gene expression and apoptotic cell counts in follicular lymphoma. Blood. 2004;103:695-697.
9. Martinka M, Comeau T, Foyle A, Anderson D, Greer W. Prognostic significance of t(14;18) and bcl-2 gene expression in follicular small cleaved cell lymphoma and diffuse large cell lymphoma. Clin Invest Med. 1997;20:364-370.
10. Møller MB, Nielsen O, Pedersen NT. Oncoprotein MDM2 overexpression is associated with poor prognosis in distinct non-Hodgkin's lymphoma entities. Mod Pathol. 1999;12:1010-1016.
11. Bilalovic N, Blystad AK, Golouh R, et al. Expression of bcl-6 and CD10 protein is associated with longer overall survival and time to treatment failure in follicular lymphoma. Am J Clin Pathol. 2004;121:34-42.
12. Viardot A, Moller P, Hogel J, et al. Clinicopathologic correlations of genomic gains and losses in follicular lymphoma. J Clin Oncol. 2002;20:4523-4530.
13. Höglund M, Sehn L, Connors JM, et al. Identification of cytogenetic subgroups and karyotypic pathways of clonal evolution in follicular lymphomas. Genes Chromosomes Cancer. 2004;39: 195-204.
14. McKelvey EM, Gottlieb JA, Wilson HE, et al. Hydroxyldaunomycin (Adriamycin) combination chemotherapy in malignant lymphoma. Cancer. 1976;38:1484-1493.
15. Carbone PP, Kaplan HS, Musshoff K, Smithers DW, Tubiana M. Report of the Committee on Hodgkin's Disease Staging Classification. Cancer Res. 1971;31:1860-1861.
16. Ek S, Björck E, Porwit-MacDonald A, Nordenskjöld M, Borrebaeck CA. Increased expression of Ki-67 in mantle cell lymphoma is associated with de-regulation of several cell cycle regulatory components, as identified by global gene expression analysis. Haematologica. 2004;89:686-695.
17. Ek S, Högerkorp CM, Dictor M, Ehinger M, Borrebaeck CA. Mantle cell lymphomas express a distinct genetic signature affecting lymphocyte trafficking and growth regulation as compared with subpopulations of normal human B cells. Cancer Res. 2002;62:4398-4405.
18. World Health Organization. WHO Handbook for Reporting Results of Cancer Treatment. Geneva, Switzerland: World Health Organization; 1979. WHO offset publication no. 48.
19. Affymetrix, Inc. Data analysis fundamentals. Available at: http://www.affymetrix.com/support/technical/manuals.affx. Accessed July 14, 2004.
20. Czader M, Mazur J, Pettersson M, et al. Prognostic significance of proliferative and apoptotic fractions in low grade follicle center cell-derived non-Hodgkin's lymphomas. Cancer. 1996;77:1180-1188.
21. Kaplan E, Meier P. Nonparametric estimation from incomplete observations. J Am Stat Assoc. 1958;53:157-181.
22. Cox D. Regression models and life tables. J Roy Stat Soc (B). 1972;34:187-202.
23. Bourne Y, Watson MH, Hickey MJ, et al. Crystal structure and mutational analysis of the human CDK2 kinase complex with cell cycle-regulatory protein CksHs1. Cell. 1996;84:863-874.

24. Minshull J, Straight A, Rudner AD, Dernburg AF, Belmont A, Murray AW. Protein phosphatase 2A regulates MPF activity and sister chromatid cohesion in budding yeast. Curr Biol. 1996;6:1609-1620.

25. Gyuris J, Golemis E, Chertkov H, Brent R. Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. Cell. 1993;75:791-803.

26. Schrader C, Meusers P, Brittinger G, et al. Topoisomerase IIalpha expression in mantle cell lymphoma: a marker of cell proliferation and a prognostic factor for clinical outcome. Leukemia. 2004;18:1200-1206.

27. Takahashi M, Yang XJ, Lavery TT, et al. Gene expression profiling of favorable histology Wilms tumors and its correlation with clinical features. Cancer Res. 2002;62:6598-6605.

28. Järvinen TA, Tanner M, Rantanen V, et al. Amplification and deletion of topoisomerase IIalpha associate with ErbB-2 amplification and affect sensitivity to topoisomerase II inhibitor doxorubicin in breast cancer. Am J Pathol. 2000;156:839-847.

29. Wilson WH, Teruya-Feldstein J, Fest T, et al. Relationship of p53, bcl-2, and tumor proliferation to clinical drug resistance in non-Hodgkin's lymphomas. Blood. 1997;89:601-609.

30. Escargueil AE, Plisov SY, Skladanowski A, et al. Recruitment of cdc2 kinase by DNA topoisomerase II is coupled to chromatin remodeling. FASEB J. 2001;15:2288-2290.

31. Fang G. Checkpoint protein BubR1 acts synergistically with Mad2 to inhibit anaphase-promoting complex. Mol Biol Cell. 2002;13:755-766.

32. Martin-Lluesma S, Stucke VM, Nigg EA. Role of Hec1 in spindle checkpoint signaling and kinetochore recruitment of Mad1/Mad2. Science. 2002; 297:2267-2270.

33. Zur A, Brandeis M. Timing of APC/C substrate degradation is determined by fzy/fzr specificity of destruction boxes. EMBO J. 2002;21:4500-4510.

34. Rudner AD, Murray AW. Phosphorylation by Cdc28 activates the Cdc20-dependent activity of the anaphase-promoting complex. J Cell Biol. 2000;149:1377-1390.

35. Maxwell CA, Keats JJ, Crainie M, et al. RHAMM is a centrosomal protein that interacts with dynein and maintains spindle pole stability. Mol Biol Cell. 2003;14:2262-2276.

36. Mohapatra S, Yang X, Wright JA, Turley EA, Greenberg AH. Soluble hyaluronan receptor RHAMM induces mitotic arrest by suppressing Cdc2 and cyclin B1 expression. J Exp Med. 1996; 183:1663-1668.

37. Miller TP, Grogan TM, Dahlberg S, et al. Prognostic significance of the Ki-67-associated proliferative antigen in aggressive non-Hodgkin's lymphomas: a prospective Southwest Oncology Group trial. Blood. 1994;83:1460-1466.

38. Martin AR, Weisenburger DD, Chan WC, et al. Prognostic value of cellular proliferation and histologic grade in follicular lymphoma. Blood. 1995; 85:3671-3678.

39. Llanos M, Alvarez-Arguelles H, Aleman R, Oramas J, Diaz-Flores L, Batista N. Prognostic significance of Ki-67 nuclear proliferative antigen, bcl-2 protein, and p53 expression in follicular and diffuse large B-cell lymphoma. Med Oncol. 2001; 18:15-22.

40. Wolowiec D, Berger F, Ffrench P, Bryon PA, Ffrench M. CDK1 and cyclin A expression is linked to cell proliferation and associated with prognosis in non-Hodgkin's lymphomas. Leuk Lymphoma. 1999;35:147-157.

41. Jin YH, Park CK. Expression of cyclin B1 and cdc2 in nodal non-Hodgkin's lymphoma and its prognostic implications. J Korean Med Sci. 2002; 17:322-327.

42. Juan G, Traganos F, James WM, et al. Histone H3 phosphorylation and expression of cyclins A and B1 measured in individual cells during their progression through G2 and mitosis. Cytometry. 1998;32:71-77.

43. de Vos S, Hofmann WK, Grogan TM, et al. Gene expression profile of serial samples of transformed B-cell lymphomas. Lab Invest. 2003;83:271-285.