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Bax mutations are an infrequent event in indolent lymphomas and in mantle cell lymphoma

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ABSTRACT

Background and Objectives. **The Bax gene is one of the most important genes involved in apoptosis regulation. Recently, it has been proposed that inactivating mutations of this death agonist may contribute to the pathogenesis of human tumors. This study was aimed at defining the status of the Bax gene in indolent lymphomas.**

Design and Methods. **Fifty paraffin-embedded biopsies from patients with indolent lymphomas (10 small lymphocytic lymphomas, 5 immunocytomas, 20 follicular lymphomas and 15 marginal zone lymphomas) and 10 mantle cell lymphomas (MCL) were studied. All six exons of the Bax gene, together with their flanking sequences, underwent mutational analysis by PCR-SSCP followed by direct sequencing of positive cases. Moreover, Bax protein expression was investigated in all samples by immunohistochemical analysis.**

Results. **All analyzed cases showed wild type Bax gene alleles and variable levels of Bax protein expression.**

Interpretation and Conclusions. **This study indicates that deregulation of apoptotic control in indolent lymphomas and MCL is not caused by Bax mutations and that other molecular mechanisms must, therefore, be involved.**

Key words: Bax, lymphomas, apoptosis

Bax gene is a central member of the Bcl-2 apoptosis regulator gene family. Members of this family can be divided into two groups: death antagonists, including Bcl-2, Bcl-xL, Mcl-1 and A1, that protect the cells against apoptosis, and death agonists such as, Bax, Bak, Bad, and Bcl-xS, that make cells sensitive to apoptosis.[1-5] The different members of the Bcl-2 family can form homodimers or heterodimers with other members of the same family through the highly conserved amino acid residues of the homologous domains, BH-1, BH-2 and BH-3.[1,3]

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The ratio of death agonists to antagonists determines the susceptibility to death stimuli.[2] *In vitro* studies showed that the ratio between Bcl-2/Bax protein determines the susceptibility to cell death following a death signal. Bax overexpression in cell lines determined Bax/Bax homodimerization and acceleration of apoptotic death, whereas the overexpression of Bcl-2 determined prevalence of Bcl-2/Bax or Bcl-2/Bcl-2 heterodimers with death repression.[2,5] Several studies have indicated that the BH-3 domain is important for the formation of Bax homodimers, while BH-1 and BH-2 domains are important for heterodimerization between Bax and Bcl-2 or other Bcl-2 family members.[6] Bax and other members of the Bcl-2 family regulate the induction of apoptosis through control of the activation of caspases, by a mechanism involving the release of mitochondrial cytochrome c.[5] Bax-deficent mice exhibit lymphoid hyperplasia suggesting that Bax may function as a tumor suppressor in human hematopoietic cells.[7] Moreover, it has recently been postulated that mutations of Bax may be present in several human neoplasias in which the inactivation of normal Bax protein could result in the prevalence of death antagonists.[8-13] The most frequent Bax mutations are: 1) missense mutations in the BH domains that cause loss of Bax pro-apoptotic function or alter the interaction between this gene and the other Bcl-2 family members; 2) frameshift mutations in deoxyguanosine residues (G8 tract) within the Bax coding sequence, that cause a complete absence of Bax protein.[5,8-12,14] Using PCR-SSCP methods inactivating mutations of Bax have been observed in 21% of cell lines derived from hematologic tumors and Burkitt's lymphoma but not in T-cell acute lymphocytic leukemia (T-ALL) *in vivo*.[8,10,12] Indolent lymphomas are a group of lymphoid neoplasias that, according to the REAL classification, is histologically composed of 4 classes: follicular lymphoma, marginal zone lymphoma, well differentiated small lymphocytic lymphoma/LLC and immunocytoma.[15] Although the histologic pattern is similar to mantle cell

Table 1. List of primers and PCR conditions used for amplification of the coding exons of Bax.

Fragment	*Sequence of primers*	*Annealing temperature*	*$MgCl_2$*
Exon 1 (207 bp)	5'-CGTTCAGCGGGGCTCTCA-3' 5'-CAGGCCGGTAGGAAGGAT-3'	57°C	1 mM
Exon 2/3 (400 bp)	5'-CCCCTAGAACCCAAGAGTC-3' 5'-GGCTGAGAGTCCTGTGTCC-3'	60°C	1 mM
Exon 4 (209 bp)	5'-TCTCCTGCAGGATGATTGC-3' 5'-TCCCCAGGTCCTCACAGAT-3'	57°C	1 mM
Exon 5 (192 bp)	5'-CAGGCAGTGGGGACAAGGTT-3' 5'-GCGGTGGTGGGGGTGAGGAG-3'	63°C	1 mM
Exon 6 (237bp)	5'-CCCCTGGCCGAGTCACTGAA-3' 5'-AATGCCCATGTCCCCCAATC-3'	62°C	2 mM
Exon 3 (G8) (94 bp)	5'-ATCCAGGATCGAGCAGGGCG-3' 5'-ACTCGCTCAGCTTCTTGGTG-3'	60°C	1.5 mM

lymphoma with proliferation of a population of small lymphocytes with clumped chromatin, inconspicous nucleoli, and barely visible cytoplasm, indolent lymphomas exhibit different immunophenotypic, genetic and clinical features.[15-18] For these reasons and to relate clinical and pathologic aspects better, several authors suggest that the classification of this group of lymphomas shoud be completely revised.[19-21] The aim of the present report is to improve the definition of the apoptotic control of this group of neoplasms, through mutational analysis and protein expression analyses of the Bax gene.

Design and Methods

Sample and DNA extraction

We examined samples from 10 patients with mantle cell lymphomas and 50 with indolent lymphomas, including 10 small lymphocytic lymphomas, 5 immunocytomas, 20 follicular lymphomas, and 15 marginal zone lymphomas, extranodal MALT-type. Histology was defined according to REAL classification[18] in the department of Pathology of Università Cattolica del Sacro Cuore in Rome. The percentage of malignant cells in all samples was 70% or greater. Three slides, 10 μm thick, were cut from tissues embedded in paraffin blocks, treated with xylene and washed twice with ethanol. Genomic DNA was extracted with a DNA extraction kit (QIAGEN, Hilden, Germany) and its amplificability was tested for six exons of the p53 gene, used as a housekeeping gene. As a control, DNA was extracted from blood of normal donors by standard phenol/cloroform. A positive mutated control for the G8 tract, the DNA from cell line CEM, which has a G7 tract in an exon 3 allele of Bax, was used.

PCR-SSCP and sequence analysis to detect Bax mutations

Table 1 show the sequence and sites of primers used to amplify the six coding exons of Bax, and the PCR conditions.[22] Each 30 μL of PCR reaction sample contained 100-200 ng of DNA, 0.4 μmol/L primers, 200 μmol/L dNTPs, 0.4 U Taq DNA polymerase (Promega, Madison, WI, USA), and 2 μCi ^{32}P dCTP (3,000 μCi/mmol; Dupont, Delaware, USA). PCR products were diluted fivefold in loading buffer containing formamide (95%), denaturated at 95°C for 10 minutes, and placed on ice for two minutes. Finally, two or three μL of the mixture were loaded onto 6% denaturing and non-denaturing (including glycerol) polyacrylamide gel.

Electrophoresis was performed at 400 Volts for 18-24 hours. Gels were then dried and exposed overnight at room temperature, or at –80°C for 2-3 hours, with Kodak X-OMAT AR film (Kodak, Rochester, NY, USA) and an intensifying screen. The samples which showed abnormal bands on the gel were subjected to direct sequencing using a Sequinase kit according to the manufacturer's instructions (Amersham, Uppsala, Sweden). Following electrophoresis on 6% polyacrylamide (8 mol/L urea) gel, samples were analyzed by autoradiography.[23,24]

Immunohistochemistry

Bax protein was detected using the anti-Bax p19 polyclonal antibody (Santa Cruz, San Diego, CA, USA) directed against the amino terminal portion of the human Bax. After incubation with the primary antibody, followed by avidin-biotin peroxidase complex, the reaction was revealed using tyramide amplification and diaminobenzidine as a chromogen (Dako Catalyzed Amplification System, Dakopatts, Golstrup, The Netherlands).

Results

Mutational analysis of the Bax gene

Using PCR-SSCP analysis, we analyzed samples from 50 indolent lymphomas and 10 mantle cell lymphomas for mutations of all six exons of the Bax gene. All cases showed wild type Bax alleles for all exons of the gene, independently of histologic subtype. The only abnormal band was found in exon 3 of the DNA of the cell line CEM, that showed a single nucleotide deletion (G7) in a simple tract of 8 deoxyguanosine residues (G8) encompassing codons 38 to 41 of human Bax (Figures 1 and 2).

Immunohistochemical expression of Bax protein

All 60 cases studied displayed expression of Bax protein. Using, as an internal positive control

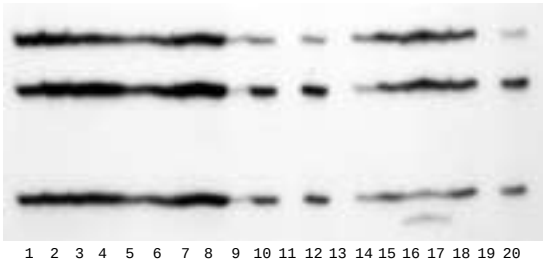

Figure 1. PCR-SSCP analysis of point mutations for a 94-base pair region encompassing the G(8) tract in exon 3 of Bax. Genomic DNA from follicular lymphoma (lanes 1-5), marginal zone lymphoma (lanes 5-10), mantle cell lymphoma (lanes 11-13), well differentiated small lymphocytic lymphoma (lane 14), immunocytoma (lane 15), DNA from CEM cell line (lane 16-17) and DNA from normal donors (lanes 18 and 20) was subjected to PCR-SSCP analysis. Electrophoresis was carried out in a gel containing 10% glycerol at room temperature. DNA from the CEM cell line shows a different pattern of bands with respect to normal controls in lanes 18 and 20, caused by a single nucleotide deletion, G(7), in this cell line. The negative control and unamplified samples, in lanes 19, 11 and 13, produce white lines.

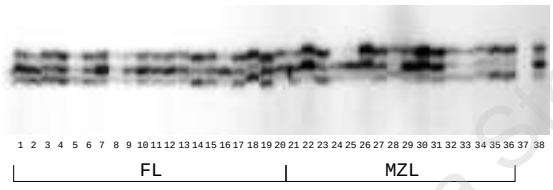

Figure 2. PCR-SSCP analysis of point mutations for a 143-base pair region encompassing the G(8) tract in exon 3 of Bax. The figure shows a PCR-SSCP analysis, in denaturing conditions, using genomic DNA from follicular lymphomas (FL) and marginal zone lymphomas (MZL). The migration patterns of FL and MZL samples were similar to those of a normal donor in lane 38. Lane 37: negative control.

(4+), the excretory ducts of salivary glands, present in MALT type extranodal marginal zone lymphoma, we compared the relative levels of Bax expression between different specimens. Follicular lymphoma and small lymphocytic lymphoma showed strong expression of Bax protein (3+); while marginal zone lymphoma, extranodal MALT type, and immunocytoma showed an intermediate Bax immunoreaction (2+); weak Bax expression was present in mantle cell lymphoma and splenic marginal zone lymphoma (1+).

Discussion

This study indicates that Bax is rarely involved, if at all, in the molecular pathogenesis of indolent lymphomas and mantle cell lymphomas. An intriguing point is the apparent discrepancy between the high frequency of Bax mutations in cell lines and the absence of mutations found in primary biopsies. This relatively high percentage of Bax mutations coud be associated with the immortalization process of fresh primary neoplasias. In fact, Bax mutations would constitute a selective *in vitro* growth advantage, since the small fraction of Bax defective cells would acquire a better capacity to adjust to the new environment. This phenomenon has been postulated also in T-ALL, in which Bax mutations are rare *in vivo* and relatively common in cell lines.[10,12,25] The low incidence of frameshift Bax mutations in indolent and mantle cell lymphomas further supports the notion that microsatellite instability (MSI) is not a feature of pathogenesis of these lymphomas.[26-28] In solid colorectal and gastric tumors with replication error prone (RER^+), microsatellite repeats, localized within the coding sequence of genes such as Bax or IGFIIR or TGFB-RII, are preferentially targeted and cluster with tumor cases displaying the RER^+ phenotype as consequence of MSI.[9,11] This is not surprising since the dominant type of genetic damage in hematologic tumors consists of specific translocations juxtaposing genes that are normally distant in the genome, thereby activating proto-oncogenes and/or creating new fusion proteins.

This study suggests that the deregulation of the apoptotic process in low-grade lymphomas is not due to Bax alterations and that other molecular mechanisms are implicated. It was recently postulated that microenvironmental signals are responsible at least for the control of survival of follicular lymphoma (FL) cells *in vitro*. These FL cells would be activated via a CD40/CD40 ligand interaction resulting in an increase in the level of Bcl-XL expression and a promotion of survival.[29] A similar event could be possible in other types of low-grade lymphomas, in which the microenvironment and the interactions between neoplastic and non-neoplastic cells (including inflammatory cells) might play a more relevant role in determining the apoptotic choice of tumor cells. To establish a possible incidence and prognostic significance of Bax protein expression, Gascoyne *et al*. examined the immunohistochemical expression of Bax protein in diffuse non-Hodgkin's lymphomas with a large cell component (DLCL) and correlated this analysis with clinical data. We showed that the highest expression of Bax protein is found in follicular and small lymphocytic lymphomas; intermediate expression in marginal zone lymphomas, extranodal MALT type, and in immunocytomas, while splenic marginal zone lymphomas and mantle cell lymphoma were char-

acterized by low Bax protein expression. By itself, Bax expression cannot be considered a major prognostic marker in indolent lymphomas. To improve the understanding of the role of this protein in the apoptotic process in this group of lymphomas, the correlation between Bax, Bcl-2 and other members of this family should be studied. Despite the absence of Bax mutations, the different Bax expression in indolent lymphomas may be caused by alterations in pre- or post-transcriptional regulatory mechanisms: in particular, p53 gene dysregulation may have an important role.[30]

Finally, our study shows that the analysis of Bax does not offer clues for a more precise characterization of indolent lymphomas, despite the heterogeneity of this group of diseases, suggesting the need for further studies on other pathologic and clinical markers.

Contributions and Acknowledgments.

MM and LML designed the study. MM, together with FD'A and FP, analyzed the samples and prepared the manuscript; RR, GL and AC were involved in critically revising the intellectual content of the manuscript; LML gave the final approval for its submission. The order in which the names of the Authors appeared is based on the importance of their contributions. LML was a major contributor to the study design and the manuscript writing and therefore appears last.

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Disclosures

Conflict of interest: none.

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Potential implications for clinical practice

- Cytogenetic[31,32] and molecular characterization of indolent lymphomas might provide new prognostic criteria.

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