Bcl-2 Associated Athanogene-1 Overexpression in Diffuse Large B-Cell Lymphoma

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Abstract

Background: Apoptosis and cell cycle regulation play an important role in pathogenesis and tumor progression in patients with Diffuse Large B-Cell Lymphoma (DLBCL). Bcl-2 associated athanogene-1 (BAG-1) is an antiapoptotic protein as well as a regulator of cell growth. There is no conclusive evidence about BAG-1 protein expression in this disease. Objective: To investigate the expression level of BAG-1 protein in DLBCL. Methods: Thirty patients diagnosed from 1997-2004, as having DLBCL, were selected. Also 30 normal lymph nodes were included as normal counterparts in this study. BAG-1 expression was determined by immunohistochemical staining in both DLBCL and normal lymph node samples. Results: Of the 30 DLBCLs examined, 100% were positive for nuclear and 83% were positive for cytoplasmic BAG-1 staining. Of the 30 normal lymph nodes investigated, 20% were positive for nuclear and 0% were positive for cytoplasmic BAG-1 staining. Nuclear staining in DLBCL samples was significantly higher than those of normal lymph nodes (100% versus 20%, p <0.001). Besides, cytoplasmic staining in DLBCL samples was significantly higher than those of normal lymph nodes (83% versus 0%, p <0.001). There was no association between BAG-1 staining and patients’ overall survival. Conclusion: Our data indicated that BAG-1 protein was deregulated in this disease similar to some other malignancies such as breast and colon cancer. Overexpression of BAG-1 in DLBCL suggests that this protein probably plays an important role in the pathogenesis of DLBCL. Besides, higher nuclear BAG-1 staining might be correlated with poor prognosis.

Keywords: BAG-1, DLBCL, Immunohistochemistry, Apoptosis

INTRODUCTION

Diffuse large B cell lymphoma (DLBCL) is the most common type of lymphoid malignancy and accounts for 30-40% of adult non-Hodgkin lymphomas (NHLs) (1,2). Because of their marked biological heterogeneity and highly variable clinical course,
numerous markers detectable by immunohistochemistry and linked to different biological aspects of these tumors have been studied. They included mainly markers of proliferation (3), cell cycle regulation (4), apoptosis and others (5). Bcl-2 associated athanogene-1 (BAG-1) is a multifunctional protein that interacts with a wide range of cellular targets to regulate growth control pathways important for normal and malignant cells including apoptosis, proliferation, transcription, metastasis and motility (6,7). It exists as multiple isoforms (BAG-1L, BAG-1M and BAG-1S), which are differentially localized in the cell. BAG-1L is predominantly nuclear, BAG-1S is predominantly a soluble cytoplasmic protein and BAG-1M is distributed between the two compartments (8,9). Localization of BAG-1 isoforms in the cell can be regulated. BAG-1 interacts with a diverse array of molecular targets including Bcl-2 apoptosis regulator (10), Retinoblastoma susceptibility protein (Rb) and p53 (11,12). A major role of BAG-1 is to prevent release of proapoptotic factor, cytochrome c from mitochondria, which is important for the activation of caspases in response to many apoptotic inducers (13). Previous studies have shown that BAG-1 might be an important downstream target of survival signaling pathways. It is also important to note that regulation of BAG-1 expression appears to be highly cell type-specific (14,15). The expression of BAG-1 is frequently altered in malignant cells (7). Pathways regulated by BAG-1 play key roles in cancer development and progression and determine response to therapy. There has been considerable interest in determining the clinical significance of BAG-1 expression in malignant cells (16); for example BAG-1 expression in the nuclei of Esophageal Squamous Cell Carcinoma (ESCC) plays an important role in tumor development and may be useful for predicting the prognosis after surgery (17). Although it is possible that BAG-1 has multiple targets, inactivation of p53 might be one way in which it can protect cells from apoptosis (18). It is also demonstrated that in Hela cells with BAG-1 underexpression, p53 expression increases. This indicates that BAG-1 may promote apoptosis by enhancing the function of p53 (19). Genetic alterations and/or disregulation of p53 and Rb are frequently detected in DLBCL (1). According to the above studies and effects of BAG-1 protein on growth control pathways in normal and malignant cells and its significance as a prognostic marker (6,7,16), our hypothesis is that abnormal BAG-1 expression may be observed in DLBCL compared to normal cells and different levels of nuclear and cytoplasmic BAG-1 immunostaining may be useful as a prognostic marker for determining the DLBCL clinical outcome and making decisions on therapy. To test this hypothesis we investigated BAG-1 protein expression on paraffin-embedded tissue sections of DLBCL patients and studied its relation to overall survival (OS).

MATERIALS AND METHODS

Thirty DLBCL tumor blocks collected from 1997-2004 from Omid Hospital of Isfahan, and thirty normal lymph nodes used as normal counterparts were included in this study. The sections of breast cancer and DLBCL samples with strong BAG-1 expression were used as positive controls. The diagnosis of DLBCL was based on paraffin-embedded tissue sections. The DLBCL samples were morphologically characterized by the large size of the cells, their vesicular nuclei with prominent nucleoli and their relatively abundant cytoplasm. All of the DLBCL tissue samples had been fixed in 4% buffered formalin, processed and embedded in paraffin according to the normal schedule used in the laboratory. To
confirm the DLBCL diagnosis, Hematoxylin & Eosin as well as CD20, CD15, CD30, EMA and ALK IHC slides (Figure 1) were prepared from every specimen and studied by a pathologist. Five μm thick sections were cut from each block, transferred to poly-L-lysine coated slides and dried overnight at 37°C. The sections were deparaffinized in Xylene (5 minutes at room temperature) and rehydrated through the graded concentrations (100, 100, 95, 85, 70, 50) of ethanol to distilled water. For optimal results, heat – induced epitope retrieval was performed in a high pH buffer (Dako, Denmark) and kept in a microwave at 700 W for 20 min.

![Image](CD 20 x 1000) ![Image](CD 20 x 400)

**Figure 1.** A representative of CD20 IHC staining of a patient’s sample, confirming DLBCL diagnosis.

Immunohistochemical staining was performed using universal LSAB2 kits (Dako, Denmark). Primary antibody was a mouse monoclonal IgG antibody specific for human BAG-1 (clone KS-6C8, Dako, Denmark), diluted 1/100 v/v in antibody dilution buffer (Dako, Denmark) before use. Blocking reagent was applied on each slide for 20 min followed by 10 min incubation with diluted primary antibody. The sections were then incubated with anti-mouse IgG antibody for 10 min followed by incubation with HRP streptavidin for another 10 min. Staining of the sections was performed upon incubating them with Diaminobenzidine (DAB) chromogen (Dako, Denmark -K3465) for 5 min. All of the incubations were performed in humid chambers and at room temperature. After each staining procedure, the slides were rinsed three times in PBS (pH 7.2). Finally, the sections were lightly counterstained in Mayer’s Hematoxylin and mounted in histomount (Zymed, 00-8000). The slides were read in a blind fashion under microscope, and the results were recorded as follows: Immunoscoring was performed separately for both normal lymph node and DLBCL samples. Since subcellular localization is clearly important for BAG-1 function, cytoplasmic and nuclear stainings were ranked independently on a score system of staining intensity with categories of 0, none; 1+, light; 2+, moderate; 3+, heavy; and 4+, intense. The percentage of immune positive tumor cells was determined by counting a minimum of 200 cells from at least three representative high power fields. Cells were considered positive only when a micropunctate pattern of staining was seen in nucleus, cytoplasm or both. H-scores were then calculated as the product of intensity (0 to 4) X distribution (0% to 100%), with H-scores ranging from 0 to 400. Two tumor sections from each DLBCL sample were immunostained and scored separately to minimize the effects of immunohistochemical heterogeneity. However BAG-1 immunostaining patterns did not significantly differ among pairs of sections from the same DLBCL sample (data not shown). The pathologist (M.M.) scoring the stained samples was unaware of the clinical histories of the patients. All samples were reread in a blind fashion by the pathologist, and the mean H-score of both tumor sections was determined. To set cutoffs for dichotomization of data into high (positive) and low (negative)
expression groups, the mean H-score data for the entire data of normal lymph node samples were calculated. The H-score > 0.5 and H-score > 0 were determined by this approach to be appropriate for use as cutoffs for nuclear BAG-1 positivity and cytoplasmic BAG-1 positivity, respectively (20,21).

Statistical analyses were performed using SPSS software (Version 11.5). The Fisher test was used to compare both nuclear and cytoplasmic BAG-1 protein expression between DLBCL and normal lymph node groups. Pearson correlation coefficients were calculated for the evaluation of the association between BAG-1 staining levels and OS.

RESULTS

BAG-1 expression. BAG-1 immunostaining was compared between DLBCL and normal lymph node samples. 100% of the DLBCL samples were positive for BAG-1 expression. As for the pattern of BAG-1 expression, all samples with DLBCL (100%) had the nuclear staining pattern and 25 (83%) had cytoplasmic staining pattern. Patterns of BAG-1 immunostaining are illustrated in Figure 2. In normal group, 6 samples (20%) had the nuclear staining pattern and none (0%) had cytoplasmic staining pattern in the B cells of the germinal center (Figure 3). There were significant differences (p<0.001) between nuclear BAG-1 staining in DLBCL and normal cases (100% versus 20%), and significant differences (p<0.001) between cytoplasmic BAG-1 staining in DLBCL and normal cases (83% versus 0%) (Table 1). In DLBCL group, there was an association between nuclear and cytoplasmic BAG-1 staining (p<0.05). Our study demonstrated overexpression of BAG-1 in patients with DLBCL compared to the normal lymph nodes. There was no correlation (p>0.05) between different levels of nuclear and cytoplasmic BAG-1 staining and the OS.

![Image](https://example.com/image1.jpg)

**Figure 2.** BAG-1 immunostaining of DLBCL samples by Immunohistochemistry. Different levels of BAG-1 expression observed in different samples, a) light b) moderate c) intense. Resolution scale: 300dpi

![Image](https://example.com/image2.jpg)

**Figure 3.** BAG-1 immunostaining of normal lymph node samples by Immunohistochemistry demonstrating the lack of BAG-1 expression. Resolution scale: 300dpi
**Table 1. Expression of BAG-1 in Normal lymph node and DLBCL samples**

<table>
<thead>
<tr>
<th>Proteins</th>
<th>cutoff</th>
<th>Normal lymph node</th>
<th>DLBCL</th>
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<tr>
<td></td>
<td></td>
<td>No (%)</td>
<td>No (%)</td>
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<tr>
<td>BAG-1, cytosolic</td>
<td>≥0.5</td>
<td>0/30</td>
<td>25/30</td>
<td>0.001</td>
</tr>
<tr>
<td>BAG-1, nuclear</td>
<td>&gt;0.5</td>
<td>6/30</td>
<td>30/30</td>
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