The prognostic significance of the immunohistochemical expression of p53, bcl-2, c-erb B-2 and cathepsin-D in ovarian cancer patients receiving platinum with cyclophosphamide or paclitaxel chemotherapy

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Summary

Purpose: To evaluate the prognostic significance of the immunohistochemical expression of p53, bcl-2, c-erbB-2 and cathepsin-D in epithelial ovarian cancer (EOC).

Methods: We analyzed 100 patients with ovarian carcinoma, FIGO Stage IC-IV who underwent primary cytoreductive surgery and received adjuvant chemotherapy with cyclophosphamide and a platinum analogue (CP) (n = 46) or paclitaxel and a platinum analogue (TP) (n = 54). Immunohistochemical expression was studied on paraffin-embedded tissue from the primary tumor.

Results: After a median follow-up of 55 months median progression-free survival (PFS) and overall survival (OS) were 16 and 41 months, respectively. Positive bcl-2 staining and absence of cathepsin-D expression were associated with an increased complete response rate in the CP group ($p = 0.011$ and $p = 0.003$) but not in the TP group. PFS and OS were not associated with the expression of any of the markers studied. FIGO stage ($p = 0.006$) and histology ($p = 0.047$) were the only independent prognostic factors for survival.

Conclusion: Bcl2 and cathepsin D expression are associated with response to CP but not TP chemotherapy. P53, bcl-2, c-erb B-2 and cathepsin D expression was not correlated with PFS and OS in our study.

Key words: Ovarian cancer; p53; bcl-2; c-erb B-2; Cathepsins; Paclitaxel.

Introduction

Ovarian cancer is the fifth leading cause of death from cancer in the developed western world and remains the most lethal gynecologic cancer [1]. The majority of patients with ovarian carcinoma, present with advanced disease that has spread outside the pelvis. The current management of advanced ovarian carcinoma includes cytoreductive surgery, followed by combination chemotherapy. Despite the progress that has been achieved by incorporating paclitaxel into first-line regimens, the majority of these women will develop drug-resistant recurrences and will die of their disease [2].

Many chemotherapeutic agents that are active against ovarian carcinoma, including platinum analogues, seem to ultimately exert their action through induction of the molecular pathways that mediate programmed cell death, or apoptosis [3, 4]. The role of apoptosis in the development of resistance to chemotherapy has been increasingly investigated and key genes have been recognized as important determinants of this event [5]. The product of the bcl-2 gene is an important regulator of apoptosis, firstly identified from its involvement in the most common translocation in B-cell follicular lymphoma the t (14; 18), as a result of which the bcl-2 oncogene is juxtaposed to the immunoglobulin heavy chain gene on chromosome 14. Bcl-2 is then activated and its product enhances cell survival inhibiting programmed cell death [6]. Bcl-2 belongs to a large family of genes highly conserved in evolution that includes many other important members such as bax, bad and bik that antagonize inhibition of apoptosis, by binding to bcl-2 [7, 8]. Hence, the balance of expression of various members of the BCL family determines the extent to which cell death is promoted or prevented. Apart from the Bcl family the best characterized regulator of apoptosis, so far, is wild-type p53 [9, 10]. It has been reported that cells with loss of normal p53 protein function, or elevated levels of bcl-2, are relatively resistant to cytotoxic agents, such as platinum analogues [11, 12].

The c-erb B-2 oncogene encodes for a 185,000 molecular weight transmembrane phosphoglycoprotein with intrinsic tyrosine kinase activity. Amplification or overexpression of the c-erbB-2 oncogene may lead to increased overall tyrosine kinase activity and thus, more aggressive tumor growth [13]. Overexpression of c-erb B-2, may also influence sensitivity to chemotherapy [14].

Cathepsin-D is a lysosomal aspartyl endopeptidase with a proteolytic effect on extracellular matrix. Overexpression of cathepsin-D in human breast cancer has been strongly associated with more aggressive tumor growth, lymph node metastasis and poorer patient survival [15]. In ovarian cancer the existing studies on the prognostic significance of cathepsin-D expression have produced conflicting results [16, 17].

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Paclitaxel is a taxane derivative, which is very effective in ovarian cancer [2], is widely used for first-line treatment, in combination with cisplatin or carboplatin, in patients with ovarian carcinoma. Recent data suggest that its activity is independent of p53-mediated apoptosis [18], which indicates that it might overcome platinum resistance.

The purpose of this study was to evaluate the expression of p53, bcl-2, c-erb B-2 and cathepsin-D in advanced stage ovarian cancer patients, and to correlate this expression with the response to chemotherapy and patient outcome. We also studied the possible implications of the administration of paclitaxel in the prognostic significance of these markers.

Patients and Methods

Patients

One hundred consecutive patients with Stage IC-IV ovarian carcinoma who were diagnosed and treated at our institution between January 1994 and December 1997 were included in the study. Patients were followed-up at our institution and paraffin-embedded tissue from laparotomy was available for immunohistochemistry. Tumors were staged according to the International Federation of Obstetrics and Gynecology (FIGO) staging system [19]. Laparotomy and debulking surgery was performed in all cases.

Combination chemotherapy was initiated within a median of two weeks after surgery. The first 46 patients were treated with the combination of cyclophosphamide and cisplatin (75 mg/m^2) or carboplatin (AUC 6), while in the remaining 54 patients cyclophosphamide was substituted for paclitaxel (175 mg/m^2). In all cases chemotherapy was administered every three weeks, for a maximum of six cycles. Chemotherapy was discontinued in case of progressive disease or unacceptable toxicity. Standard criteria for response were used [20]. Recurrence was diagnosed by a > 100% increase in the CA-125 levels above upper normal limits, confirmed on two separate occasions four weeks apart, and/or abnormal imaging findings.

Following chemotherapy patients were followed-up every six months with physical examination, CA125 measurement, chest X-ray and CT scan of the abdomen and pelvis.

Immunohistochemistry

Serial 3 mm sections from paraffin-embedded tissue from the primary tumor were used for immunohistochemistry. After dewaxing, sections were rehydrated and endogenous peroxidase activity was blocked using a 1% solution of hydrogen peroxide in methanol. Sections for p53 and cathepsin-D were microwaved for 15 minutes in a citrate buffer solution with a pH 6.0 for heat mediated antigen retrieval. Primary monoclonal antibodies against c-erb B-2 (CB11), p53 (DO-7) and cathepsin-D (C5) (YLEM, Rome, Italy) were applied to each section at a dilution of 1:70, 1:50 and 1:80, respectively, and incubated for one hour. The monoclonal antibody against bcl-2 (Dakopatts, Glostrup, Denmark) was added at a dilution of 1:70 for one hour. Bound primary antibodies were then detected using a biotin-labeled rabbit antiserum against mouse immunoglobulins and a streptavidin-biotin peroxidase complex (DAKO). The peroxidase reaction was developed using diaminobenzidine tetrahydrochloride. Positive and negative controls were used for each antibody. Positive controls consisted of invasive carcinomas with known intense immunostaining. Primary monoclonal antibodies were substituted for serum immunoglobulins from non-immunized mice in negative controls.

All sections were analyzed twice by a pathologist (SM), who was blinded to other clinicopathological characteristics. Specimens with differences in their grading were restained, reassessed and the third result was considered the final. A four-level classification system was used. A distinct nuclear staining was considered a positive reaction for p53, while cytoplasmic staining was evaluated for bcl-2 and cathepsin-D. A reaction of at least 5% of tumor cells was used as the cut-off point for positivity. For c-erbB-2 a four-level grading system (0-3) according to the extent of membranous staining and the percentage of positive tumor cells was used [21]; 0 and 1+ were considered as weak staining and 2+ and 3+ were considered as intense reactions. One hundred high power fields were studied to grade each specimen.

Statistical methods

All analyses were carried out using the SPSS for Windows Version 11.0 statistical software (SPSS Inc, Chicago, IL, USA). Associations between categorical parameters were determined using X^2. PFS and OS were estimated using the Kaplan-Meier method. The Cox proportional hazards method was used for univariate and multivariate analysis. A p value < 0.05 was considered the level of statistical significance.

Results

Patients

Baseline characteristics of the 100 patients included in the study are shown in Table 1. Most patients had Stage III or IV (72%) and grade 3 (54%) tumors and had > 2 cm (72%) residual disease after debulking surgery.

Immunohistochemistry

Fifty cases (50%) were p53 positive (Figure 1a). Normal tissue showed no p53 staining. Twenty-two cases (22%) showed positive bcl-2 immunostaining. Bcl-2 immunostaining was focal (Figure 1b). Normal ovarian tissue elements also stained positive for the bcl-2 protein. Seventy-six (76%) cases stained positive for c-erb B-2 [9 (9%): 3+, 34 (34%): 2+, and 33 (33%): 1+]. In the majority of cases with 2+ c-erb B-2 staining > 50% of neoplastic cells were positive. Staining for c-erb B-2 was cytoplasmic and membranous (Figure 1c). Seventy-three (73%) cases were positive for cathepsin-D. Cathepsin-D immunostaining was located in the cytoplasm and had a characteristic granular appearance (Figure 1d). There was no association between immunohistochemical expression and baseline characteristics apart from a trend of more frequent positive p53 staining in grades 2 and 3 (p = 0.083) (Table 1).

Response to chemotherapy

After primary debulking surgery 79 patients had measurable disease and were evaluable for response to chemotherapy. In 57 cases (72%) a complete clinical response was achieved. In 14 cases (18%) response was partial and eight patients had stable or progressive disease during the course of chemotherapy. Table 2 shows types of response in the two treatment groups in relation to immunohistochemical expression. No correlation was found in the paclitaxel group. In contrast, bcl-2 and cathepsin-D expression were associated with response in the cyclophosphamide group: all bcl-2 positive patients showed a com-

1a)

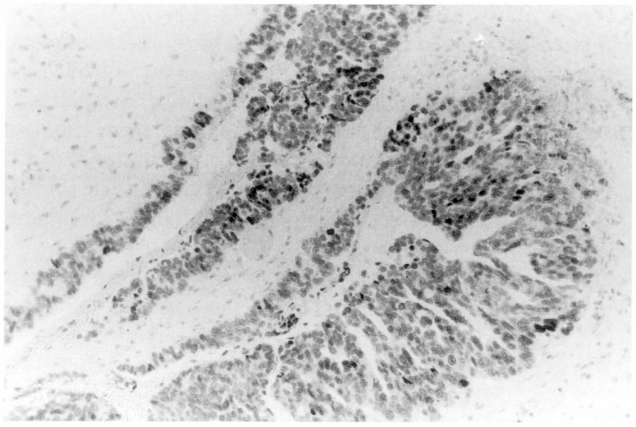
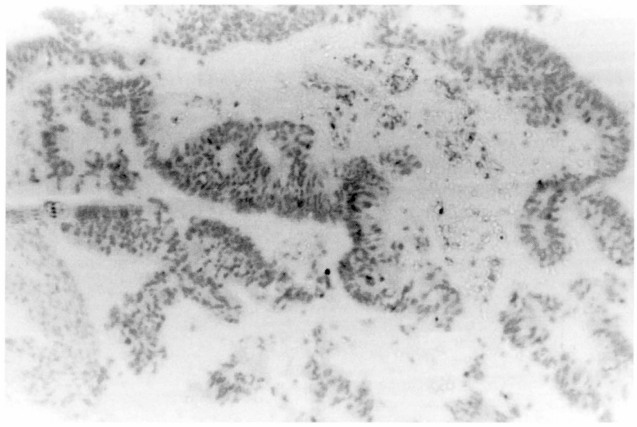


Fig. 1b)



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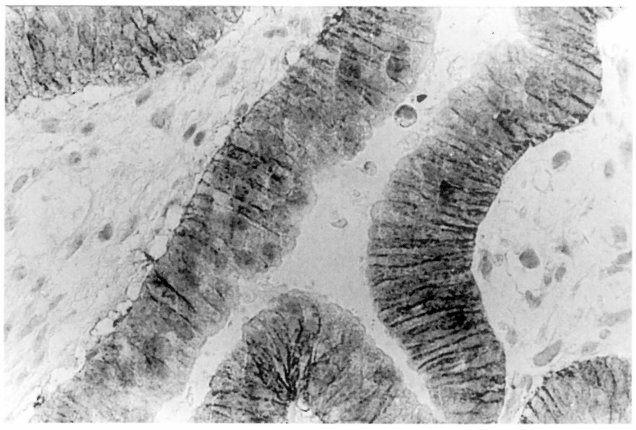

Fig. 1d)

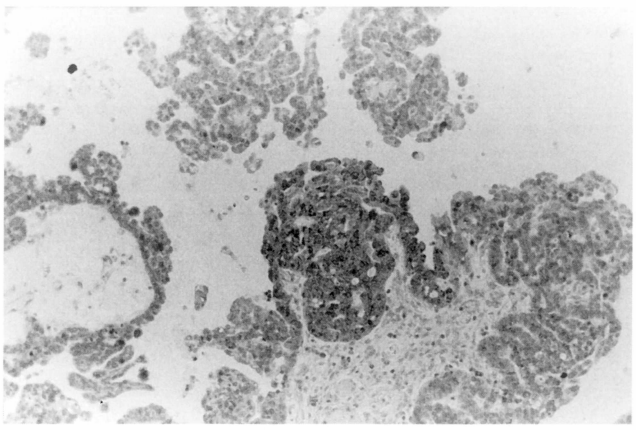

Figure 1. — **a)** p53 nuclear staining of a serous papillary ovarian carcinoma (x 150); **b)** Bcl-2 cytoplasmic staining of an endometrioid carcinoma (x 150); **c)** C-erb B-2 membranous staining of an endometrioid carcinoma (x 400); **d)** Cathepsin-D granular cytoplasmic staining of an endometrioid carcinoma (x 400).

Table 1. — *Patient characteristics and correlation with immunohistochemical expression.*

Characteristics	n	p53 + ve			bcl-2 + ve			c-erb B-2 + ve*			CD + ve		
		n	(%)	p	n	(%)	p	n	(%)	p	n	(%)	p
Age				NS			NS			NS			NS
< 60	48	21	43.8		9	18.8		18	37.6		35	72.9	
≥ 60	52	29	55.8		13	25		25	48		38	73.1	
FIGO Stage				NS			NS			NS			NS
IC	10	3	30		3	30		4	40		8	80	
II	8	4	50		2	25		2	25		4	50	
III	68	34	50		14	20.6		29	42.6		51	75	
IV	14	9	64.3		3	21.4		8	57.2		10	71.4	
Residual disease				NS			NS			NS			NS
< 2 cm	28	13	46.4		8	28.6		10	35.7		21	75	
> 2 cm	72	37	51.4		14	19.4		33	45.8		52	72.2	
Histology				NS			NS			NS			NS
Serous	50	25	50		13	26		22	44		36	72	
Endometrioid	17	8	47.1		4	23.5		9	52.9		12	70.6	
Mucinous	20	9	45		5	25		6	30		14	70	
Clear cell	7	3	42.9		0	0		3	42.9		7	100	
Undifferentiated	6	5	83.3		0	0		3	50		4	66.7	
Grade				.083			NS			NS			NS
1	7	1	14.3		2	28.6		4	57.1		7	100	
2	39	18	46.2		9	23.1		18	46.1		30	76.9	
3	54	31	57.4		11	20.4		21	38.9		36	66.7	
Chemotherapy				NS			NS			NS			NS
CyP	46	23	50		11	23.9		19	41.3		30	65.2	
PP	54	27	50		11	20.4		24	44.5		43	79.6	

+ve: positive; *: positive = intense expression (+2 and +3); CD: cathepsin-D; CyP: Cyclophosphamide + cisplatin or carboplatin; PP: Paclitaxel + cisplatin or carboplatin.

Table 2. — *Response to chemotherapy in the two treatment groups.*

Immunohistochemical expression	CyP (n = 31)			PP (n = 45)		
	CR (%)	PR + NR (%)	p	CR	PR + NR (%)	p
p53 positive	8 (44.4)	7 (53.8)	NS	16 (44.4)	6 (33.3)	NS
bcl-2 positive	7 (38.8)	0 (0)	.011	7 (19.4)	3 (33.3)	
c-erb B-2 (+2,+3)	7 (38.8)	7 (53.8)	NS	15 (41.6)	5 (55.5)	NS
cath-D positive	7 (38.8)	12 (92.3)	.003	27 (75)	8 (88.8)	NS

CR: complete response; PR: partial response; NR: no response.

plete response to treatment, while only 11 of the 24 bcl-2 negative patients achieved a complete response (p = 0.011); in addition, all but one of the 12 cathepsin-D negative patients achieved a complete response as opposed to seven of the 19 positive patients (p = 0.003).

Progression free survival (PFS) and overall survival (OS)

Median follow-up of the whole population was 55 months. Median PFS was 16 months (95% confidence intervals [CI]: 13.14-18.86). There was no correlation between immunohistochemical expression and PFS. Moreover no correlation was found when patients were stratified according to treatment group. Median OS was 41 months (95% CI: 27.7-54.28). Expression of bcl-2, cerB-2 and cathepsin D was not correlated with OS, while there was a trend for improved survival in p53 negative patients (52 vs 32, p = 0.0741) (Figure 2). No correlation was found when patients were stratified accord-

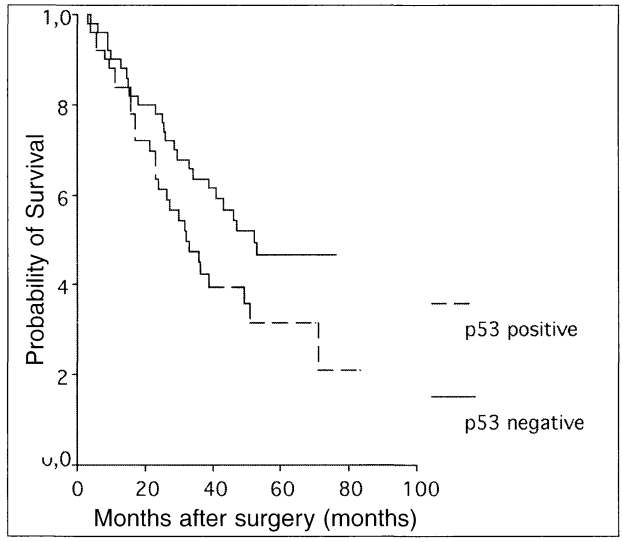

Figure 2. — Survival curves in patients with positive (+) and negative (–) p53 expression.

ing to treatment group. In addition, the combination of the expression of p53 and bcl-2 did not offer any other prognostic information regarding PFS and OS.

Univariate analysis showed that age (p = 0.0242), FIGO stage (p < 0.00001), grade (p = 0.0251), histology (p = 0.0332) and cytoreduction (p = 0.0036) were associated with survival. Multivariate analysis including the above factors as well as p53 expression (p = 0.0741 in univariate analysis) showed that only histology (p = 0.047) and stage (p = 0.001) were independent prognostic factors (Table 3).

Table 3. — *Multivariate analysis for survival.*

Characteristic	RR	95% CI	p
Age			0.464
≤ 60	1		
> 60	1.253	0.686-2.276	
Histology			
Serous	1		
Endometrioid	0.634	0.262-1,535	
Mucinous	0.795	0.359-1.762	
Clear cell	5.463	1.701-17.540	
Undifferentiated	0.791	0.241-2.597	
Grade			0.082
1	1		
2	6.268	0.808-48.610	
3	5.542	0.704-43.642	
Stage			0.006
IC	1		
II	0.580	0.051-6.602	
III	6.468	1.410-29.657	
IV	9.532	1.905-47.690	
Cytoreduction			0.484
< 2 cm	1		
≥ 2 cm	0.708	0.270-1.861	
p53			0.150
– ve	1		
+ ve	1.515	0.857-2.680	

Discussion

The aim of our study was the evaluation of the prognostic significance of the products of four genes which have been implicated in carcinogenesis and tumor behavior in many human malignancies. Furthermore, we studied the impact of taxane treatment on the prognostic significance of these factors. The expression of the proteins was studied immunohistochemically. The percentage of positive cases we found is in agreement with those reported in previous studies for ovarian carcinoma [14, 16-18, 22-25]. In earlier experimental studies in cell culture systems derived from ovarian and other malignant tumors, the possible role of p53 and bcl-2 in the development of resistance to chemotherapy has been investigated. It was shown that overexpression of p53 and bcl-2 confers resistance to chemotherapeutic agents [12, 26, 27]. Surprisingly, positive bcl-2 staining has been shown to be a favorable prognostic factor [22]. We found that bcl-2 expression was associated with a higher CR rate in patients who were treated with cyclophosphamide and platinum analogues but not in patients treated with paclitaxel. This result is in concert with results of a previous study showing that paclitaxel activity does not require a functional p53 gene [18].

P53 immunohistochemical expression has been associated with poorer prognosis in various carcinomas as well as ovarian cancer [23] expression, although opposite results have also been published [24]. In our series, p53 expression was associated with poorer OS, although this difference was not statistically significant. Furthermore, there was no difference in the prognostic significance of the apoptosis-related markers between the two different treatment groups. The lack of prognostic significance of p53 and bcl-2 is in concert with results of previous studies [23, 24, 28]. It has been suggested that the interaction between p53 and bcl2 expression rather than each separate immunostaining result represents an important prognostic factor [23, 25, 28]. We found no prognostic significance of any of the possible p53/bcl-2 expression combinations. Nevertheless, the small number of patients included in our series represents a serious limitation, especially for subgroup analyses and, therefore, these results should be viewed with caution.

It has been shown that cathepsin-D is overexpressed significantly more often in metastatic ovarian deposits compared to the primary tumor [29]. Therefore, cathepsin-D can be regarded as an indicator of tumor aggressiveness. Recent evidence also suggested that cathepsin-D has a role to play in the mechanisms of drug sensitivity and development of chemoresistance as a mediator of interferon-γ and TNF-α induced apoptosis [30]. In our study cathepsin-D expression predicted poor response to CP but no TP combination. It, therefore, seems that paclitaxel efficacy is independent of cellular cathepsin-D. Nevertheless, this difference was not reflected in PFS or OS, indicating that other factors might interfere with long-term retention of response to chemotherapy.

In early studies c-erb B-2 overexpression was associated with poorer survival in patients with ovarian cancer [14, 31]. In accordance with more recent data [32, 33], we failed to show any differences in response to chemotherapy, PFS and OS between groups with strong and weak expression of c-erb B-2. The grouping system we used may represent a drawback, since only very strong mem-

branous expression (+3) is accepted as the cut-off point for overexpression, which has yielded the most frequent prognostic associations in human malignancies [34]. Nevertheless, this group included only nine patients in our study, which is in agreement with the small percentages generally reported for ovarian carcinoma [21]. Due to the small number of cases with +3 expression we stratified patients with no or weak (+1) expression vs strong (+2,+3) in order to perform meaningful statistical analysis.

Conclusion

In conclusion, bcl-2 and cathepsin-D expression seems to be associated with resistance to cyclophosphamide and cisplatin but not taxane-containing chemotherapy. The true value of these factors as a means of selection for appropriate treatment can only be answered in properly designed prospective randomised trials.

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