

Evaluation of the Prognostic Value of Cellular Inhibitor of Apoptosis Protein in Epithelial Ovarian Cancer Using Automated Quantitative Protein Analysis

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Abstract

Purpose: The cellular inhibitor of apoptosis protein (cIAP) is regarded as an important prognostic biomarker in cancer. Here, we sought to determine the prognostic value of cIAP protein levels in epithelial ovarian cancer using a novel method of compartmentalized *in situ* protein analysis.

Methods: A tissue array composed of 150 advanced-stage ovarian cancers, treated with surgical debulking followed by platinum/paclitaxel-based combination chemotherapy, was constructed. For evaluation of protein expression, we used an immunofluorescence-based method of automated *in situ* quantitative measurement of protein analysis.

Results: The mean follow-up time for the entire cohort was 34.4 months. Patients with tumors bearing high cIAP membranous expression had a 3-year survival rate of 31% compared with 73% for patients with low cIAP expressing tumors ($P = 0.0020$). In multivariable analysis, adjusting for well-characterized prognostic variables, low membranous cIAP expression level was the only significant prognostic factor for overall survival.

Conclusions: Our results indicate that cIAP protein levels have prognostic value in ovarian cancer patients. Modulation of cIAP levels may improve clinical outcome in ovarian cancer. (Cancer Epidemiol Biomarkers Prev 2006;15(6):1179-83)

Introduction

Ovarian cancer is ranked fifth in incidence of cancers among women in the United States (1). Despite the fact that it is highly curable if diagnosed early, ovarian cancer is more fatal than all the other gynecologic malignancies combined. At an early stage, when the disease is confined to the ovary, the 5-year survival rate is 95% (1); however, fewer than one third of the cases are detected at this early stage.

The current management of patients with advanced disease (stages III and IV) involves optimal surgical debulking followed by chemotherapy. The current standard chemotherapeutic approach for ovarian cancer patients includes platinum-based (plus or minus taxanes) regimens. Whereas the majority of patients respond initially, 60% to 80% of them still die of the disease. Traditional clinicopathologic factors do not accurately classify patients in relation to prognosis. The most established marker for ovarian cancer is CA-125, which is detectable in the serum of >80% of women with ovarian carcinomas (2). However, CA-125 is reliable only in monitoring response to treatment or disease recurrence and not as a diagnostic or prognostic marker (3). Therefore, considerable interest lies in identifying molecular prognostic indicators to guide treatment decisions.

Apoptosis in ovarian cancer cells, in response to chemotherapy, is mediated via activation of effector caspases 3, 6, and 7 resulting from the cleavage of their proteolytically inactive proforms (4, 5). The intrinsic pathway of apoptosis is subsequently initiated, which involves the release from the mitochondria of multiple death-promoting molecules such as holocytochrome *c* and Smac (6). Proteolytic activities of

caspase 3 are inhibited by the inhibitor of apoptosis family of proteins (X-linked IAP, cIAP1, cIAP2, and survivin; refs. 7, 8). Whereas X-linked IAP exerts its function by targeting active caspase 3 to proteasome 26S for degradation via ubiquitination (8), cIAP function is associated with Akt signaling pathway (9). Specifically, Akt phosphorylation results in cIAP stabilization, increased cIAP-1 levels, and inhibition of apoptosis whereas the hypophosphorylated Akt leads to decreased cIAP-1 levels and apoptosis.

Here, we sought to determine the association of cIAP levels with survival in a cohort ovarian cancer tissue microarray using a novel *in situ* quantitative method of protein expression and correlated those data with clinical and pathologic data.

Materials and Methods

Patient Population. Inclusion criteria were newly diagnosed epithelial ovarian cancer patients (Fédération Internationale des Gynaecologistes et Obstétristes stages III and IV) who underwent surgical resection in the Department of Gynecology of Alexandra University Hospital in Athens between 1996 and 2003 and treated postoperatively with chemotherapy regimens that all included carboplatin and paclitaxel. In all cases, an effort was made for optimal surgical cytoreduction and adequate staging, which included, at least, total abdominal hysterectomy with bilateral salpingo-oophorectomy, inspection and palpation of all peritoneal surfaces and retroperitoneal area, biopsies of suspect lesions for metastases, infracolic omentectomy, and peritoneal washings. Included patients had stage III/IV disease according to the Fédération Internationale des Gynaecologistes et Obstétristes staging system. Grading was done by evaluation of tumor architecture, the amount of solid neoplastic areas, nucleus/cytoplasm ratio, and nuclear pleomorphism. The tumors were subdivided into three groups, well-differentiated (G1), moderately differentiated (G2), and poorly differentiated (G3), according to the WHO system (10).

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Table 1. Demographic, clinical, and pathologic data

	n	cIAP membranous expression class		
		Low	High	P*
Differentiation				
Well	13	11	2	0.367
Moderate	41	39	2	
Poor	73	64	9	
Not recorded	1	1	0	
Initial histology				
Serous	91	84	7	0.196
All others	37	31	6	
FIGO stage				
III	99	87	12	0.389
IV	29	28	1	
Residual disease				
≤2 cm	28	25	3	1.000
>2 cm	100	90	10	
Clinical response to chemotherapy				
PR + CR	78	69	9	0.765
All others	50	46	4	

Abbreviations: PR, partial remission; CR, complete remission; FIGO, Fédération Internationale des Gynaecologues et Obstétristes.

*Significant at the 0.05 level.

Chemotherapy was instituted 2 to 3 weeks after surgery. All patients received chemotherapeutic regimens that included platinum (cisplatin or carboplatin) and paclitaxel. Gynecologic examination, CA-125 assay, and radiological investigations, if necessary, were done monthly for the clinical assessment of response, which was recorded according to WHO criteria (10). Follow-up examinations were done every month.

Tissue Microarray Construction. A tissue microarray consisting of tumors from each patient in the cohort was constructed at the Yale University Tissue Microarray Facility. Following institutional review board approval, the tissue microarray was constructed as previously described (11), including 150 cases. Tissue cores 0.6 mm in size were obtained from paraffin-embedded formalin-fixed tissue blocks from the Alexandra University Hospital Department of Pathology archives. H&E-stained slides from all blocks were first reviewed by a pathologist to select representative areas of invasive tumor to be cored. The cores were placed on the recipient microarray block using a Tissue Microarrayer (Beecher Instrument, Silver Spring, MD). All tumors were represented with 2-fold redundancy. Previous studies have shown that the use of tissue microarrays containing one to two histospots provides a sufficiently representative sample for analysis by immunohistochemistry (11-15). Addition of a duplicate histospot, although not necessary, does provide marginally improved reliability. The tissue microarray was then cut to yield 5- μ m sections and placed on glass slides using an adhesive tape transfer system (Instrumedics, Inc., Hackensack, NJ) with UV cross-linking.

Quantitative Immunohistochemistry. Tissue microarray slides were deparaffinized and stained as previously described (15). In brief, slides were deparaffinized with xylene followed by ethanol. Following rehydration in distilled water, antigen retrieval was accomplished by application of proteinase K and incubation for 30 minutes. Endogenous peroxidase activity was blocked by incubating in 0.3% hydrogen peroxide in methanol for 30 minutes. Nonspecific antibody binding was then blocked with 0.3% bovine serum albumin for 30 minutes at room temperature. Following these steps, slides were incubated with primary antibody at 4°C overnight. Primary antibody to cIAP1/2 (sc-12410, Santa Cruz Biotechnology, Santa Cruz, CA) was used at 1:700 dilution in 0.3% bovine serum albumin/TBS. This antibody has been validated in previous studies using immunohistochemistry and Western blot analysis of normal and neoplastic tissue (16-19). Subse-

quently, slides were incubated with goat anti-mouse secondary antibody conjugated to a horseradish peroxidase-decorated dextran polymer backbone (Envision, DAKO Corp., Carpinteria, CA) for 1 hour at room temperature. Tumor cells were identified by use of anticytokeratin antibody cocktail (rabbit anti-pancytokeratin antibody cocktail (rabbit anti-pancytokeratin antibody z0622; DAKO) with subsequent goat anti-rabbit antibody conjugated to Alexa 546 fluorophore (A11035, Molecular Probes, Eugene, OR). We added 4',6-diamidino-2-phenylindole to visualize nuclei. Target cIAP molecules were visualized with a fluorescent chromogen (Cy-5-tyramide; Perkin-Elmer Corp., Wellesley, MA). Cy-5 (red) was used because its emission peak is well outside the green-orange spectrum of tissue autofluorescence. Slides were mounted with a polyvinyl alcohol-containing aqueous mounting media with antifade reagent (*n*-propyl gallate, Acros Organics, Vernon Hills, IL).

Automated Image Acquisition and Analysis. Automated image acquisition and analysis using automated quantitative protein analysis (AQUA) has previously been described (20). In brief, monochromatic, high-resolution (1,024 × 1,024 pixel; 0.5 μ m) images were obtained of each histospot. We distinguished areas of tumor from stromal elements by creating a mask from the cytokeratin signal. 4',6-Diamidino-2-phenylindole signal was used to identify nuclei and the cytokeratin signal was used to define cytoplasm. Overlapping pixels (to a 99% confidence interval) were excluded from both compartments. The cIAP signals were scored on a normalized scale of 1 to 255, expressed as pixel intensity divided by the target area. AQUA scores for each subcellular compartment (nuclear and cytoplasmic signal) were recorded. AQUA scores for duplicate tissue cores were averaged to obtain a mean AQUA score for each tumor.

Statistical Analysis. Histospots containing <10% tumor, as assessed by mask area (automated), were excluded from further analysis. AQUA scores represent expression of a target protein on a continuous scale from 1 to 255. It is often useful to categorize continuous variable to stratify patients into high versus low categories. Several methods exist to determine a cut point, including biological determination, splitting at the median, and determination of the cut point which maximizes

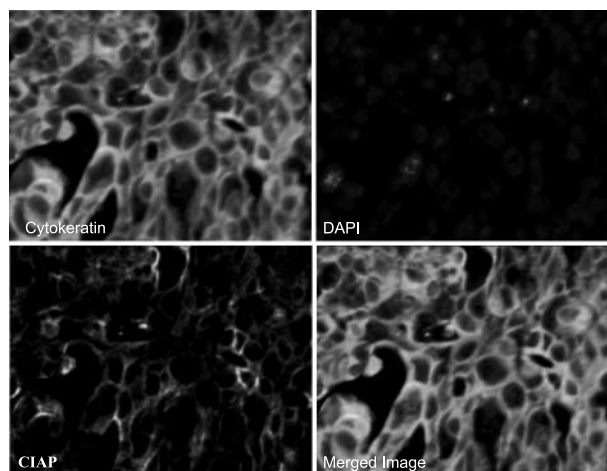


Figure 1. Protein expression of cIAP was determined using a quantitative method of *in situ* expression analysis (AQUA) based on immunofluorescence. Digital images of each tumor spot were captured using Cy3-labeled anticytokeratin antibody to generate a tumor mask. 4',6-Diamidino-2-phenylindole (DAPI) was used to visualize nuclei and Cy5 was used to visualize cIAP. A 3-color merge image for each tumor is also shown. Tumors displayed predominately a membranous expression pattern.

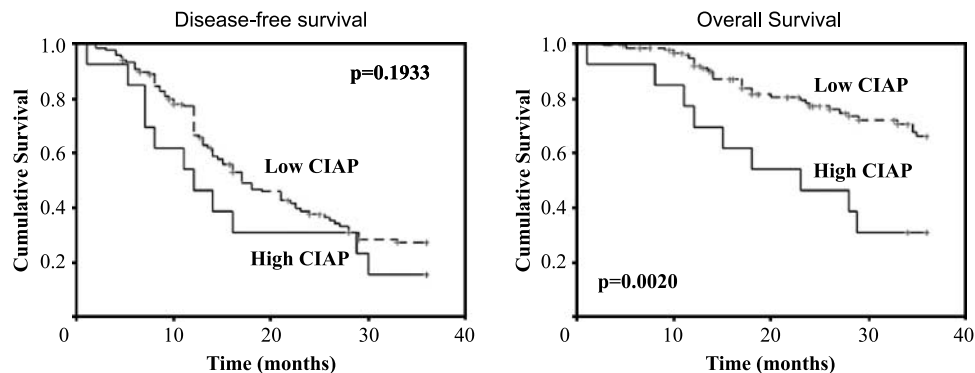


Figure 2. Kaplan-Meier survival analysis for overall survival and disease-free survival by membranous cIAP expression levels. Patients with low membranous cIAP levels had improved overall survival.

effect difference between groups. If the latter method (the so-called "optimal P -value" approach) is used, a dramatic inflation of type-I error rates can result (21). A recently developed program, X-Tile, allows determination of an optimal cut point while correcting for the use of minimum P -value statistics (22). As the AQUA technology is new, there are no established cut points available for quantitative protein expression. Therefore, for categorization of cIAP expression levels, the X-Tile program was used to generate an optimal cut point. This approach has been successfully applied to AQUA data analysis (23). Two methods of statistical correction for the use of minimal P -value approach were used. First, the X-Tile program output includes calculation of Monte Carlo P values for the optimal cut point generated. Cut points that yield Monte Carlo $P < 0.05$ are considered robust and unlikely to represent type I error. Second, the Miller-Siegmund minimal P -value correction referenced by Altman et al. (21) was used. This approach is accepted in the statistical literature, but relatively unknown in the medical/biological research community. Briefly, when making multiple comparisons to find the minimum P values using the log-rank test, the false-positive rate (i.e., the percentage of times a marker that has no true prognostic value will be found to have $P < 0.05$) can approach 40%.

Altman's statistical adjustment generates a minimum P value corrected to yield a true false-positive rate of 5%. The corrected P value (P_{cor}) is calculated as follows: $P_{\text{cor}} = \phi(\zeta) [\zeta - (1/\zeta)] \log[e] [(1 - \epsilon)(2)/\epsilon(2)] + 4\phi(\zeta)/\zeta$.⁵ Our calculations were done using an ϵ of 0.10. Disease-free survival and overall survival were subsequently assessed by Kaplan-Meier analysis with log-rank for determining statistical significance, and only P_{cor} was reported. This approach has been successfully applied to AQUA data analysis. All survival analyses were done at 3-year cutoffs. Confidence intervals were assessed by univariate and multivariate Cox proportional hazards model.

Overall survival was defined as time from first day of chemotherapy to death from any cause. Progression-free survival was defined as time from first day of chemotherapy to the first of either death from any cause or disease progression (assessed by CA-125 increase and/or imaging studies). Performance status was dichotomized into "0" versus all others, and histologic type into serous versus all others. Although several cutoff values of residual volume tumor have been proposed, it has been reported that gradual gradations of residual disease can affect ovarian cancer prognosis. Our patient population was divided into two groups according to the extent of residual disease at first surgery: ≤ 2 cm and > 2 cm. Comparisons of cIAP expression with Fédération Internationale des Gynaecologistes et Obstétristes stage and grade were

made by Mantel-Haenszel χ^2 test. Comparisons of cIAP expression with performance status, histology, clinical response, and residual disease were made by Fisher's exact test. Comparison of cIAP expression status with age was made using Pearson correlation. All calculations and analyses were done with SPSS 12.0 for windows (SPSS, Inc., Chicago, IL).

Results

Clinical and Pathologic Variable Analysis. One hundred-fifty patients were included in the study. Mean follow-up time (range) for the entire cohort was 34.4 months (range, 1-91.7 months). There were 117 (77.5%) stage III and 33 (22.5%) stage IV. One hundred-three (61%) patients had tumors of serous histology. Initial histologic grade was 14 well differentiated (9%), 49 moderately differentiated (33%), and 87 poorly differentiated (58%). One hundred-three (61%) patients had tumors of serous histology. Following initial surgical debulking, residual disease by size was distributed as follows: 38 (25%) with < 2 cm and 112 (75%) with > 2 cm. For clinical response to initial therapy, complete response or partial response was recorded in 86 (57%) patients and stable disease/no response in 64 (43%) patients. Demographic and clinicopathologic variables for the cohort are summarized in Table 1.

Generation of Optimal Cut Point by X-Tile Analysis. Of the 150 patients included in this study, 128 (85.3%) had sufficient tissue for analysis of cIAP protein expression by AQUA. Tissues deemed insufficient had $< 10\%$ tumor mask within the histospot, as represented on the tissue microarrays. As visualized by fluorescent immunohistochemistry, cIAP displayed predominantly strong membranous staining (Fig. 1). Using the X-Tile program, an optimal cut point for cIAP membranous expression was determined at 59.44 AQUA units, with a Monte Carlo P value of 0.04 as determined by X-Tile. Monte Carlo $P < 0.05$ indicates robust and valid cut point selection. Patients with cIAP membranous expression ≤ 59.44 were classified as low expressers ($n = 115$) and patients with cIAP membranous expression > 59.44 were classified as high expressers ($n = 13$). There was no association between cIAP membranous expression and any of the clinicopathologic variables.

Univariate Survival Analysis

Overall Survival. The expression status of cIAP membranous expression was evaluated for association with overall survival using Kaplan-Meier survival analysis with log-rank statistic for determining significance. This analysis showed that high expression is associated with inferior 3-year overall survival rates. As shown in Fig. 2, patients with high cIAP membranous expression had a 3-year survival rate of 31% compared with 73% for patients with low cIAP tumors ($P = 0.0020$). Univariate Cox regression revealed a hazard ratio for high cIAP tumors of 3.0 ($P = 0.002$).

⁵ Where ϕ indicates the probability density function. P_{min} is the minimum P values generated by evaluating multiple cut points. zeta is the $(1 - P_{\text{min}}/2)$ quantile of the standard normal distribution. Epsilon denotes the proportion of values excluded from consideration as an optimal cut point.

Table 2. Univariate 3-year survival analysis (Kaplan-Meier log-rank)

	Mean survival time (mo)	Cumulative survival (%)	P
cIAP AQUA analysis	Progression-free survival		
	High membranous cIAP	16	15.4
	Low membranous cIAP	20	34.8
	Overall survival		
	High membranous cIAP	22	30.8
Low membranous cIAP	31	73.0	0.1933
			0.0020 [†]

*Significant at the 0.05 level.

†Significant at the 0.01 level.

Progression-Free Survival. The expression status of cIAP was also evaluated for association with disease-free survival. Kaplan-Meier analysis showed that patients with high cIAP expressing tumors had a worse 3-year disease-free survival, but this did not reach significance ($P = 0.193$). Univariate Cox regression revealed an increased risk for patients with high cIAP tumors, but this also failed to reach statistical significance (hazard ratio, 1.5; $P = 0.201$). Results of the univariate survival analysis are summarized in Table 2.

Multivariable Survival Analysis. Using the Cox proportional hazards model, we did multivariable analysis to assess the predictive value of membranous cIAP expression. cIAP expression by AQUA was analyzed for overall survival and progression-free survival. We also included the following known prognostic variables in the regression model: grade, residual disease, response to chemotherapy, and initial histology. Residual disease (95% confidence interval, 1.72-6.93; $P = 0.0001$) and response to chemotherapy (95% confidence interval, 1.13-2.83; $P = 0.013$) were significant predictor variables of disease-free survival. For overall survival, only cIAP level (95% confidence interval, 1.3-6.031; $P = 0.009$) was a significant predictor. Results of multivariable survival analyses are summarized in Table 3.

Discussion

In the present study, we show that cIAP protein levels, evaluated by *in situ* proteomic analysis, predict for patient outcome in a cohort of chemotherapy-treated advanced-stage ovarian cancer patients. To the best of our knowledge, our study is the only one of its kind that evaluated tumor cIAP protein levels in relation to patient outcome in ovarian cancer.

Phosphorylation and subsequent activation of Akt leads to translocation of nuclear factor κ B (NF- κ B) transcription factor to the nucleus where it interacts with promoter gene targets to enhance transcription (24). The *cIAP-2* promoter contains two NF- κ B DNA-binding elements located at -210 and -147 (24). Thus, *cIAP-2* gene transcription is regulated by Akt/NF- κ B pathway.

Mabuchi et al. (25) did *in vitro* and *in vivo* studies in ovarian cancer models and showed that paclitaxel transiently activates

Table 3. Multivariate 3-year survival analysis by Cox regression

Variable	Hazard ratio (95% confidence interval)	P
Overall survival		
Grade	1.10 (0.69-1.77)	0.688
Initial histology	0.86 (0.42-1.76)	0.674
Residual disease	2.31 (0.87-6.14)	0.092
Clinical response to chemotherapy	2.14 (1.06-4.30)	0.034*
cIAP membranous high expression	2.80 (1.30-6.03)	0.009 [†]

*Significant at the 0.05 level.

†Significant at the 0.01 level.

NF- κ B via the phosphatidylinositol 3-kinase/Akt pathway and that combination therapy with paclitaxel and an NF- κ B inhibitor increases the therapeutic efficacy of paclitaxel. In addition, a study in human ovarian surface epithelial cells bearing BRCA1 185delAG mutation suggests that the increased initial response to chemotherapy was due to decreased cIAP levels through phosphorylated Akt down-regulation (26). Taken together, these preclinical studies indicate that cIAP levels determine chemotherapy-induced apoptosis in ovarian cancer cells and that cIAP levels are regulated by Akt.

Despite advances in chemotherapy, >50% of women with ovarian cancer still die of the disease. In the era of molecular therapies, the identification of molecular targets for therapy becomes extremely important. Numerous retrospective studies have reported prognostic markers in ovarian cancer using conventional immunohistochemistry. These studies are limited by the nonquantitative nature of conventional immunohistochemistry combined with the use of arbitrary immunohistochemical cut points. As a consequence, the majority of them have uncertain clinical and/or biological value. The advantage of the present study is the use of an *in situ* quantitative method of protein analysis to measure cIAP protein levels, which avoids the biases of pathologist-based scoring.

DNA microarray has the advantage of providing the molecular profile of the tumors without biological prejudice. Many genes identified by this technology are of unknown biological significance and it is even uncertain whether they are expressed at the protein level. In the present study, our goal was to evaluate whether cIAP can be used as a molecular target in ovarian cancer. Therefore, we used AQUA to answer this question. Indeed, we were able to show that cIAP levels in ovarian cancer predict patient outcome. If these results are validated in a prospective setting then, modulation of cIAP protein levels may prove a useful therapeutic strategy in ovarian cancer. Based on the aforementioned preclinical data, Akt inhibitors may decrease cIAP protein levels, but this remains a conjecture.

In summary, our results, if validated in a second cohort, are important for two reasons: First, they show that cIAP levels have prognostic significance in ovarian cancer. Second, they have important implications for treating patients with advanced ovarian cancer: As most of these patients, despite initial response to chemotherapy, relapse and die, novel therapies are desperately needed. Novel targeted therapies, such as Akt or NF- κ B inhibitors, may decrease cIAP levels and improve the outcome of patients with ovarian cancer.

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