The apoptosis regulators p53, bax and PUMA: Relationship and impact on outcome in early stage (FIGO I-II) ovarian carcinoma after post-surgical taxane-based treatment

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Abstract. The objective of this study was to evaluate the prognostic effect of the apoptosis regulators p53, bax and PUMA for recurrent disease and disease-free survival (DFS) in a series of 105 patients in FIGO-stages I-II with epithelial ovarian cancer, all treated with post-surgical platinum-taxane chemotherapy. For the detection of positivity of the biological markers p53, bax and PUMA the techniques of tissue microarrays and immunohistochemistry (IHC) were used. In tumors the frequency of p53 positivity was 24%, that of bax positivity was 83%, and strong positivity was found for PUMA (43%). The bax status was related to tumor grade (P=0.029). Positive staining for bax was related to strong positivity of PUMA in the tumors (P=0.004). The p53, bax or PUMA status alone or concomitant (p53 bax, p53 PUMA and bax PUMA) were not related to age, histopathological subtype, serous/non-serous tumors or type of the staging procedure at primary surgery. In survival analysis p53-positive tumors (P=0.014) and concomitant p53-positive and weak PUMA-positive tumors (P=0.015) were significantly correlated with shorter DFS. Concomitant p53-negative and bax-positive tumors were significantly correlated with longer survival (P=0.019). FIGO-stage (OR=6.0) and p53 status (OR=4.1) were predictive factors for tumor recurrence in logistic regression analysis and independent prognostic factors (HR=2.4 for both) in multivariate Cox regression analysis. In a separate Cox multivariate regression analysis the p53 bax status (HR=2.2) was an independent prognostic factor for DFS. The p53 PUMA status (HR=0.4) was not an independent prognostic factor, however, a borderline significance (P=0.07) was noted. Our results indicate that FIGO stage and p53 status alone were independent predictive factors for recurrence and prognostic factors for survival. Furthermore, p53 bax status was an independent prognostic factor for survival in this study.

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Introduction

Epithelial ovarian cancer is still the leading cause of mortality among women with gynecologic malignancies. Nearly 30% of the patients will present with stage I-II disease with the favorable 5-year survival being 70-90%. However, nearly one-third of patients initially diagnosed with early-stage ovarian cancer will recur (1-3). Primary surgery with comprehensive surgical staging is the cornerstone of treatment for all epithelial ovarian cancer followed by platinum- and taxane-based chemotherapy (4,5). Clinicopathological parameters have been proven insufficient to define prognostic subgroups and to predict response to chemotherapy (6).

Nowadays, it is accepted that cancer can arise as a cell cycle defect, where injured or mutated cells are allowed to progress through the cell cycle (7,8). Apoptosis, a goal of cancer treatment is controlled by regulators, which have either an inhibitory effect on programmed cell death or block the protective effect of inhibitors (9,10). The tumor suppressor gene p53 and its protein p53, has a crucial function as an apoptosis regulator (11). As a transcription factor, wild-type p53 can limit cell proliferation after DNA damage by arresting the cell cycle or inducing apoptosis by activating the expression of bax, PUMA and other apoptosis regulators (11,12). The mitochondrial pathway plays an essential role in apoptosis induction and is controlled by the bcl-2 family of anti-apoptotic proteins and the pro-apoptotic proteins bax and PUMA (BH3-only protein) (13). Positive staining for p53 has been associated with worse survival rates (14,15) and high bax expression was a favourable prognostic factor in two studies (6,16). PUMA was shown to be an independent predictor of overall survival in a study on patients with colorectal cancer (17). As survival after recurrence in stage I-II ovarian cancer is comparable to those with recurrent advanced-stage disease (1), identification of new prognostic factors to improve treatment is necessary. The present study was undertaken to evaluate the prognostic effect of the apoptosis regulators p53, bax and PUMA in a series of 105 patients in FIGO-stages I-II with epithelial ovarian cancer, all treated with post-surgical platinum-taxane chemotherapy.

Materials and methods

Patients and treatment. This study assessed 105 patients in FIGO-stages I-II epithelial ovarian cancer, who were a part of a patient population of 131 patients in earlier published studies (18,19). The study was approved by the local Ethics Committee (decision ref. UPS-03-477). All patients underwent primary surgery and postoperative adjuvant taxane carboplatin chemotherapy and were recruited in the Uppsala-Örebro Medical region between January 2000 and December 2004. Adequate (optimal or modified) surgical staging according to the EORTC surgical staging categories in early ovarian cancer (20) was undertaken in 33 (31%) out of the 105 cases, and in the remaining 72 (69%) patients surgical staging was regarded as inadequate according the same guidelines. Patients' characteristics are demonstrated in Table I. All patients in the study received paclitaxel 175 mg/m^2 and carboplatin (AUC=5) at 3-week intervals, usually in 4-6 courses 4-6 weeks after primary surgery. The mean follow-up time was 67 months (range 9-110 months).

Table I. Patient characteristics (n=105).

Characteristics	n (%)
Age (median)	58.0 (range 25-84)
WHO performance status	
0	37 (90.4)
1	66 (9.6)
FIGO-stage	
IA	31 (29.5)
IB	5 (4.7)
IC	52 (49.5)
II	17 (13.3)
Histopathology	
Serous	42 (40.0)
Mucinous	17 (16.2)
Endometrioid	32 (30.5)
Clear cell	12 (11.4)
Anaplastic	2 (1.9)
FIGO-grade	
Grade 1	29 (27.6)
Grade 2	32 (30.5)
Grade 3	44 (40.9)

Clinical sampling and tissue microarray construction of ovarian cancer tissue. Tissue samples of ovarian cancers were obtained at the time of primary surgery. The tissue microarrays were constructed as described previously (21) from core tissue biopsy specimens (diameter 0.6 mm). Tumor tissues were embedded in paraffin and 5 μm sections stained with haematoxylin-eosin were obtained to select representative areas for biopsies. Two core biopsies were obtained from each specimen. The presence of tumour tissue on the arrayed samples was verified by the haematoxylin-eosin-stained section. The specimens were then reviewed, classified and graded by a single pathologist.

Immunohistochemistry. Five micrometer sections were cut from each multi-tissue block and were placed on coated slides and dried overnight at 37˚C. The sections were pretreated by heat-induced epitope retrieval in target-retrieval solution (Dako, Glostrup, Denmark), pH 6 or EDTA buffer pH 9, for 7+7 min in a microwave oven (99˚C). Blocking with peroxidase was performed for 5 min. The slides were counterstained with hematoxylin for 2 min. The following monoclonal primary antibodies were used: DO-7, directed against p53 protein (dilution 1:1,000; Dako) and PUMA-α that was directed against PUMA protein (dilution 1:50; Abcam, Cambridge Science, Cambridge, UK). For bax protein, polyclonal bax antibody (dilution 1:100; Dako) was used as a primary antibody.

The immunostainings were performed in an Autostainer automated machine (Dako) using the REAL EnVision detection system (Dako). The immunohistochemical analyses and interpretation were performed at the Department of Pathology, Halmstad Medical Central Hospital.

Interpretation of immunohistochemical staining. The immunohistochemical (IHC) stainings were interpreted by the two authors (IS and TS). At the time of evaluation, no information was available on the specific diagnosis and prognosis for the individual cases. A semi-quantitative analysis (22) was used and the stainings were graded as negative, +, ++ and +++ for p53, bax and PUMA. The staining for p53 was considered to be positive when strong and widespread granular staining of the nuclei of the tumour cells was found. Staining for bax were interpreted as positive when strong granular and punctuate staining of the cytoplasm in most of the tumour cells was shown. As completely negative staining of cytoplasm for PUMA hardly was detected in this series of patients and our findings were limited to weak or moderate/strong staining of the cytoplasm; the cut-off value was considered between + (weak positive) and ++/+++ (strong) for statistical analyses.

Statistical analyses. The Pearson's Chi-square test was used for testing proportional differences in univariate analyses. The survival curves were generated by using the Kaplan-Meier technique and differences between these curves were examined by the log-rank test. All tests were two-sided and the level of statistical significance was P≤0.05. The Statistica 10 (StatSoft™) statistical package was used for the analyses. For multivariate analyses the logistic regression model was used with recurrence as the end point and Cox regression model was used with disease-free survival (DFS) as the endpoint.

Results

Patients. The total number of recurrences in the complete series was 29 out of 105 (28%). Recurrent disease was significantly associated with FIGO-substages (P=0.0007), FIGO-grade (P=0.043), but not with the histopathological subtype (P=0.880), or serous/non-serous tumors (P=0.532). However, the type of staging procedure at the primary surgery reached a borderline significance (P=0.053). In the complete series both the 5-year DFS rate and the overall survival rate (OS) were 70%.

Table II. Tumor expression of the p53-protein, bax-protein and PUMA-protein vs. clinicopathological features (n=105).

	$p53^+$ n (%)	$p53^-$ n (%)	bax^+ n (%)	bax^- n (%)	$PUMA^{++}$ n (%)	$PUMA^+$ n (%)
Positivity	25 (24)	80 (76)	84 (83)	20 (17)	45 (43)	59 (57)
Histopathology						
Serous	13 (52)	29 (36)	36 (43)	6 (30)	21 (47)	20 (34)
Non-serous	12 (48)	51 (64)	48 (57)	14 (70)	24 (53)	39 (66)
P-value	0.160		0.292		0.187	
Tumor grade						
G1+G2	13 (52)	47 (59)	52 (62)	7 (35)	28 (68)	82 (63)
G3	12 (48)	33 (41)	32 (38)	13 (65)	13 (32)	19 (37)
P-value	0.552		0.029		0.578	
FIGO-stage						
IA-IB	5 (20)	31 (39)	28 (33)	7 (35)	12 (27)	24 (41)
IC	12 (50)	50 (50)	45 (54)	7 (35)	25 (55)	26 (44)
II	8 (24)	9 (11)	11 (13)	6 (30)	8 (18)	9 (15)
P-value	0.029		0.138		0.327	
Disease-free survival						
Disease-free survival	11 (44)	65 (81)	63 (75)	13 (65)	31 (69)	44 (75)
Dead of disease or alive with recurrent disease	14 (56)	15 (19)	21 (25)	7 (35)	14 (31)	15 (25)
P-value	0.0003		0.365		0.522	

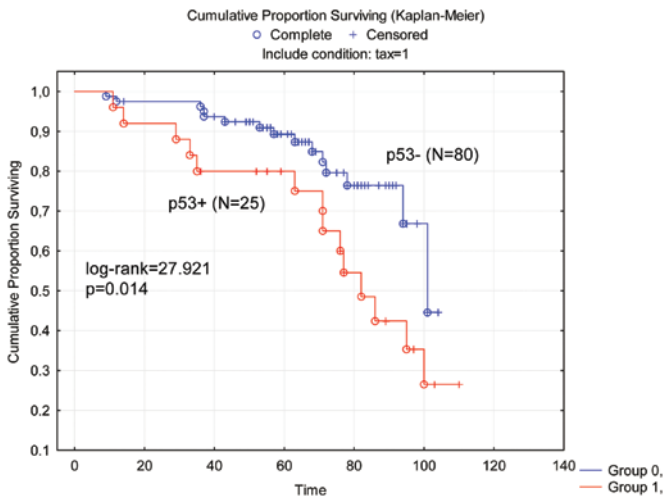

Figure 1. Kaplan-Meier plots of disease-free survival for patients demonstrated significantly (P=0.014) differences for patients according the p53 status.

Results from immunohistochemistry. Age, histopathological subtype, serous/non-serous tumors, and type of staging procedure were not related to p53, bax or PUMA status alone or combined in this study.

p53 positivity was observed in 25 out of 105 (24%) tumors. The p53 status was significantly (P=0.029) associated with both FIGO-stage and disease-free survival (P=0.0003) (Table II). Survival analysis (Fig. 1) demonstrated a significant (P=0.014) difference in patients according to their p53 status. Patients with p53-negative tumors had a 5-year DFS of 90% compared to an 80% survival for patients with p53-positive tumors. Patients with p53-positive tumors continued to die after the 5-year follow-up and DFS at 100 months was only 28%.

Bax positivity was observed in 84 out of 104 (83%) available tumors. The bax status was only related to tumor grade (Table II). Thus, well- and medium-differentiated tumors (G1 and G2) usually stained bax-positive in contrast to poorly differentiated tumors (G3), that predominantly stained bax-negative (P=0.029). Strong positivity for PUMA was found in 45 out of 104 (43%) available tumors. The PUMA status was not related to any of the clinicopathological features evaluated (Table II). The bax-status and the PUMA status alone were not related to survival.

Relationship between p53, bax and PUMA. The staining of p53 in carcinomas was not related to the staining of bax (P=0.487) or PUMA (P=0.140) in tumors. However, bax positivity was significantly (P=0.004) related to strong positivity of PUMA in tumors. Thus, strong positivity for PUMA was found in 42 out of the 83 (51%) bax-positive tumors compared to only 3 out of the 20 (15%) bax-negative tumors.

The complete series of 105 patients was split into four subgroups according to p53 and bax-status and p53 and PUMA-status of tumors, and the distribution of the subgroups were analysed according to clinicopathological features as before. It was noted that among the 65 tumors of concomitant p53 negativity and bax positivity, most (90%) tumors were classified to sub-stages IA-IC contrary to 3 out of the 6 (50%) tumours of concomitant p53 positivity and bax negativity that belonged to FIGO-stage II (Table III). Survival analysis

Table III. Status of the p53/bax proteins vs. clinicopathological features (n=104).

	$p53^+/bax^+$ n (%)	$p53^+/bax^-$ n (%)	$p53^-/bax^+$ n (%)	$p53^-/bax^-$ n (%)	P-value
Positivity	19 (18)	6 (7)	65 (62)	14 (13)	
Histopathology					0.107
Serous	9 (47)	4 (67)	27 (41)	2 (14)	
Non-serous	10 (53)	2 (33)	88 (59)	12 (86)	
Tumor grade					0.092
G1+G2	10 (53)	3 (50)	42 (65)	4 (36)	
G3	9 (47)	3 (50)	23 (35)	10 (64)	
FIGO-stage					0.025
IA-IB	5 (26)	0 (00)	23 (35)	7 (50)	
IC	9 (47)	3 (50)	36 (55)	4 (29)	
II	5 (27)	3 (50)	6 (10)	3 (21)	
Disease-free survival					0.002
Disease-free survival	9 (47)	2 (33)	54 (83)	11 (79)	
Dead of disease or alive with recurrent disease	10 (53)	4 (67)	11 (17)	3 (21)	

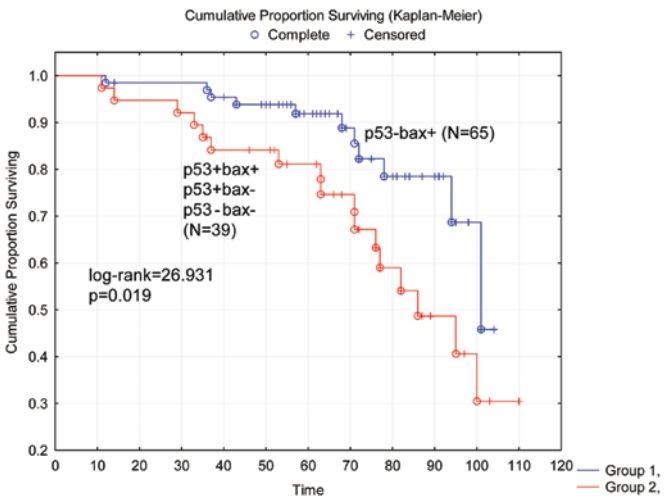

Figure 2. Kaplan-Meier plots show a significantly better (P=0.019) disease-free survival for the subgroup of patients with concomitant $p53^-$ and bax^+ tumors compared to the other three subgroups regarded as one group ($p53^+/bax^+$, $p53^+/bax^-$ and $p53^-/bax^-$).

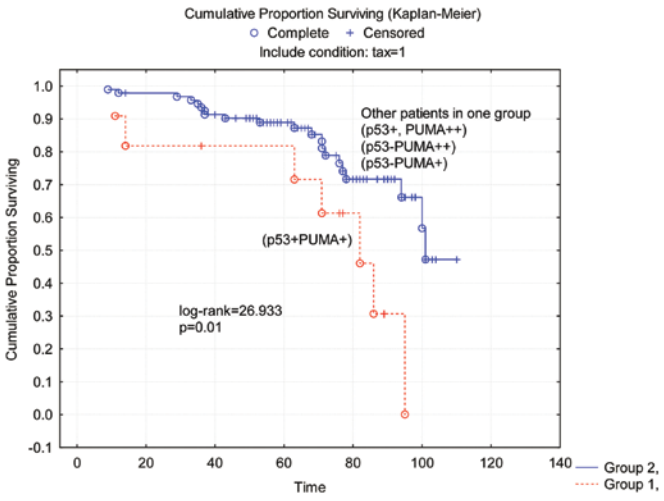

Figure 3. Kaplan-Meier plots show a significantly worse (P=0.015) disease-free survival for the subgroup of patients with concomitant $p53^+$ and weak PUMA-positive tumors compared to other three subgroups regarded as one group ($p53^+/PUMA^{++}$, $p53^-/PUMA^{++}$ and $p53^-/PUMA^+$).

showed borderline significance (P=0.054) in survival (DFS) for patients in the four subgroups based on the p53-bax status. However, a separate survival analysis (Fig. 2) showed a significantly elevated (P=0.019) survival for the subgroup of patients with concomitant p53-negative and bax-positive tumors compared to other three subgroups regarded as one group ($p53^+/bax^+$, $p53^+/bax^-$ and $p53^-/bax^-$). Association of the p53 PUMA status to FIGO-stage distributions reached borderline significance (P=0.056) (Table IV). Survival analysis for p53 PUMA status after four subgroups was not significant. However, a separate survival analysis (Fig. 3) showed significant worse (P=0.015) DFS for the subgroup of patients with concomitant p53-positive and weak PUMA-positive tumors compared to other three subgroups regarded as one group ($p53^+/PUMA^{++}$, $p53^-/PUMA^{++}$ and $p53^-/PUMA^+$).

Multivariate analysis. In a logistic regression analysis of predictive factors for tumor recurrences the FIGO-stage OR=6.0 and p53-status OR=4.1 were significant and independent factors (Table V).

In a multivariate Cox regression analyses with DFS as the endpoint (Table VI) FIGO-stage with the hazard ratio (HR)=2.4 and p53-status with HR=2.4 were significant prognostic factors. In a separate multivariate regression analysis (Table VII) both p53 bax-status HR=2.3 and FIGO-stage HR=2.3 were independent prognostic factors. However, the

Table IV. Status of the p53-PUMA proteins vs. clinicopathological features (n=104).

	$p53^+/PUMA^{++}$ n (%)	$p53^+/PUMA^+$ n (%)	$p53^-/PUMA^{++}$ n (%)	$p53^-/PUMA^+$ n (%)	P-value
Positivity	14 (13)	11 (10)	31 (30)	48 (47)	
Histopathology					0.235
Serous	7 (50)	6 (55)	14 (45)	14 (29)	
Non-serous	7 (50)	5 (45)	17 (55)	34 (71)	
Tumor grade					0.840
G1+G2	8 (57)	5 (45)	19 (61)	27 (56)	
G3	6 (43)	6 (55)	12 (39)	21 (44)	
FIGO-stage					0.056
IA-IB	4 (29)	1 (9)	8 (26)	23 (48)	
IC	6 (43)	6 (55)	19 (61)	20 (42)	
II	4 (28)	4 (36)	12 (13)	5 (10)	
Disease-free survival					0.003
Disease-free survival	7 (50)	4 (36)	24 (77)	40 (83)	
Dead of disease or alive with recurrent disease	7 (50)	7 (64)	7 (23)	8 (17)	

Table V. Predictive factors for tumor recurrences (logistic regression analysis) (n=105).

Variables	B	SE	OR	95% CI	P-value
Age	0.019	0.023	1.019	0.973-1.068	0.407
Stage (I vs. II)	1.792	0.628	6.006	1.725-20.915	0.005
Staging[a]	-1.053	0.619	0.349	0.102-1.193	0.092
p53[b]	1.416	0.539	4.123	1.414-12.026	0.009

[a]Adequate staging performed/not performed; [b]p53-negative vs. positive.

Table VI. Cox proportional hazard analysis of disease-free survival rate (n=105).

Variable	B	SE	HR	95% CI	P-value
Age	0.024	0.019	1.024	0.987-1.063	0.201
Stage (I vs. II)	0.0871	0.398	2.390	1.093-5.223	0.028
Tumor grade[a]	0.370	0.401	1.448	0.660-3.179	0.355
p53[b]	0.894	0.381	2.447	1.157-5.170	0.019

[a]G1+G2 vs. G3 tumors; [b]p53-negative vs. positive.

Table VII. Cox proportional hazard analysis of disease-free survival rate (n=104).

Variable	B	SE	HR	95% CI	P-value
Age	0.025	0.019	1.025	0.987-1.064	0.190
Stage (I vs. II)	0.827	0.399	2.288	1.045-5.006	0.038
Tumor grade[a]	0.231	0.414	1.260	0.558-2.841	0.577
$p53^-/bax^{+b}$	0.812	0.403	2.254	1.023-4.964	0.043

[a]G1+G2 vs.G3 tumors; [b]$p53^-/bax^+$ vs. others ($p53^+/bax^-$, $p53^+/bax^+$, $p53^-/bax^-$).

p53 PUMA status (analyzed together with age, stage and tumor grade) was not an independent prognostic factor for DFS HR=0.40, 95% confidence interval (CI) 0.19-1.09; P=0.07).

Discussion

In the present study on patients with early-stage ovarian cancer after post-surgical taxane-platinum treatment it was possible to indentify a subgroup of patients with concomitant p53-negative and bax-positive tumors, with DFS of 92% at 5 years and 70% at 8.3 years without any relation to histological subtype or type of surgical staging. Most (62%) patients in this study belonged to this favourable subgroup. For patients in the other three subgroups in one group ($p53^+/bax^+$, $p53^+/bax^-$ and $p53^-/bax^-$) DFS was 82 and 32% at 5 years and 8.3 years, respectively. Furthermore, it was possible to indentify a subgroup of patients with concomitant p53 positivity and weak PUMA staining of tumors, with 5-year DFS of 80% and very poor outcome after a long follow-up time. On the contrary, survival for patients with p53-positive and concomitant strong PUMA-positive tumors was not different from patients with p53-negative tumors without regard to the PUMA-status of tumors. Therefore it seems that positive expression of bax or strong expression of PUMA in p53-positive tumors can protect from recurrent disease. p53-status alone was considered as the strongest prognostic biological factor in this study without any regard to different surgical staging procedures. Thus, the evaluated biomarkers in the study were not predominantly identifying missed patients with occult advanced disease, who were not adequately staged. Patients with p53-positive tumors continued to die of disease after the 5-year checkpoint and survival at 100 months from diagnosis was only 43%. The risk of late recurrent disease for patients with p53 overexpression of tumors also was noted in the GOG-157 study (23). A strong correlation has been observed between a p53 mutation and DO-7 expression of the p53 protein, the antibody used in the present study (23,24). The frequency of p53 positivity was 24% in this study and previous studies (14,15,23,24) have reported positive staining in 20-51% of tumors belonging to patients in FIGO-stages I-II. The frequency of 83% for bax positivity in the present study could be compared with the frequency of 27 and 85%, respectively from studies (16,25) limited to patients in early stages or 57% in a study (26) from patients in all stages. Lack of clinical studies on ovarian cancer with regard to the BH3-only protein PUMA for comparison could be observed. PUMA expression of 91% of tumors on patients with stage II-III primary colon carcinomas (17) could be compared to strong positivity for PUMA in 43% of tumors in our study.

FIGO-stage was the strongest predictive factor for recurrent disease in this study and this has been confirmed by studies on patients in FIGO-stages I-II both after complete surgical staging (1,27) and apparent staging (5). However, the fact, that p53 status and p53/bax status both were independent prognostic factors for DFS in multivariate analysis was the most striking finding noted in our study. A hazard ratio (HR) of 2.5 for p53 status means 2.5 times increased risk for a patient with a p53-positive tumor to have a recurrent disease or die of disease. An HR of 1.47 for p53 status was found in a meta-analysis (28) including 62 studies with overall survival as endpoint. Furthermore, the p53-bax status with HR of 2.2 indicated more than two times increased risk for recurrent disease or to die of disease for patients with tumors of concomitant p53-negativity and bax-positivity were moving to the other three subgroups of the p53 bax-status of tumors. In multivariate analyses a HR of 0.4 for the p53 PUMA-status, meant a 60% reduced risk for recurrence or to die of disease for a patient with p53-negative tumors or tumors of concomitant positivity for p53 and strong positivity for PUMA compared to patients with tumors of p53 positivity and weak PUMA positivity.

The functional link between p53, bax and PUMA in this study could be explained by the central role of the p53 tumor suppressor protein in induction of both the cell cycle arrest and the apoptosis. In addition, the p53 protein also regulates the death effector bax activation by inducing the BH3-only protein PUMA in response to taxane, a microtubule-damaging agents (MDA) (13,29). Bax plays an essential role in the mitochondrial pathway of apoptosis. Mitochondrial translocation of BH3-only proteins (such as PUMA) has been suggested as a critical step in bax activation during apoptosis. PUMA is essential for both p53-dependent apoptosis induced by DNA-damage and is also involved in p53-independent cell death (30-32).

The intact function of the p53 tumor suppressor protein in p53-negative tumors might activate the tumor suppressor bax protein in bax-positive tumors by means of a strong presence of PUMA and facilitating a taxane-mediated apoptosis that could account for the favorable prognostic effect for subgroup of patients with concomitant p53-negative and bax-positive tumors. In summary the p53 status alone and the bax p53 status were independent prognostic factors for outcome (DFS) of patients with epithelial ovarian cancer in FIGO-stages I-II after primary surgery and adjuvant platinum-taxane chemotherapy. For the first time to our knowledge, it was found, that the BH3 only protein PUMA seems to have prognostic effect on epithelial ovarian cancer in clinical studies, at least in combination with p53. Thus favorable prognostic effect of intact (negative) p53 status alone or combined with positive bax status or strong presence of PUMA, respectively, in tumors was observed in the present study.

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