Expression of AIF and HtrA2/Omi in Small Lymphocytic Lymphoma and Diffuse Large B-Cell Lymphoma

Shaoying Li, MD; Mei Wan, MD, PhD; Xu Cao, PhD; Yongsheng Ren, MD, PhD

**Context.**—The pathogenesis of non-Hodgkin lymphoma may involve deregulation of apoptosis. In response to apoptotic stimuli, several proapoptotic proteins are released into the cytoplasm from the mitochondria, including second mitochondria-derived activator of caspases/direct inhibitor of apoptosis protein binding protein with low pI (Smac/DIABLO), apoptosis-inducing factor (AIF), and high temperature requirement protein A2 (HtrA2/Omi). Apoptosis-inducing factor promotes apoptosis through a caspase-independent pathway, whereas Smac/DIABLO and HtrA2/Omi do so through both caspase-dependent and caspase-independent pathways. Smac/DIABLO was reported to be strongly positive in diffuse large B-cell lymphoma (DLBCL) and virtually absent in small lymphocytic lymphoma/chronic lymphocytic leukemia (SLL/CLL). Little is known about the expression of AIF and HtrA2/Omi in lymphomas.

**Objective.**—To evaluate the expression of AIF and HtrA2/Omi in SLL and DLBCL.

Apoptosis or programmed cell death is a tightly regulated and selective physiologic process that maintains the optimal number of cells in tissues by removing redundant, damaged, or functionally abnormal cells. It is essential for normal tissue homeostasis, cellular differentiation, and development. Deregulation of apoptosis is thought to be a hallmark of human cancer. Proper regulation of apoptosis is extremely important in the hematopoietic system, a cellular compartment with an intrinsic proliferative capacity and a high cell turnover rate. Apparently, in addition to oncogenic mutation and stimulation, apoptosis plays an important role in the pathogenesis of hematologic neoplasm. For example, it is believed that small lymphocytic lymphoma/chronic lymphocytic leukemia (SLL/CLL) results from decreased apoptosis, which is supported by overexpression of antiapoptotic proteins such as BCL2, tumor necrosis factor α, and TOSO (also known as Fas inhibitory molecule 3), and lack of expression of proapoptotic protein such as second mitochondrial-derived activator of caspases/direct inhibitor of apoptosis protein binding protein with low pI (Smac/DIABLO) and others. Diffuse large B-cell lymphomas (DLBCLs) are a heterogeneous group of tumors and may associate with either low or high levels of apoptosis. Difference in resistance to apoptosis is possibly responsible for the different response of DLBCLs to chemotherapy and therefore the different prognosis.

**Results.**—Apoptosis-inducing factor was strongly and diffusely expressed in 19 of 23 (83%) cases of DLBCL with comparable expression pattern between germinal center–like and non-germinal center–like subgroups. Apoptosis-inducing factor was weakly positive in 15 of 20 (75%) cases of SLL/CLL with increased intensity in pseudofollicles. In contrast, HtrA2/Omi was weakly expressed in SLL/CLL (17 of 20; 85%) and DLBCL (18 of 23; 78%).

**Conclusions.**—The different expression level and pattern of AIF and HtrA2/Omi in SLL/CLL and DLBCL may suggest different apoptotic mechanisms involved in the pathogenesis and prognosis of these diseases. HtrA2/Omi does not appear to be a major player in the regulation of apoptosis of DLBCL and SLL/CLL.

(Arch Pathol Lab Med. 2011;135:903–908)
temperature requirement protein A2 (HtrA2/Omi), cytochrome c, and endonuclease G. Once released into the cytosol, these mitochondrial proteins activate both caspase-dependent and caspase-independent cell death pathways. To protect against inadvertent damage and death, cells have evolved a system of checks and balances. The members of evolutionarily conserved inhibitor of apoptosis protein (IAP) family are endogenous caspase inhibitors and inhibit apoptosis. 18 At least 3 classes of IAPs composed of a total of 8 proteins have been identified in humans including the best known cIAP1, cIAP2, and XIAP. 19 Overexpression of IAPs has been found in a wide variety of cancer cell lines and primary tumor samples. 20,21 There is growing evidence that IAPs play a role in cancer pathogenesis and resistance to chemotherapy and radiotherapy. 22,23

Apoptosis-inducing factor is a 57-kDa protein encoded by “programmed cell death 8” gene located on the X chromosome in humans. 24 It has been known that AIF has both apoptosis and nicotinamide adenine dinucleotide oxidase activities. 25 In physiologic situations, AIF is a nicotinamide adenine dinucleotide oxidase with a local redox function in the mitochondria. It acts as a free radical scavenger and protects cells from cell death induced by oxidative stresses. 26,27 The mitochondrial level of AIF has been recently associated with tumorigenicity of various carcinoma cell types. 28 Apoptosis-inducing factor promotes apoptosis through a caspase-independent pathway. Upon release from mitochondria, AIF is translocated to the nucleus where it binds to the chromosome via its C-terminal nuclear localization sequence and causes DNA condensation and fragmentation. 29,30 Apoptosis-inducing factor lacks intrinsic nuclease activity and its DNA degrading activity depends on the recruitment of downstream nucleases. Studies have showed the cooperation of AIF with endonuclease G, a DNAse of mitochondrial origin that is involved in the caspase-independent pathway, in Caenorhabditis elegans and mammals. 29,30 Dual functions of AIF have been proposed: The prosurvival function of AIF plays a crucial role in tumorigenesis, whereas the apoptotic activity of AIF might contribute to cancer cell death induced by some agents including chemotherapy. HtrA2/Omi is a 37-kDa serine protease located in the mitochondrial intermembrane space that plays an important physiologic role in mitochondrial homeostasis. 30 Upon apoptotic stimulation, it is released into the cytosol, where it contributes to apoptosis through both caspase-dependent and caspase-independent pathways. Similar to Smac/DIABLO, HtrA2/Omi binds to and neutralizes IAPs (XIAP, cIAP1, and cIAP2) via its N-terminal IAP-binding motif, therefore releasing the inhibition of IAP to caspase-3, -7, and -9. It also exerts its proapoptotic function via its protease activity, independent of IAP and caspase. 21,32

Because AIF and HtrA2/Omi both play important roles in apoptosis, analysis of their expression is necessary to understand the pathogenesis of cancer. The expression of AIF and HtrA2/Omi has been studied in some tumors or tumor cell lines, such as colorectal carcinoma, esophageal squamous carcinoma EC9706 cells, gastric carcinoma, and renal cell carcinoma. 33–38 However, little is known about their expression in lymphomas. In this study, we have used immunohistochemical method to assess the expression of AIF and Omi/HtrA2 in SLL/CLL and DLBCL.

### MATERIALS AND METHODS

This study was approved by the University of Alabama at Birmingham institutional review board. The study group included 43 surgically resected lymphomas, which included 23 DLBCL cases and 20 cases of SLL/CLL. The control group included 10 cases of benign/reactive lymph nodes. These cases were retrieved from surgical pathology archives of the University of Alabama at Birmingham Hospital. The clinical history, pathology reports, and hematoxylin-eosin–stained slides were reviewed to confirm the diagnosis.

Immunohistochemical staining was performed on serial paraffin-embedded sections of human lymphoid tissue using UltraVision One detection system (HRP polymer and DAB Plus chromogen, Thermo Fisher Scientific, Fremont, California). The sections were then incubated with either AIF antibody (rabbit polyclonal, 1:50 dilution, Epitomics, California) overnight at 4°C. After washing in Tris buffered saline 0.025% Triton X-100, the slides were incubated in hydrogen peroxide block for 10 minutes. After washing in Tris buffered saline buffer, they were incubated with Ultra V Block for 5 minutes to block nonspecific binding. The sections were then incubated with either AIF antibody (rabbit polyclonal, 1:50 dilution, Cell Signaling, Massachusetts) or HtrA2/Omi antibody (rabbit monoclonal, 1:50 dilution, Epitomics, California) overnight at 4°C. The expression levels of AIF and Omi/HtrA2 were graded from 0 to 3+.

### RESULTS

The patients’ age, sex, and tumor location are summarized in Table 1. The cases were of DLBCL include 8 GCB, 9 non-GCB, and 6 remaining cases of undetermined subtypes. The SLL/CLL includes 2 cases with either
prolymphocytic or large cell transformation. Apoptosis-inducing factor immunostaining, when present, was detected in the cell cytoplasm with a fine or coarse granular pattern. In reactive lymph nodes, moderate to strong AIF immunoreactivity was detected in the lymphoid cells of follicular centers of 10 benign/reactive lymph nodes (Figure 1, A). Some follicular dendritic cells and histiocytes also showed cytoplasmic

Figure 1. Immunohistochemical staining of apoptosis-inducing factor in diffuse large B-cell lymphoma and small lymphocytic lymphoma/chronic lymphocytic leukemia. A, Reactive lymph node with follicular hyperplasia with the arrow showing a germinal center (original magnification ×500). B, Diffuse large B-cell lymphoma (original magnification ×500). C, Small lymphocytic lymphoma/chronic lymphocytic leukemia with the arrow showing a proliferation center (original magnification ×200). D, The area without proliferation center in C (original magnification ×500). E, The proliferation center in C (original magnification ×500). F, The area of small lymphocytic lymphoma/chronic lymphocytic leukemia with prolymphocytic transformation (original magnification ×500).
stain. The frequency of AIF expression in DLBCL and SLL/CLL is summarized in Table 2. Apoptosis-inducing factor was strongly and diffusely expressed in 15 of 23 cases of DLBCL (Figure 1, B). There is no difference in the AIF expression level and pattern between GCB and non-GCB subgroups of DLBCL. The expression of AIF was detected in 15 of 20 SLL/CLL with most cases showing weak positivity (Figure 1, C). Unlike DLBCL, SLL/CLL demonstrates a heterogeneous AIF expression pattern characterized by predominantly positive staining in large cells and increased intensity in pseudofollicles (Figure 1, C through E). A strong and diffuse staining of AIF was noted in the areas with either prolymphocytic or large cell transformation in 2 CLLs/SLLs (Figure 1, F).

In contrast, HtrA2/Omi immunostain was only weakly expressed in 17 of 20 cases of SLL/CLL (85%), 18 of 23 cases of DLBCL (78%), and the follicular center and mantle zone of 10 benign lymph nodes (Figure 2, A through C). It is difficult to tell if it is true positivity or background

<table>
<thead>
<tr>
<th></th>
<th>Diagnosis</th>
<th>No. of Cases</th>
<th>AIF</th>
<th>+</th>
<th>+</th>
<th>Positive, %</th>
<th>P*</th>
<th>HtrA2/Omi</th>
<th>+</th>
<th>+</th>
<th>Positive, %</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DLBCL</td>
<td>23</td>
<td>15</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>83</td>
<td>0</td>
<td>1</td>
<td>17</td>
<td>3</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>SLL/CLL</td>
<td>20</td>
<td>2</td>
<td>2</td>
<td>11</td>
<td>5</td>
<td>75</td>
<td>0</td>
<td>0</td>
<td>17</td>
<td>3</td>
<td>85</td>
</tr>
</tbody>
</table>

Abbreviations: DLBCL, diffuse large B-cell lymphoma; SLL/CLL, small lymphocytic lymphoma/chronic lymphocytic leukemia.

* P value by 2-tailed Fisher exact test to compare the positive % of AIF and HtrA2/Omi between DLBCL and SLL/CLL.
staining. There is no increased intensity of HtrA2/Omi in 2 cases of SLL/CLL with either prolymphocytic or large cell transformation (Figure 2, D).

Overall, the expression of AIF was stronger in DLBCL than in SLL/CLL, but there was no significant difference in the frequency of expression between these 2 lymphomas ($P > .05$). For HtrA2/Omi expression, both the frequency and intensity showed no significant difference ($P > .05$).

**COMMENT**

Deregulation of apoptosis is not only involved in the development of hematopoietic malignancies but also frequently associated with the resistance to anticancer therapies, including chemotherapy, radiation, or immunotherapy.$^{39}$ The apoptosis machinery of cells is tightly regulated by proapoptotic and antiapoptotic proteins. Knowing the expression pattern of these proapoptotic and antiapoptotic proteins is the necessary step to understand the pathogenesis of hematopoietic malignancies and further aids the development of targeted therapy. To our knowledge, the expression of 2 proapoptotic proteins, AIF and HtrA2/Omi, in lymphomas has not been examined to date. In our present study, we have shown that AIF is expressed in germinal center lymphoid cells and some histiocytes and dendritic cells of normal/reactive lymph nodes. It is detectable in more than 80% of DLBCLs and SLLs/CLLs with the expression level much higher in DLBCL than in SLL/CLL. HtrA2/Omi is similarly and weakly expressed in all normal/reactive lymph nodes, DBCLs, and SLLs/CLLs.

SLL/CLL results from an accumulation of malignant small B cells due to an imbalance between cell proliferation and death rates. This imbalance may be caused by increased cell proliferation, decreased death, or a combination of both processes. It is believed that both low proliferative rate and decreased cell death or a defect in apoptosis play a role in clinical indolent non-Hodgkin lymphomas such as SLL/CLL. Our results of weak expression of AIF and HtrA2/Omi in SLL/CLL combined with previous studies of total lack of Smac/DIABLO expression$^7$ and variable expression of cIAP1 and cIAP2 in SLL/CLL$^{31}$ appear to support the notion that this lymphoma is characterized by defective apoptosis. However, the weak and cytoplasmic expression of AIF may indicate that AIF does not play a critical role as either a prosurvival or an apoptotic factor in CLL/SLL. Interestingly, because the expression of AIF is largely located in large cells and proliferation centers of SLL/CLL, and highly and diffusely increased in the areas of either prolymphocytic or large cell transformation, the increased level or diffuse staining pattern of AIF in those areas of SLL/CLL may indicate a potential role of AIF in promoting cell proliferation and transformation to an aggressive disease course.

Clinically aggressive non-Hodgkin lymphomas such as DLBCL are highly proliferative and may be associated with either low or high level apoptosis. It has been shown that Smac/DIABLO is highly expressed in approximately 53% of DLBCLs.$^9$ Our current study demonstrated that AIF is strongly expressed in most cases of DLBCL. High frequency and level of AIF and Smac/DIABLO in DLBCL may indicate that both caspase-dependent and caspase-independent apoptotic pathways are functionally active in this lymphoma. Because apoptosis is strictly regulated by multiple proapoptotic and antiapoptotic proteins, the actual apoptotic rate in DLBCL also depends on other factors like IAPs. Our data indicate no difference of AIF expression between GCB and non-GCB subgroups of DLBCL.

An important fact about AIF is its double functions.$^{25}$ Normally, AIF is located in the mitochondria and involved in the oxidative phosphorylation process. After apoptotic stimuli, it is released to the cytoplasm from mitochondria and further translocates to the nucleus to induce apoptosis. Apparently, translocation to the nucleus is critical for its proapoptotic function. Both previous studies in other tumors and our results in SLL/CLL and DLBCL showed AIF expression in the cytoplasm. Is there any unknown factor that inhibits its translocation to the nucleus? Does this possible inhibition contribute to the defect of apoptosis? Alternatively, the strong cytoplasmic expression of AIF may represent a major prosurvival function of AIF in tumorigenesis and progression in DLBCL, while the apoptotic activity of AIF may contribute to cell death induced by apoptotic stimuli such as chemotherapy, and the nuclear expression of AIF may be a temporary phenomenon that is unable to be detected in this study. A lot of details are still unclear and much work needs to be done to further elucidate the regulation of AIF functions.

Apoptosis-inducing factor, a major player in caspase-independent apoptosis, is a very promising drug target. In lymphomas with low or defective expression of AIF, such as SLL/CLL, administration or overexpression of AIF or its agonist and use of reagents that can increase its mitochondrial release and translocation to nuclei theoretically could induce tumor cell apoptosis and serve as an anticancer therapy. Several drugs, such as BZL101 and Antiprimod, that induce AIF to release from mitochondria have undergone phase I or II clinical trial and demonstrated promising antitumor activity.$^{40}$ Both in vitro study and animal studies have shown that Antiprimod has a potential in the treatment of multiple myeloma, mantle cell lymphoma, and other advanced cancers.$^{41}$ Because it is thought that SLL/CLL commonly demonstrates decreased apoptosis and the resistance of DLBCL to chemotherapy may be due to an apoptotic defect, we postulate that Antiprimod may sensitize these lymphoma cells to apoptosis induced by chemotherapy or immunotherapy; therefore, it may enhance chemotherapy and/or immunotherapy for SLL/CLL and DLBCL, particularly for chemotherapy-refractory DLBCL. However, extensive and detailed studies are required before any possible conclusion can be made.

HtrA2/Omi plays a pivotal role in both caspase-dependent and caspase-independent apoptotic pathways, which makes it a valuable therapeutic target. Our results of low expression of HtrA2/Omi in DLBCL and SLL/CLL suggest that administration of HtrA2/Omi or its agonist, or use of reagents that increase its mitochondrial release, theoretically could induce tumor cell apoptosis. This may make it a possible therapeutic target for developing new drugs for these lymphomas.

In summary, the different expression of AIF and HtrA2/Omi in CLL/SLL and DLBCL suggests different apoptotic mechanisms involved in the pathogenesis of these diseases. High frequency and level of AIF and Smac/DIABLO (previous studies) in DLBCL indicate that both caspase-dependent and caspase-independent apoptotic pathways are functionally active in this lymphoma.
Weak expression of AIF and lack of Smac/DIABLO (previous studies) in CLL/SLL suggests that apoptosis in this neoplasm is not active. Weak expression of HtrA2/Omi in CLL/SLL, DLBCL, and benign/reactive lymph nodes suggests that HtrA2/Omi may not be a major proapoptotic protein in these disease entities. The results may help us to better understand the pathogenesis of these lymphomas and also may benefit new drug development for these lymphomas. Additional studies are required to further elucidate the roles of these proapoptotic proteins in the pathogenesis of these lymphomas.

References