Prognostic significance of bcl-xL gene expression and apoptotic cell counts in follicular lymphoma

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Abstract
Bcl-xL, a member of the Bcl-2 family, exerts an anti-apoptotic effect on lymphocytes. To assess its clinical significance in follicular lymphoma patients, real-time quantitative RT-PCR analysis of bcl-xL gene expression was investigated in whole lymph node sections and laser microdissected lymphoma cells of 27 patients. Compared to 10 patients with reactive follicular hyperplasia, bcl-xL gene was overexpressed in follicular lymphoma patients with a higher level in microdissected lymphoma cells. Bcl-xL gene level correlated with the number of apoptotic lymphoma cells labeled by TUNEL assays (r=-0.7736). Clinically, high bcl-xL level was significantly associated with multiple extranodal involvement (P=0.0020), elevated lactate dehydrogenase level (P=0.0478), and high-risk international prognostic index (P=0.0235). Moreover, bcl-xL gene overexpression was linked to short overall survival (P=0.0129). The value of bcl-xL gene expression as a prognostic marker in follicular lymphoma should thus be considered.

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Introduction

Follicular lymphoma is characterized primarily by defects in cell apoptosis rather than cell proliferation. Bcl-2, involved in follicular lymphoma, prevents cell apoptosis by blocking the mitochondrial pathway. The long isoform of bcl-x, bcl-xL, acts as another important anti-apoptotic factor of bcl-2 family and is expressed in follicular lymphoma. Experimentally, functional differences exist between bcl-2 and bcl-xL. In bcl-2-deficient mice there is massive death of mature lymphocytes, while immature lymphocytes undergo apoptosis in bcl-x-deficient mice. In vitro, bcl-2 maintains survival of resting T cells when bcl-xL prevents apoptosis of activated T cells. Importantly, when bcl-xL is downregulated, even constitutive level of bcl-2 is maintained, follicular lymphoma cells undergo apoptosis. Therefore, bcl-xL seems to play a key role in follicular lymphoma. However, the relation between bcl-xL gene expression and clinical features in patients with follicular lymphoma has not yet been reported.

In the present study, we assessed bcl-xL gene expression and apoptotic cell counts in 27 follicular lymphoma patients using real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) and terminal deoxytransferase-catalyzed DNA nick end labeling (TUNEL) assays. Bcl-xL gene overexpression in lymphoma cells was related to low number of apoptotic cells. Both factors reflect poor disease outcome in follicular lymphoma patients.

Patients and Methods

Patients

Twenty-seven follicular lymphoma patients, 16 males and 11 females, aged from 21 to 82 years (median 51 years), with available frozen tumor specimen at diagnosis were included in this study. Histological diagnoses were established according to the WHO classifications. Ten age- and sex-matched patients with reactive follicular hyperplasia were referred as controls.

Written informed consent was obtained from all patients for this study, in accordance with our Institution's regulation.

Tissue samples

Lymph nodes, surgically removed for diagnostic purposes, were immediately cut into two parts: one was fixed in formaldehyde and further processed for paraffin embedding, the other was snap frozen and stored in −80°C. A section was cut from each tissue block for systemically light microscopic control.
Laser microdissection

Seven-µm frozen sections were prepared, immediately fixed in 70% ethanol, and stained with hematoxylin-eosin. The sections were dehydrated through a graded series of ethanol and xylene and air-dried. For each patient, approximately 1500 lymphoma cells, corresponding to an average surface of 450,000µm², were laser microdissected (PALM, Bernried, Germany) and catapulted into tubes for RNA extraction.

RNA extraction and cDNA synthesis

Total RNA was extracted from 10 frozen sections of 10 µm thickness using the acid-guanidinium thiocyanate-phenol-chloroform method. First-strand cDNA was synthesized from 1µg of total RNA using Superscript II reverse transcriptase (Invitrogen Corporation, CA, USA) and random hexamers according to the manufacturer's instructions.

Total RNA from microdissected lymphoma cells was independently extracted and immediately reverse transcribed following the same protocol.

Real-time quantitative RT-PCR

Quantitative PCR was performed on ABI PRISM 7700 system using the Pre-Developed TaqMan Assay Reagent specific to human bcl-xL and human transcription factor IID/TATA binding factor (TBP) gene expression quantification (PE Applied Biosystems, Warrington, UK), according to the manufacturer's instructions. TBP gene was used as an endogenous control.

Quantification results were expressed in terms of the threshold cycle value (CT) according to the baseline adjusted to 0.05. Jurkat cell, which expresses bcl-xL gene, was considered as calibrator. The comparative CT method (PE Applied Biosystems) was used to quantify relative bcl-xL expression compared to Jurkat cell. Briefly, the CT values were averaged for each triplicate. The differences between the mean CT values of bcl-xL and those of TBP was calculated ΔCTsample=CT bcl-xL - CT TBP, and the same as ΔCT for Jurkat cell (ΔCTcalibrator =CT bcl-xL – CT TBP). Final results, the sample/calibrator ratio, expressed as N-fold differences of bcl-xL expression in the samples compared with Jurkat cell, were determined as $2^{-(\Delta CT_{sample}-\Delta CT_{calibrator})}$.

TUNEL assay
Cell apoptosis was confirmed by in situ detection of fragmented DNA, using TUNEL assay, on deparaffinized 5-µm-thick sections, treated with proteinase K (20 µg/mL) for 15 minutes at room temperature.

The number of apoptotic lymphoma cells was assessed blindly by 2 pathologists, who did not know the bcl-xL levels, on an Olympus Provis AX 70 microscope, with wide-field eyepiece number 26.5. At ×400 magnification, this wide-field eyepiece provided a field size of 0.344mm². Results are expressed as the mean number of cells per field at ×400 magnification.

Statistical analyses

Patient characteristics were compared using Chi-square and Fisher’s exact tests for categorical variables, and Wilcoxon’s test for continuous variables. Overall survival was measured from the date of diagnosis to either death from any cause or the stopping date of December 31, 2002. When the stopping date was not reached, the data were censored at the date of the last follow-up evaluation. Survival functions were estimated using the Kaplan-Meier method and compared by Log-rank test. Multivariate survival analysis was performed using a Cox regression model. Differences were considered significant when the two-sided P value was < 0.05. All statistical analyses were performed using SAS 8.2 (SAS Institute Inc, Cary, NC, USA) and Splus 2000 (MathSoft Inc, Berkeley, CA, USA) softwares.

Results and Discussion

Follicular lymphoma cells overexpressed bcl-xL

Compared to the patients with reactive follicular hyperplasia, bcl-xL gene was overexpressed in whole lymph node sections [median 5.5 (range 1.2-28.1) vs 2.3 (1.6-3.2), P=0.0115].

Since lymph node sections contain not only lymphoma cells, but also stromal cells, lymphoma cells were further isolated by laser microdissection. The higher level of bcl-xL in microdissected lymphoma cells compared with whole lymph node sections [median 11.0 (range 1.9-59.6) vs 5.5 (1.2-28.1), P=0.0379] demonstrated that bcl-xL was expressed by lymphoma cells.

Bcl-xL gene overexpression corresponded to low numbers of apoptotic lymphoma cells
The number of the apoptotic lymphoma cells on TUNEL assays (Figure 1) ranged from 5 to 108 (median 38) and inversely correlated with bcl-xL gene expression level both on lymph node sections (r=-0.7552) and microdissected lymphoma cells (r=-0.7736).

This is in accordance with experimental data showing that bcl-xL gene prevents apoptosis.\(^2\) Bcl-xL-overexpressing mice showed enhanced survival of B cells,\(^{12}\) while bcl-x-deficient mice had extensive lymphocyte apoptosis.\(^5\) In vitro, B lymphocytes expressing high level of bcl-xL were resistant to Fas-induced cell apoptosis.\(^{13}\) In contrast, downregulation of bcl-xL enhanced TGF\(\beta\)-induced apoptosis of cultured B lymphoma cells.\(^{14}\)

Bcl-xL reflected poor disease outcome in follicular lymphoma

Bcl-xL overexpression and low numbers of apoptotic lymphoma cells were associated with multiple extranodal sites, elevated LDH level, and high risk international prognostic index in the 27 follicular lymphoma patients (Table 1). Moreover, both factors were significantly related to short overall survival (Figure 1).

To our knowledge, this is the only study of bcl-xL gene expression on disease outcome in follicular lymphoma patients. Experimental data on B lymphoma cells demonstrated that bcl-xL gene overexpression caused resistance to apoptosis induced by anti-IgM or by chemotherapeutic agents.\(^{15,16}\) This mechanism might favor lymphoma development and result in poor prognosis in follicular lymphoma patients. Further studies are needed to assess the value of bcl-xL as a therapeutic target in high risk follicular lymphoma patients.
References


Figure 1  TUNEL assays on two follicular lymphoma cases.
Figure legend

**Figure 1  TUNEL assays on two follicular lymphoma cases.**

Case 1 (A and B) Follicular lymphoma with high level of bcl-xL gene expression.
A: A malignant follicle (surrounded by dotted line) is negative. TUNEL assay x 200.
B: Higher magnification of the malignant follicle: Typical malignant centrocytic cells, with a cleaved nucleus (arrows) are negative. TUNEL assay x 700.

Case 2 (C and D) Follicular lymphoma with low level of bcl-xL gene expression.
C: A malignant follicle (surrounded by dotted line) is formed of cells stained by TUNEL assay, when normal lymphocytes around the follicle (arrows) are negative. TUNEL assay x 300.
D: Higher magnification of the malignant follicle: Typical malignant centrocytic cells, with a cleaved nucleus (arrows) stained. TUNEL assay x 800.

E: Kaplan-Meier survival curve for follicular lymphoma patients according to bcl-xL gene expression. The overall survival of the patients with bcl-xL gene level above the median (red line) was significantly shorter than that of patients with bcl-xL level below and equal to the median level (green line).

F: Kaplan-Meier survival curve for follicular lymphoma patients according to the number of apoptotic cells. The overall survival of the patients with number of apoptotic lymphoma cells less than median (red line) was significantly shorter than that of patients with number of apoptotic cells more than or equal to the median (green line).
Table 1 Bcl-xL gene expression, apoptotic lymphoma cell counts and their relation to clinical characteristics in follicular lymphoma patients (n=27)

<table>
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<th>Characteristics</th>
<th>No. (%)</th>
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<td>1.9-59.6</td>
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<tr>
<td>0-1</td>
<td>16 (59%)</td>
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<td>5.4-59.6</td>
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A - Sample/Calibrator Ratio
TUNEL - terminal deoxytransferase-catalyzed DNA nick end labeling
LDH - lactate dehydrogenase
IPI - international prognostic index
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