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Bcl-2 Family of Proteins in Indolent B-Cell Non-Hodgkin's Lymphoma: Study of 116 Cases

Beamon Agarwal and Kikkeri N. Naresh*

Department of Pathology, Tata Memorial Hospital, Mumbai (Bombay), India

The bcl-2 family of proteins comprises both antagonists and agonists of apoptosis. We have investigated whether subsets of indolent B-cell non-Hodgkin's lymphoma (IB-NHL) differ in the expression of the bcl-2 family members; 116 cases of IB-NHL, composed of chronic lymphocytic leukemia (CLL, *n* = 48), follicular lymphoma (FL, *n* = 38), marginal zone B-cell lymphoma (MZBCL, *n* = 15), and mantle cell lymphoma (MCL, *n* = 15), were investigated for expression of bcl-2, bcl-X, mcl-1, bax, and bak proteins by immunohistochemistry. Expression of bcl-2 and bcl-X proteins was moderate/high among most IB-NHLs. Expression of mcl-1 was low/absent in most cases of CLL and MCL and low/moderate in most cases of FL and MZBCL. Most MCLs did not express bax protein. Bax expression was absent/low among most cases of CLL and low/moderate among most cases of FL and MZBCL. Expression of bak was moderate/low among most cases of CLL, MZBCL, and MCL but was absent/low among most cases of FL. The different subsets of IB-NHLs differ in their expression of mcl-1, bax, and bak proteins. Am. J. Hematol. 70: 278–282, 2002. © 2002 Wiley-Liss, Inc.

Key words: apoptosis; lymphoma; bcl-2; bcl-X; mcl-1; bax; bak; tissue array

INTRODUCTION

The bcl-2 family of proteins, composed of bcl-2, bcl-Xl, bcl-w, mcl-1, A1, and NR13 as antagonists of apoptosis and bax, bak, bok, bcl-Xs, bid, bad, bik, blk, hrk, and bim as agonists of apoptosis, are expressed in a wide variety of tissues and the tumors derived thereof [1]. The mechanisms of action of some of these proteins, principally homodimerization and heterodimerization, have been extensively investigated. While bax homodimers promote apoptosis, bcl-2 homodimers and bcl-2/bax heterodimers inhibit apoptosis. Similarly, bcl-Xl inhibits apoptosis while bcl-Xs and bak proteins inhibit the anti-apoptotic function of bcl-Xl [1–3].

The expression pattern of the members of the bcl-2 protein family varies in the subsets of non-malignant lymphoid cells. Non-Hodgkin's lymphoma (NHL), a heterogeneous group of neoplasms of B, T, and NK cells, is known to express bcl-2, bcl-Xl, bax, mcl-1, and bak proteins [4,5]. NHLs can be broadly divided into indolent and aggressive categories. However, even among indolent lymphomas, the biologic behavior varies among the well-defined lymphoma subtypes [6]. It is possible that differences in the apoptotic machinery are responsible for the difference in the biology of these lymphomas.

To address the above-mentioned issue, we investigated the expression of the bcl-2 protein family members (bcl-2, bcl-X, mcl-1, bax, and bak proteins) in 116 cases of indolent B-NHLs (IB-NHL) with the intent to (i) identify differences in the patterns of expression between the different subsets and (ii) identify inter-relationships between the expression of the bcl-2 protein family members.

MATERIALS AND METHODS

The 116 cases of IB-NHL were selected from an earlier study performed for the establishment of the prevalence of NHL subsets in India [7]. They included 48 cases of chronic lymphocytic leukemia (CLL), 38 cases of follicular lymphoma (FL), 15 cases of marginal zone B-cell lymphoma (MZBCL) (11 involving the lymph node (LN) and 4 involving extranodal sites), and 15 cases

*Correspondence to: Kikkeri N. Naresh, M.D., Professor of Pathology, Department of Pathology, Tata Memorial Hospital, Dr. E. Borges Marg, Parel, Mumbai [Bombay], India. E-mail: knnaresh@hotmail.com

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TABLE I. Immunohistochemistry Parameters

Antibody	Company	Incubation	Dilution	Clonality
bcl-2	DAKO, Denmark	1 hr at 37°C	1:25	Monoclonal
bcl-X	DAKO, Denmark	Overnight at room temperature	1:25	Polyclonal
mcl-1	DAKO, Denmark	1 hr at 37°C	1:100	Polyclonal
bax	Immunotech, Marseille, France	Overnight at room temperature	Ready to use	Monoclonal
bak	Oncogene Research Products, Calbiochem, San Diego, CA	1 hr at 37°C	1:20	Monoclonal

of mantle cell lymphoma (MCL). The immunophenotypic characteristics of the various categories were as follows: B-CLL, $CD20^+$, $CD5^+$, $CD23^+$, $CD10^-$, $CD3^-$, Bcl-2^+, $CD43^+$; FL, $CD20^+$, $CD74^+$, $CDw75^+$, $CD5^-$, $CD23^-$, $CD10^+$, $CD3^-$, Bcl-2^+, $CD43^-$; MCL, $CD20^+$, $CD5^+$, $CD23^-$, $CD10^-$, $CD3^-$, Bcl-2^+, $CD43^+$, cyclin $D1^+$; MZBL, $CD20^+$, $CD5^-$, $CD23^-$, $CD10^-$, $CD3^-$, Bcl-$2^{+/-}$, $CD43^-$. Section of a reactive LN was used as a control for immunohistochemical evaluation.

Preparation of Histology Material

To provide uniform conditions for immunohistochemistry and facilitate immunostain operations in one batch, tissue arrays were prepared. After the original hematoxylin and eosin sections were screened and a 3–4 mm^2 area representing the lesion on the slide was identified, the corresponding area in the paraffin block was punched out. Representative material from 24 cases was reembedded in paraffin to constitute one paraffin block (one tissue array), and from these tissue arrays 4-μm-thick paraffin sections were cut on poly-L-lysine-coated slides.

Immunohistochemistry

The paraffin sections were immunostained using mouse monoclonal antibodies to bcl-2 (DAKO, Denmark), bcl-X (DAKO), mcl-1 (DAKO), bax (Immunotech, Marseille, France), and bak (Oncogene Research Products, Calbiochem, San Diego, CA). The deparaffinized tissue sections were treated in a microwave oven in citrate buffer pH 6.0 for 10 min for antigen retrieval. The dilutions of the primary antibodies used, incubation time, and incubation temperature are provided in Table I. The reaction was localized by the peroxidase-labeled streptavidin biotin procedure (LSAB+ kit, DAKO, Denmark). Sections from a reactive LN served as positive controls for all immunostains, while those without addition of primary antibody served as negative controls. After mounting, the immunostained slides were marked by horizontal and vertical lines on the coverslips to facilitate identification of individual tissue bits (cases). The intensity of the reaction was scored on a scale of 0–3.

Statistical Analysis

To evaluate differences in the intensity of expression of various proteins and of the apoptotic indices among the subsets of B-NHL, one-way ANOVA analysis and Scheffe's post-hoc test were used. The inter-relationship between the expression of the various proteins was tested by bivariate correlation and linear regression analysis. SPSS statistical software, version 10, for Windows was used for the analysis.

RESULTS

Signal Localization

The bcl-2, bcl-X, and bax expression was noted predominantly as a diffuse cytoplasmic signal. The mcl-1 expression was noted as diffuse and granular positivity in the cytoplasm. The bcl-2 expression in most cases, and bcl-X and mcl-1 expression in a few cases, was also noted on the nuclear membrane. The bak immunostaining was seen as fine or coarse granular positivity in the cytoplasm, with the positivity being more concentrated toward one of the poles.

We did not notice any significant heterogeneity in the expression of the various proteins between tumor cells in a given case. However, the intensity of staining varied between cases (Figure 1).

Expression of Bcl-2 Family Members in Various IB-Cell NHLs

Most CLLs, FLs, and MCLs had moderate to high expression of bcl-2 and bcl-X. The MZBCLs had high expression of bcl-2 and low to intermediate expression of bcl-X . While most CLLs and MCLs were either negative or had low expression of mcl-1, the majority of FLs and MZBCLs expressed mcl-1 to varying extents. FLs were predominantly negative for bak and showed low to intermediate expression of bax. The MZBCLs had absence to low expression of bax and low to intermediate expression of bak. The CLLs and MCLs were mostly negative for bax and had low to intermediate expression of bak. None of the cases were negative for expression of all member of the bcl-2 family (Tables II and III).

We found a significant difference in the expression of bax (one-way ANOVA significance = 0.018) protein among different IB-NHLs. Specifically, FLs had a significantly higher bax expression as compared to MCL ($P = 0.018$).

Inter-relationships Between Expression of the Bcl-2 Protein Family Members in IB-NHLs

Among IB-NHLs, statistically significant correlations were as follows: bcl-2 expression with bcl-X ($P =$

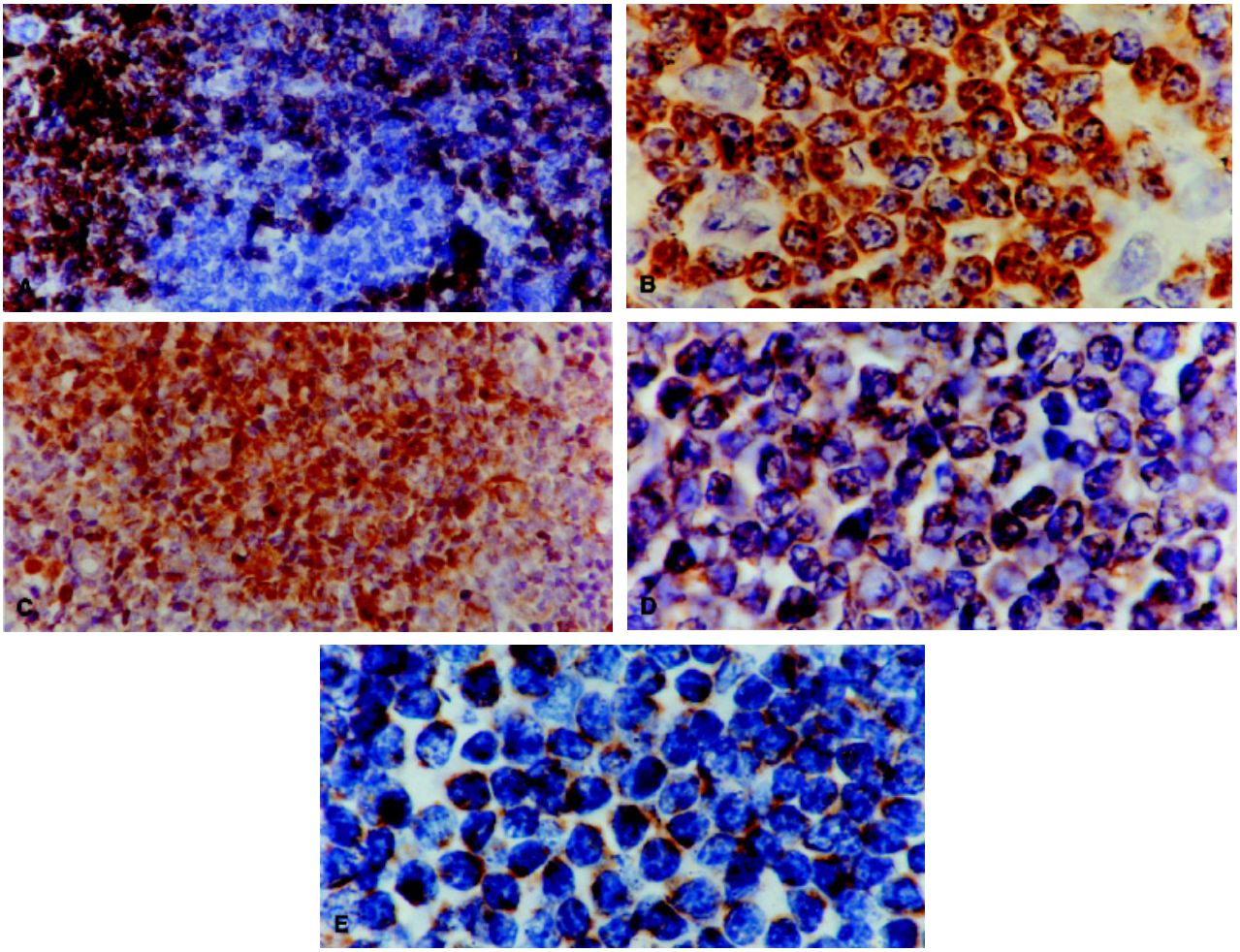

Fig. 1. (a) bcl-2 expression in mantle cell lymphoma; the tumor cells expressing bcl-2 surround a germinal center, which is negative for bcl-2. (b) bcl-X expression in chronic lymphocytic leukemia. (c) mcl-1 expression in follicular lymphoma; the neoplastic follicle shows grade 2 expression. (d) bax expression in marginal zone B-cell lymphoma. (e) bak expression in chronic lymphocytic leukaemia. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE II. Expression of Anti-Apoptotic Proteins in B-Cell NHLs (Values Represent Percentage of Cases)*

	bcl-2				bcl-X				mcl-1			
Subtype	0	1	2	3	0	1	2	3	0	1	2	3
CLL	0.0	12.5	**22.9**	**64.6**	6.3	35.4	**31.3**	**27.1**	**29.2**	**31.3**	33.3	6.3
FL	2.6	7.9	**34.2**	**55.3**	10.5	26.3	**34.2**	**28.9**	13.2	**31.6**	**31.6**	23.7
MZBCL	20.0	6.7	13.3	**60.0**	13.3	33.3	**40.0**	13.3	13.3	**40**	**26.7**	20
MCL	6.7	0.0	**40.0**	**53.3**	13.3	20.0	**40.0**	**26.7**	**33.3**	**26.7**	20	20

*CLL, chronic lymphocytic leukemia; FL, follicular lymphoma; MZBCL, marginal zone B-cell lymphoma; MCL, mantle cell lymphoma; 0, negative; 1, mildly intense; 2, moderately intense; 3, highly intense.

0.011) and bak ($P = 0.001$); bcl-X expression with mcl-1 ($P = 0.007$); bax expression with mcl-1 ($P = 0.031$); and bak expression with mcl-1 ($P = 0.004$). All correlations were direct, and we found no inverse relationships.

We further checked for the relative over-expression of the pro-apoptotic in comparison to the anti-apoptotic proteins. Three of the bcl-2 negative cases, four of bcl-X negative cases, and eight of mcl-1 negative cases expressed bax. Two of three bcl-2 negative/bax positive cases were MZBCLs, and the other case was a FL. Three of four bcl-X negative/bax positive cases were FLs, and the other case was a CLL. Among the eight mcl-1 negative/bax positive cases, four were CLLs, three were FLs,

TABLE III. Expression of Pro-Apoptotic Proteins in B-Cell NHLs (Values Represent Percentage of Cases)*

	Bax				Bak			
Subtype	0	1	2	3	0	1	2	3
CLL	**52.1**	29.2	14.6	4.2	33.3	**29.2**	**35.4**	2.1
Follicular	34.2	**36.8**	**23.7**	5.3	**50.0**	28.9	13.2	7.9
MZBCL	40.0	**53.3**	6.7	0.0	40.0	**13.3**	**46.7**	0.0
MCL	**80.0**	20.0	0.0	0.0	40.0	**20.0**	**40.0**	0.0

*CLL, chronic lymphocytic leukemia; FL, follicular lymphoma; MZBCL, marginal zone B-cell lymphoma; MCL, mantle cell lymphoma; 0, negative; 1, mildly intense; 2, moderately intense; 3, highly intense.

and one was a MZBCL. While none of the bcl-2 negative cases expressed bak, one of the bcl-X negative cases (CLL) expressed bak. Six cases (five CLLs and one MCL) were mcl-1 negative/bak positive.

DISCUSSION

The bcl-2 family of proteins contributes to the cell's decision to undergo or not to undergo apoptosis on confrontation with death signals, and this is a crucial intermediate phase in the death or survival induction pathway that is distinct from the initiators and the effectors of apoptosis. This decision for apoptosis or survival is not dependent on the levels of any one of the members but is a net result of the titration of the several components of the bcl-2 family [1–3]. Some of the better-established interactions are (i) interactions of bcl-2 and bax–bax homodimers promoting death and bcl-2 homodimers and bcl-2–bax heterodimers promoting survival; (ii) inhibition of bax by formation of heterodimers with bcl-2, bcl-Xl, and mcl-1; (iii) in spite of bak not having a direct pro-apoptotic function, it functions as an anti-anti-apoptotic protein by binding to bcl-2 and bcl-Xl; and (iv) formation of bax homodimers by either sequestration or release bcl-2 and bcl-Xl by bid and bad depending upon the phosphorylation status.

Non-neoplastic lymphoid cells show remarkable changes in the pattern of expression of the bcl-2 family of proteins depending on the stage of differentiation [8]. As noted in our study, lymphoma subsets also differ in the expression of these proteins, but the pattern deviates from that of their "normal" counterparts. While normal germinal center B cells express bcl-Xl, mcl-1, bax-α, and bad, FL cells express bcl-2, bcl-Xl, and mcl-1 [9]. The normal mantle cells have high amounts of bcl-2, low amounts of bcl-X, bax, and bak, and do not express mcl-1. In contrast, the MCLs express higher amounts of bcl-X and a marginally higher mcl-1.

Earlier studies have shown that bcl-2 is expressed in most CLLs, in a majority of FLs, and in low-grade mucosa-associated lymphoid tissue NHLs [10,11]. Further, a majority of the NHLs express bcl-X with bcl-Xl as the predominant form [12]. There has been some discrepancy in the reported expression of bax among NHLs. While some reports claim bax expression in 100% of NHLs, others portray a more conservative estimate [11–13].

In our study, while the expression of bcl-2 and bcl-X appears to be similar among IB-NHLs (except that MZBCL had relatively lower levels of bcl-X), bax expression was more variable. FL and MZBCL had relatively high bax expression; bax expression was intermediate in B-CLL and lowest in MCL. It is noteworthy that median survival of these patients follows a similar trend, with FL and MZBCL having the best survival, B-CLLs having an intermediate survival, and MCL having the worst survival [6]. It is also well known that MCLs respond poorly to chemotherapy [14]. This poor response could be related to the negative bax expression seen in an overwhelming majority (80%) of MCLs. Among the other pro-apoptotic proteins, a higher bak expression has been reported in CLL and MCL [15]. Our study also documented a higher expression of bak among the cases of CLL, MCL, and MZBCL.

The upregulation of the bcl-2 protein in FLs is a consequence of t(14;18) [16]. Variant translocations of the bcl-2 gene with breakpoint in the 5′ region and juxtaposition to the light chain genes have been found in approximately 10% of CLLs [17]. Hypomethylation of the bcl-2 gene has also been observed in CLLs [18]. However, the biologic basis of the alterations in the levels of expression of the other bcl-2 family members in B-NHLs is less clear. In FLs and in reactive follicles, high bcl-Xl levels are thought to be maintained through CD40 signaling via CD40L-positive reactive T cells [19]. The mechanism of bcl-Xl upregulation in other IB-NHLs that have scant CD40L-expressing reactive T cells needs investigation. Further, Epstein-Barr virus (EBV) latent membrane protein-1 has been shown to upregulate mcl-1 transcription [20]. However, this is unlikely to be the factor responsible for its upregulation in NHLs without apparent EBV association (most IB-NHLs are not associated with EBV). Further, the modulation of the levels of bax and bak in human lymphomas is largely unknown.

One other way to address the issue whether the variations in the expression of the bcl-2 family of proteins in

innate or induced by the environment would be to examine the expression levels of these molecules in different compartments, such as LN, peripheral blood, and bone marrow. Higher expression of CD20 and CD44H has been demonstrated in the peripheral blood B-CLL cells as compared to the cells populating the LNs [21,22]. We have not come across any study addressing the differences in the expression of the bcl-2 family of proteins between peripheral blood and LN samples in patients of B-CLL. The results and the implications could be quite intriguing.

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