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,

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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).

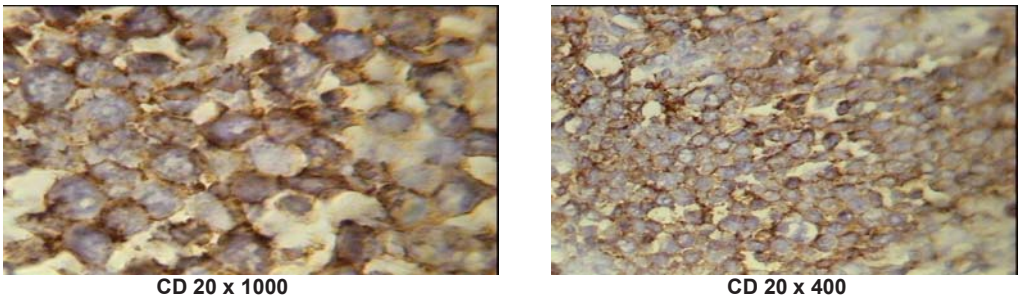
According to the above studies and effects of BAG-1 protein on growth control pathways in normal and malignant cells and its significance as a prognostic marker (6,7,16), our hypothesis is that abnormal BAG-1 expression may be observed in DLBCL compared to normal cells and different levels of nuclear 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.



CD 20 x 1000 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).

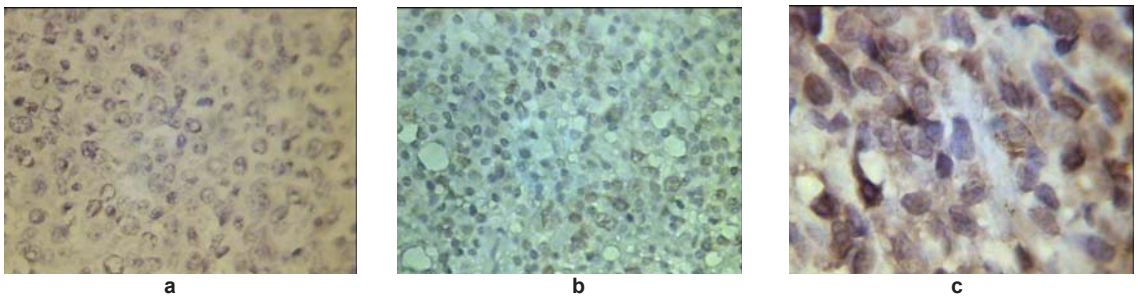
Finally, the sections were lightly counterstained in Mayer's Hematoxylin and mounted in histomount (Zymed, 00-8000). The slides were read in a blind fashion under microscope, and the results were recorded as follows: Immunoscoring was performed separately for both normal lymph node and DLBCL samples. 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.



a b c

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

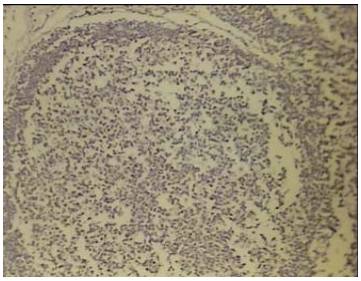


Figure 3. BAG-1 immunostaining of normal lymph node samples by Immunohistochemistry demonstrating the lack of BAG-1 expression. Resolution scale: 300dpi

Table 1. Expression of BAG-1 in Normal lymph node and DLBCL samples

Proteins	Normal lymph nodes			DLBCL		p
	cutoff	No	%	No	%	
BAG-1, cytosolic	>0	0/30	0	25/30	83	<0.001
BAG-1, nuclear	>0.5	6/30	20	30/30	100	<0.001

DISCUSSION

Immunohistochemical studies of BAG-1 protein expression in some human cancers showed a relationship between its expression and clinical outcome. In majority of invasive breast carcinomas, overexpression of BAG-1 was important for both multi-step oncogenesis and resistance of cancer cells to apoptosis induced by DNA-damaging alkylating agents. It is possible that nuclear expression of BAG-1 is associated with a shorter disease-free and overall survival in the early stages of breast cancer, while cytoplasmic expression has been associated with longer survival in non-small cell lung cancer (22). Yamauchi et al (2001) demonstrated that in contrast to breast and lung cancer, nuclear expression of BAG-1 in laryngeal tumors conferred a poor failure-free survival rate after radiotherapy and suggested that nuclear BAG-1 localization predicts unfavourable outcome, thus radiation therapy should be undertaken for laryngeal carcinoma patients (22,23). BAG-1 protein was frequently deregulated in a variety of malignancies including colorectal cancer. The expression of nuclear BAG-1 was associated with poorer prognosis and was potentially a useful predictive factor for distant metastasis in colon cancer. Nuclear BAG-1 inhibited apoptosis in a colorectal adenoma-derived cell line. This indicated that nuclear localizations of the BAG-1 protein confers a survival advantage in colorectal adenoma-derived cells and that nuclear BAG-1 could potentially be an important survival factor in colorectal carcinogenesis (24).

To the best of our knowledge there is no evidence about BAG-1 protein expression in DLBCL. This is the first study to demonstrate the BAG-1 protein expression (cytoplasmic and nuclear) by immunohistochemical methods and to indicate its prognostic significance for determining the clinical outcome of DLBCL (2- year overall survival) in such patients. The data presented here showed high nuclear and cytoplasmic BAG-1 expression in DLBCL group compared to the value of nuclear and cytoplasmic BAG-1 staining in normal lymph nodes. Cytoplasmic staining of normal lymph nodes was 0%. BAG-1 expression differed widely among different samples in this study. This might be the result of biological heterogeneity in DLBCL. Although determination of BAG-1 expression levels by densitometry alleviates the subjectivity of scoring BAG-1 labeling intensity to some extent, it does not give information on whether the immunoreactivity is nuclear or cytoplasmic, therefore we used the score system (16). Our data showed low levels of cytoplasmic BAG-1 expression in this type of malignancy that is different from the studies of BAG-1 expression in breast cancer, in which relatively high levels of cytoplasmic BAG-1 expression was detected in two thirds or more of the cases (16). Immunoreactivity in neoplastic B-cells was found in cytosol, nucleus or both, whereas immunoreactivity in normal B-cells were found only in nucleus. This result was consistent with prior studies of BAG-1 immunolocalization in normal tissues in which the level of BAG-1 protein in Western blot was determined with a densitometer and expression level of BAG-1 was very low (25). It seems that our findings are similar to those demonstrated by Takayama et al who looked at differences between normal lymph

nodes (no BAG-1) and lymphoma cell lines (increased BAG-1 expression) (9). However, the use of a patient cohort in comparison to cell lines is extremely valuable. Our data was consistent with prior studies of BAG-1 expression by Western blot analysis in which the level of BAG-1 expression was significantly higher in lymphomas compared to normal lymph nodes (26). Our study demonstrated BAG-1 protein overexpression in patients with DLBCL relative to normal lymph nodes. Besides, there was no significant difference between nuclear and cytoplasmic BAG-1 staining and OS. This can be due to the small cohort of patients used. In addition to using large clinical trials, applying International Prognostic Index (IPI) instead of OS and other prognostic factors is also needed in future studies.

According to the above studies we proposed that interaction of BAG-1 with Bcl-2, p-53 and other apoptosis regulating proteins may play an essential role in the oncogenesis and disease progression of DLBCL. Since BAG-1 interacts with Bcl-2 and is unregulated by mutant p53 in vitro (20), it would be interesting to determine if their expression is correlated with each other and with other clinical prognostic factors in DLBCL. Our data indicated that BAG-1 protein was deregulated in this disease similar to some other malignancies. Our data showed that different levels of BAG-1 staining was not correlated with OS of patients with DLBCL. However, examination of more DLBCL samples is required. Numerous studies with other carcinomas revealed that different immunostaining patterns (nuclear and cytoplasmic) of BAG-1 may be associated with distinct clinical behavior. It is possible that BAG-1 may play a role as a prognostic factor in DLBCL, thus we also suggest further investigations to clarify the exact relationship between different immunostaining patterns of BAG-1 and different prognostic factors in large cohort of patients with DLBCL.

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REFERENCES

1 DeLevalL,HarrisNL.VariabilityinimmunophenotypeindiffuselargeB-celllymphomaanditsclinicalrelevance. Histopathology. 2003;43:509-28.
2 Tzankov A, Pehrs AC, Zimpfer A, Ascani S, Lugli A, Pileri S et al. Prognostic significance of CD44 expression in diffuse large B cell lymphoma of activated and germinal centre B cell-like types: a tissue microarray analysis of 90 cases. J Clin Pathol. 2003; 56:747-52.
3 Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U, Stein H. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol. 1984; 133:1710-5.
4 Leroy K, Haioun C, Lepage E, Le Metayer N, Berger F, Labouyrie E et al. p53 gene mutations are associated with poor survival in low and low-intermediate risk diffuse large B-cell lymphomas. Ann Oncol. 2002; 13:1108-15.
5 Sanchez E, Chacon I, Plaza MM, Munoz E, Cruz MA, Martinez B et al. Clinical outcome in diffuse large B-cell lymphoma is dependent on the relationship between different cell-cycle regulator proteins. J Clin Oncol. 1998; 16:1931-9.
6 Townsend PA, Cutress RI, Sharp A, Brimmell M, Packham G. BAG-1: a multifunctional regulator of cell growth and survival. Biochim Biophys Acta. 2003; 1603:83-98.
7 Townsend PA, Stephanou A, Packham G, Latchman DS. BAG-1: a multi-functional pro-survival molecule. Int J Biochem Cell Biol. 2005; 37:251-9.
8 Packham G, Brimmell M, Cleveland JL. Mammalian cells express two differently localized Bag-1 isoforms generated by alternative translation initiation. Biochem J. 1997; 328:807-13.
9 Takayama S, Krajewski S, Krajewska M, Kitada S, Zapata JM, Kochel K et al. Expression and location of Hsp70/Hsc-binding anti-apoptotic protein BAG-1 and its variants in normal tissues and tumor cell lines. Cancer Res. 1998; 58:3116-31.
10 Takayama S, Sato T, Krajewski S, Kochel K, Irie S, Millan JA et al. Cloning and functional analysis of BAG-1: a novel Bcl-2-binding protein with anti-cell death activity. Cell. 1995; 80:279-84.

11 Arhel NJ, Packham G, Townsend PA, Collard TJ, Zadeh AM, Sharp A et al. The retinoblastoma protein interacts with Bag-1 in human colonic adenoma and carcinoma derived cell lines. Int J Cancer. 2003; 106:364-71.
12 King FW, Wawrzynow A, Hohfeld J, Zylicz M. Co-chaperones Bag-1, Hop and Hsp40 regulate Hsc70 and Hsp90 interactions with wild-type or mutant p53. EMBO J. 2001; 20:6297-305.
13 Takayama S, Reed JC. Molecular chaperone targeting and regulation by BAG family proteins. Nat Cell Biol. 2001;3: 237-41.
14 Packham G, White EL, Eischen CM, Yang H, Parganas E, Ihle JN et al. Selective regulation of Bcl-XL by a Jak kinase-dependent pathway is bypassed in murine hematopoietic malignancies. Genes Dev. 1998;12:2475-87.
15 Sekiya M, Adachi M, Takayama S, Reed JC, Imai K. IFN-gamma upregulates anti-apoptotic gene expression and inhibits apoptosis in IL-3-dependent hematopoietic cells. Biochem Biophys Res Commun. 1997;;239:401-6.
16 Cutress RI, Townsend PA, Brimmell M, Bateman AC, Hague A, Packham G. BAG-1 expression and function in human cancer. Br J Cancer. 2002; 87:834-9.
17 Noguchi T, Takeno S, Shibata T, Fumoto S, Uchida Y, Yokoyama S et al. Nuclear BAG-1 expression is a biomarker of poor prognosis in esophageal squamous cell carcinoma. Dis Esophagus. 2003; 16:107-11.
18 Xiong J, Chen J, Chernenko G, Beck J, Liu H, Pater A et al. Antisense BAG-1 sensitizes HeLa cells to apoptosis by multiple pathways. Biochem Biophys Res Commun. 2003; 312:585-91.
19 Yang X, Pater A, Tang SC. Cloning and characterization of the human BAG-1 gene promoter: upregulation by tumor-derived p53 mutants. Oncogene. 1999; 18:4546-53.
20 Sjostrom J, Blomqvist C, von Boguslawski K, Bengtsson NO, Mjaaland I, Malmstrom P et al. The predictive value of bcl-2, bax, bcl-xL, bag-1, fas, and fasL for chemotherapy response in advanced breast cancer. Clin Cancer Res. 2002; 8:811-6.
21 Turner BC, Krajewski S, Krajewska M, Takayama S, Gumbs AA, Carter D et al. BAG-1: a novel biomarker predicting long-term survival in early-stage breast cancer. J Clin Oncol. 2001; 19:992-1000.
22 Tang SC, Beck J, Murphy S, Chernenko G, Robb D, Watson P et al. BAG-1 expression correlates with Bcl-2, p53, differentiation, estrogen and progesterone receptors in invasive breast carcinoma. Breast Cancer Res Treat. 2004; 84:203-13.
23 Yamauchi H, Adachi M, Sakata K, Hareyama M, Satoh M, Himi T et al. Nuclear BAG-1 localization and the risk of recurrence after radiation therapy in laryngeal carcinomas. Cancer Lett. 2001; 165:103-10.
24 Barnes JD, Arhel NJ, Lee SS, Sharp A, Al Okail M, Packham G et al. Nuclear BAG-1 expression inhibits apoptosis in colorectal adenoma-derived epithelial cells. Apoptosis. 2005; 10:301-11.
25 Yang X, Hao Y, Ding Z, Pater A, Tang SC. Differential expression of antiapoptotic gene BAG-1 in human breast normal and cancer cell lines and tissues. Clin Cancer Res. 1999; 5:1816-22.
26 Xerri L, Devilard E, Bouabdallah R, Hassoun J, Chaperot L, Birg F et al. Quantitative analysis detects ubiquitous expression of apoptotic regulators in B cell non-Hodgkin's lymphomas. Leukemia. 1999; 13:1548-53.