Abstract

Purpose: We hypothesized that elevated expression in ovarian cancer of the BAG family of prosurvival proteins and associated partners would be associated with clinical features of aggressiveness in ovarian cancer.

Experimental Design: Expression patterns of BAG-1, BAG-3, BAG-4, and Bcl-xL were determined by immunohistochemical analysis of tissue samples obtained at diagnosis from 28 women with stage III or stage IV ovarian cancer treated with cisplatin, paclitaxel, and cyclophosphamide after initial cytoreduction. Association of these proteins, BAG-6, heat shock protein 70 (Hsp70), Hsp27, and Bcl-2, with clinical variables was tested in ovarian cancer tissue arrays from Gynecologic Oncology Group tissue bank.

Results: A statistically significant relationship was found between elevated cytoplasmic expression of BAG-4 and improved overall (P = 0.0002) and progression-free survival (P = 0.003) in the prospectively collected samples. Bcl-2 staining was significantly more frequent on the tissue array in lower stage (P = 0.005) and grade (P = 0.0009) tumors, whereas Hsp70 was prominent in higher grade cases (P = 0.002). Furthermore, Bcl-xL was more closely associated with serous compared with endometrioid ovarian cancers (P = 0.004).

Conclusion: Unexpectedly, cytoplasmic expression of BAG-4 and Bcl-2 marked less aggressive ovarian cancer, whereas nuclear Hsp70 suggested more aggressive behavior. Bcl-xL may play a more prominent function in the pathology of serous histology ovarian cancers compared with the endometrioid subtype. The findings presented here support involvement of these proteins in the propagation of ovarian cancer and provide a basis for the development of molecular therapeutics modulating these survival pathways.

Ovarian cancer affects over 22,000 women annually, and >70% of these women eventually die of their disease (1). The poor survival of affected women is due in part to three quarters of all patients presenting with advanced stage disease at the time of initial diagnosis (2). Complete response to standard chemotherapy with a taxane and a platinum agent varies from 50% to 85% in advanced disease, depending on the volume of tumor left after cytoreductive surgery (2). Despite this promising initial outcome, these patients have a high cancer-related mortality. New molecular prognostics and therapeutics may identify patients for novel therapies and potentially improve the durability of response. We observed increased expression of the prosurvival protein BAG-3 in platinum-resistant ovarian cancer cells (6). We therefore sought to determine the expression pattern of the BAG family of proteins and associated partners to discern relationships of these proteins with clinical features of aggressiveness in ovarian cancer.

The Bcl-2, BAG, and heat shock protein (Hsp) families of proteins directly interact with each other to regulate cell survival (see diagram in Fig. 1). The Bcl-2 family and associated proteins have been implicated in the pathogenesis of ovarian cancer (3–5). Bcl-xL and Bcl-2 may have prognostic importance in ovarian cancer (6, 7). The BAG proteins act as cochaperones for Bcl-2 and Hsp70 (8, 9). BAG-1, BAG-3, and BAG-4 [also known as silencer of death domain (SODD)] are involved in the survival of cancer cells and have been linked with aggressiveness of breast, gastric, and pancreatic cancer (8–11). Loss of BAG-4, conversely, has been observed in resistance to platinum chemotherapy (7, 12). The BAG proteins have been shown as both cytoplasmic and nuclear in location, although the functional importance of this has not been elucidated (9). Hsp27 and Hsp70 are cochaperones for the Bcl and BAG proteins and modulate induction...
of apoptosis in response to cellular stresses (13). Both Hsp27 and Hsp70 have been negatively correlated with survival in solid tumors (13, 14). Hsp27 was found to be elevated in tissue and serum from pancreatic cancer patients (14). We hypothesized, based on existing evidence, that elevated expression of the antiapoptotic BAG, Bcl, and Hsp proteins would correlate with increased aggressiveness of ovarian cancer.

In the current study, we examined members of the Bcl-2, BAG, and Hsp protein families by immunohistochemical analysis of tumor tissue. We quantified frequency of expression and subcellular localization. Expression patterns were analyzed in tissue specimens obtained at diagnosis from 28 women treated with the triple therapy regimen, from which we have up to 10-year clinical follow-up information. Details of patient outcome are reported elsewhere (15, 16).7 We also evaluated expression of these proteins in a tissue microarray of 170 ovarian cancers generated by the Gynecologic Oncology Group tissue bank. Here, we present our findings and their relationship with clinical features of histologic subtype, grade, stage, and patient survival.

**Patients and Methods**

**Patients**

Sixty-two women with advanced stage newly diagnosed ovarian cancer were treated between 1995 and 2001 using a triple-drug regimen of cisplatin, high-dose paclitaxel, and cyclophosphamide (15–19).7 Briefly, patients received cyclophosphamide (750 mg/m² i.v.) on day 1, paclitaxel (250 mg/m², 24-h infusion) beginning on day 1, and cisplatin (75 mg/m²) on day 2. Cycles were repeated every 21 days. Tissue blocks of primary and/or metastatic disease from the initial staging and cytoreductive surgery were collected for study of prognostic factors according to the National Cancer Institute Institutional Review Board–approved protocol and consent. Primary cytoreductive surgery was not done at our institution, and patients were referred for treatment; therefore, in some cases, we did not receive tissue blocks from the referring institution or the tissue received was of inadequate quality or quantity for replicate analysis of the multiple proteins included in the analysis presented here. The criterion used to select the 28 cases included in the current analysis from the 62 enrolled patients was simply the availability of tumor blocks that contained tissue adequate for this immunohistochemical staining analysis.

**Tissue array**

Replicate copies of the “stage and grade” ovarian cancer tissue array were obtained from the Gynecologic Oncology Group (GOG) tissue bank (Children's Hospital) under NIH Office of Human Subjects Protection approval. These arrays contained replicate tissue samples from 170 ovarian cancer patients with endometrioid or serous epithelial histology spanning stages I to IV and grades I to III. Each array contained samples in duplicate within the slide. No clinical outcome data are available for these anonymously presented clinical samples.

**Immunohistochemistry**

Formalin-fixed paraffin-embedded sections were analyzed for protein expression of BAG-1, BAG-3/CAIR-1, BAG-4/SODD, BAG-6, Bcl-2, Bcl-xL, Hsp27, and Hsp70 using immunohistochemistry. Staining was done manually. Antibody specifications and staining conditions are detailed.

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7 Hussain et al., submitted for publication.
Table 1. Antibodies used in the study

<table>
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<th>Antibody</th>
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<th>Clone</th>
<th>Source</th>
<th>Dilution</th>
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<td>BAG-6</td>
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<td>1/400</td>
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Abbreviations: M/P, monoclonal/polyclonal; MWO, microwave oven; RT, room temperature.

* A polyclonal rabbit antibody was generated against amino acids 1118 to 1132 of the COOH terminus of BAG-6/BAT3 isoform α. The peptide affinity-purified antibody was shown to have a single protein band by immunoblot that fully competes with cognate peptide (data not shown).

Table 2. Patient characteristics

<table>
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<th>GOG tissue array (n = 170)</th>
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<tr>
<td>Other</td>
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</table>

* Only included in stage and grade analyses.
Results

Patients and samples. Two groups of patients were studied. The first set of tissue samples was collected prospectively from patients receiving initial therapy with cyclophosphamide, high-dose paclitaxel, and cisplatin for epithelial ovarian cancer (15, 16). The regimen resulted in surgical complete remission or microscopic residual disease in 84%; the median overall survival for all women enrolled on the clinical trial is 67.8 months, and the median progression-free survival is 19.8 months. For the subset of 28 patients with tumor tissue available and adequate for the current immunohistochemistry analysis, the respective medians are 65.6 and 16.7 months, which indicate that these patients are approximately representative of the larger group from which they were selected. To further understand associations between the survival protein expression and clinical variables, a second set of samples, the GOG stage and grade tissue array, was also studied. This includes replicate samples of 170 patients with epithelial ovarian cancer of serous or endometrioid histology. Table 2 summarizes the patient characteristics in each group.

Elevated BAG-4 is associated with longer progression-free survival and overall survival in newly diagnosed patients. Seven events were tabulated on the prospective cohort of 28 evaluable cases: cytoplasmic BAG-1, nuclear BAG-1, cytoplasmic BAG-3, nuclear BAG-3, cytoplasmic BAG-4, nuclear BAG-4, and cytoplasmic Bcl-xL (representative staining in Fig. 2). The data were grouped into categories to allow for a minimum of four patients per category (see Materials and Methods). When evaluating cytoplasmic BAG-4, no case was scored 0 to 1 (<6% of cells staining with this marker). Therefore, cases were grouped into 2 to 3+ (n = 4) or 4+ (n = 24). Because of the small numbers of patients in each category, only a P value of <0.005 was considered to be statistically significant. Elevated (4+) expression of cytoplasmic BAG-4 was significantly associated with overall survival (P = 0.0002) and progression-free survival (P = 0.0026; Fig. 3).

Additional analyses were done to determine whether any marker had a relationship to treatment outcome or response. No other marker studied in this cohort of cases showed a statistically significant relationship with overall or progression-free survival. Moreover, no statistically significant associations were identified between the marker levels and response to treatment (data not shown).

Cytoplasmic Bcl-2 and nuclear Hsp70 are expressed inversely in ovarian cancer. The prospective cohort of 28 patients was relatively homogenous in stage (all stage III/stage IV), grade (78% grade 3), and histology (69% serous). Thus, we evaluated BAG proteins and other survival protein expression using the 170 ovarian cancer cases presented on the GOG stage and grade tissue array. This allowed analysis of relationships between expression and other descriptor variables of ovarian cancer.
cancer aggressiveness. This analysis covered the expression of 13 markers: both cytoplasmic and nuclear BAG-1, BAG-3, BAG-4, BAG-6, and Hsp70, and cytoplasmic Hsp27, Bcl-2, and Bcl-xL (Fig. 2). An inverse relationship was found between expression of cytoplasmic Bcl-2 and both grade ($P = 0.009$; Fig. 4A) and stage ($P = 0.005$; Fig. 4B) of ovarian cancer. Hsp70 staining followed the opposite pattern. A statistically significant relationship was found between quantity of cells expressing nuclear Hsp70 and increasing grade of tumor ($P = 0.002$). This nuclear Hsp70 staining showed a similar increase with stage of disease but did not reach statistical significance ($P = 0.09$).

**Cytoplasmic Bcl-2 and nuclear Hsp70 association with stage and grade is more pronounced in tumors of endometrioid histology.** We next queried whether these protein patterns were a general feature of epithelial ovarian cancer or if the protein expression differed by histologic subtype. The relationship of Bcl-2 with stage held true within the endometrioid ovarian tumors ($n = 71$), but was not observed within the serous tumors ($n = 78$; Fig. 4C and D). Two other markers were identified with trends toward statistically significant relationships in stage or grade when the endometrioid subtype of epithelial ovarian cancer was examined independently. Cytoplasmic Hsp27 followed a pattern similar to Bcl-2 in endometrioid tumors, decreasing in expression frequency with increasing stage of disease ($P = 0.02$). Cytoplasmic Hsp70 was more often expressed in higher grade endometrioid tumors ($P = 0.02$). No associations were observed with the serous subtype.

**Bcl-xL is differentially expressed between ovarian cancer subtypes.** The observed differences prompted us to interrogate the samples for markers that might differentiate the endometrial from the serous histology as a whole, without separating the cases by stage or grade of ovarian cancer. The staining of Bcl-xL was significantly different between the endometrial and serous subtypes of ovarian cancer specimens in the GOG tissue array (data not shown). Bcl-xL protein was present in 38% of serous tumors (mean staining $\pm$ SE, $0.38 \pm 0.10$), and was expressed more sparsely in only 18% of cases of the endometrioid subtype ($0.18 \pm 0.05$; $P = 0.0035$). None of the other analyzed markers were differentially expressed across the two subtypes of ovarian tumor specimens.

**Discussion**

The role of survival proteins in ovarian cancer etiology, progression, and clinical behavior can lead to new directions in molecular therapy. Overexpression of some BAG and Hsp proteins may provide mechanisms of resistance to platinum- and taxane-based chemotherapy (24, 25) or to the geldanamycin family of agents (26). We therefore hypothesized that elevated expression of these proteins would be associated with poor survival in patients with advanced stage ovarian cancer and would be differentially related to clinical markers of cancer aggressiveness, such as stage of disease or grade of tumor. These proteins were explored in specimens collected prospectively from women with advanced stage ovarian cancer receiving initial therapy in a National Cancer Institute trial of cisplatin, paclitaxel, and cyclophosphamide, from which we have up to 10-year follow-up information. The expression of these proteins was further analyzed using a tissue array of specimens of serous and endometrioid ovarian cancer of varied grades and stages. We found, unexpectedly, that high BAG-4 protein in the cytoplasm of tumor cells was linked to better overall survival. This line of investigation also detected an unanticipated pattern of high Bcl-2 and low nuclear Hsp70 in tumors of lower grade and stage. These findings create a pattern for prognosis of women with epithelial ovarian cancer and implicate BAG-4, Bcl-2, and Hsp70 in the pathogenesis of this disease.

We found a strong positive correlation of cytoplasmic BAG-4 with both progression-free and overall survival after triple chemotherapy with cisplatin, paclitaxel, and cyclophosphamide. BAG-4 blocks apoptosis in some instances, such as that signaled by tumor necrosis factor receptor-1 (27–29). BAG-4/SODD protein associates with the cytoplasmic tail of tumor necrosis factor-α receptors and blocks signaling by preventing multimerization for subsequent activation of apoptosis (27–29). In other situations, however, the role of BAG-4 may not be antiapoptotic. Although tumor necrosis factor-α is toxic to some ovarian cancer cells, in others, this cytokine may promote survival and invasion in part by up-regulating the expression of CD44, a membrane protein involved in cell adhesion and metastasis (30–32). One could then hypothesize that BAG-4
may be antiproliferative if it blocks growth and invasion signals triggered by tumor necrosis factor-α. BAG-4 may also maintain chemo sensitivity, especially to platinum agents, because loss of BAG-4 was observed with the development of platinum resistance (12). BAG-4 protein quantity has been shown to decrease upon development of resistance to cisplatin chemotherapy (12). The reason for the disappearance of BAG-4 was unclear, but it coincided with the appearance of an unidentified protein proposed to promote nuclear factor-κB prosurvival signals emanating from tumor necrosis factor receptor-1. Increased nuclear factor-κB signaling is present in platinum-resistant ovarian cancer, and proteasome inhibitors blocking nuclear factor-κB can restore sensitivity to platinum chemotherapy (33). These antisurvival/proliferative functions of BAG-4 may predominate in epithelial ovarian cancer, resulting in our finding that higher cytoplasmic BAG-4 portends a better prognosis and thus suggesting BAG-4 as a marker of lower invasive potential and/or increased sensitivity of the cancer cell to death upon treatment with platinum and taxane chemotherapy for the set of patients in the current study.

The BAG domain of BAG-4 can bind to either Bcl-2 or Hsp70, but not both simultaneously, and may thus serve as a molecular switch between downstream pathways triggered by these two molecules (28, 34). Overexpression of Bcl-2 in lymphomas promotes cancer cell survival and associates with increased aggressiveness (35). Paradoxically, however, expression of Bcl-2 in both breast and ovarian cancer has correlated with longer survival (36, 37) and was higher in normal ovarian tissue compared with ovarian carcinoma (5). Bcl-2 protein has been observed to decrease upon development of cisplatin resistance (12) similar to BAG-4. We found a statistically significant inverse relationship of Bcl-2 expression with both stage and grade of original tumor specimen. Our finding, in conjunction with others, supports the hypothesis that Bcl-2 is lost as ovarian tumors acquire more aggressive behavior.

Lower Bcl-2 occurred in our samples with higher nuclear accumulation of Hsp70. Hsp70 can protect cancer cells from death after stresses, such as heat shock and chemotherapy (38, 39), and has been proposed to cause resistance of ovarian cancer cells to farnesyltransferase inhibitors (4). Hsp70 is known to shuttle between the cytoplasm and the nucleus and remains in the nucleus during times of cellular stress to protect cells from death (40). We show that the level of Hsp70 in the nucleus of ovarian cancer cells was significantly higher with increasing grade of ovarian tumors in our present analysis. This finding could be interpreted as an underlying aggressive nature of tumors with elevated nuclear Hsp70 at baseline, implicating the function of this protein in protecting damaged cells from death (41). We and others have shown BAG-3 to have prosurvival function, although not correlated with subcellular localization.

Fig. 4. Bcl-2 and Hsp70 expression across stage and grade of ovarian cancer specimens. A. Bcl-2 staining decreases as grade of tumor increases, whereas nuclear Hsp70 staining increases with increasing grade. B. Bcl-2 is less frequent in advanced stage tumors; nuclear Hsp70 again follows the opposite pattern, but the trend did not reach statistical significance. C. specimens were classified by histologic subtype, and the same two markers were analyzed. The pattern of Bcl-2 decreasing with increasing grade and Hsp70 increasing was present in endometrioid histology of ovarian cancers (D), but not in serous tumors.
Cytoplasmic BAG-3 is a chaperone for Bcl-2 and Hsp proteins; in the nucleus, however, the function of BAG-3 has not been elucidated. Similarly, BAG-4 shuttles to the nucleus in conjunction with Hsp70 during times of cellular stress (45); one might expect then a similar pattern as we found with Hsp70, but this was not observed. We did not find a significant relationship between the nuclear and cytoplasmic patterns of BAG-4 nor did we observe an association of nuclear-localized BAG-4 with patient outcome. We did not find a significant association between the nuclear and cytoplasmic staining of any marker. Therefore, we cannot infer biological significance of their movement between cellular compartments.

Our data show cytoplasmic Hsp27 expression decreases in endometrioid ovarian cancer as stage increases. Because increased stage is a harbinger of worse outcome, this would suggest that Hsp27 may be a marker of better survival. Our findings are opposite to the trend previously published which associated the presence of Hsp27 with poor survival of ovarian cancer patients (38). Our findings were done in a background wherein survival could not be ascertained, and the second study did not reach statistical significance for the poor outcome finding. This provocative question needs to be investigated further.

The antiapoptotic protein Bcl-XL has been associated with chemoresistance and shorter-time progression in ovarian cancer (7). We found a differential distribution of Bcl-XL protein across the different subtypes of ovarian cancer. The highest expression occurred in serous tumors, especially those with advanced stage at diagnosis, a subset of patients for whom long-term outcome is still limited. The strong subtype association of this marker suggests that the serous variant of epithelial ovarian cancer may depend on Bcl-XL for growth and survival or that Bcl-XL may influence the development of the serous histology from an undifferentiated precursor cell. This mechanism has not been experimentally shown, but previous work has shown that exposure of endometrial cells to progestins promotes survival by preferential splicing of Bcl-x to the long form (46), thus suggesting a role for progestins in Bcl-XL production.

In summary, we have surveyed the expression of Bcl-2, BAG, and Hsp families of proteins to search for relationships with clinical features across 198 cases of ovarian carcinoma. We discovered significant associations of Bcl-2 and Hsp70 with aggressiveness of disease on the basis of tumor grade and stage at diagnosis. Importantly, cytoplasmic BAG-4 was strongly associated with long-term survival after platinum-based chemotherapy. These data provide additional insight to the potential protein interactions providing signals for proliferation and chemoresistance to ovarian cancer cells. This model points toward elevation of BAG-4 and Bcl-2 in the cytoplasm as markers of either less aggressive or more chemosensitive ovarian cancer cells, whereas nuclear Hsp70 suggests more aggressive behavior. The findings presented here support the involvement of these proteins in the propagation of ovarian cancer and provide a basis for the development of novel therapeutic agents modulating these survival pathways.

References


BAG-4/SODD and Associated Antiapoptotic Proteins Are Linked to Aggressiveness of Epithelial Ovarian Cancer

Christina M. Annunziata, Lilach Kleinberg, Ben Davidson, et al.


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