

Expression of factors involved in regulation of DNA mismatch repair- and apoptosis pathways in ovarian cancer patients

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Abstract. A major obstacle in treatment of ovarian cancer is intrinsic or acquired drug resistance causing failure of chemotherapy followed by a poor clinical outcome. Drug resistance of ovarian carcinoma can be caused by dysregulation of cellular factors involved in regulation of apoptosis and DNA repair pathways. In this study, 73 ovarian carcinoma specimens obtained before and after chemotherapy were analysed by immunohistochemistry for expression of seven proteins playing an important role in regulation of DNA mismatch repair and apoptosis. The prognostic significance of these proteins in the meaning of overall and progression-free survival was evaluated in univariate and multivariate analysis. Bcl-x_L, hMSH2, caspase-3, p21 and p53 displayed prognostic importance in univariate analysis. Furthermore, it was demonstrated that caspase-3 and p21 were also independent prognostic markers for both, overall and progression-free survival. In conclusion, these data indicate that analysis of proteins involved in DNA mismatch repair and apoptosis can be useful for prediction of clinical outcome in ovarian carcinoma patients.

Introduction

Ovarian cancer is the fifth most common malignancy and ranks fourth in causing death due to cancer among women (1). The main reason for these unfavorable circumstances is the late diagnosis at stage III or IV due to absence of clinical symptoms. Although the application of chemotherapy based on platinum-containing drugs and paclitaxel prolongs survival (2), most of the patients with advanced disease die

after the development of chemoresistant recurrent tumors. The cellular stress response on chemotherapeutic drugs is dependent on a cascade of proteins leading to cell cycle arrest, DNA damage repair and/or apoptosis. Changes in these elementary processes can lead to chemoresistance in tumor cells and are therefore needed to be investigated thoroughly.

The DNA mismatch repair (MMR) system detects nucleotide mismatches, small insertions and deletions, and different types of drug-induced DNA-adducts. Cells that have lost their MMR activity are impaired to detect and react to these types of DNA damage resulting in genomic instability and loss of essential apoptotic response. Previous studies using cancer cell lines have indicated a correlation between MMR status and sensitivity to certain classes of chemotherapeutic drugs including the observation, that MMR-deficient cells (mostly investigated proteins: hMLH1 and hMSH2) are resistant to platinum-containing drugs such as cisplatin and carboplatin (3). However, previous data obtained by immunohistochemical analyses of hMLH1 and hMSH2 in ovarian cancer patients are contradictory (4-7).

It has been shown that the *TP53* tumor suppressor gene is the most frequently mutated gene in human cancers (8). Mutated p53 protein is resistant to degradation and has a prolonged half-life which allows its detection by immunohistochemistry (9). It has also been described that the efficacy of cytostatic drugs requires functional p53 protein and that loss of its function enhances resistance to these drugs because of inefficient induction of apoptosis (10-12). Furthermore, it has been suggested that the combined inactivation of p53 and of the MMR system is required for the development of cisplatin resistance, whereas p53 plays a more important role than the failure of the MMR system alone (13). In the case of DNA damage, p53 induces the expression of several downstream genes including p21^(WAF1/CIP1) resulting in cell-cycle arrest during the G1 to S transition by inhibition of cyclin-dependent kinases (14,15). p21 is thought to be a major downstream effector of p53 and its protein expression has been detected in cells expressing wild-type p53, but not in those with non-functional p53 protein (15). Moreover, p53-independent

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pathways may also lead to an increase of p21 expression (16-18). Although p53 and p21 expression were studied extensively in ovarian carcinoma patients (19-23), the importance of p53 and p21 for the clinical outcome is not certain, because the results are conflicting (24). The p53-related protein p63 plays a key role in regulating epithelial proliferation and differentiation programs (25,26). It has been reported that p63 overexpression can mimic p53 activities such as DNA binding, activation of transcription, and induction of apoptosis (26,27), and may compromise the effectiveness of p53 gene therapy (28).

Caspases are highly specific proteases synthesized as zymogens and activated by cleavage generating large and small subunits of the mature enzyme. They are divided into two groups, upstream or initiator caspases and downstream or executioner caspases. Caspases collaborate in the proteolytic cascade by activating themselves and each other resulting in membrane blebbing, chromatin condensation, and DNA fragmentation, the hallmark changes in apoptotic processes (29). The executioner caspase-3 (also known as CPP32, Yama and apopain) is considered to be the central protein in triggering apoptosis (30) and has also been reported to be involved in drug resistance in human cancer cell lines (31-33). Scarce data are available examining the possible prognostic relevance of caspase-3 in cancer patients (34-36).

Proteins of the *BCL2* family of genes are known to modulate the programmed cell death and are also involved in development, tissue homeostasis and pathogenesis of different diseases such as cancer, viral infections, neurodegenerative disorders, and autoimmune disease (37). The anti-apoptotic protein Bcl-x_L, a functional and structural homologue of Bcl-2, is supposed to be responsible for modulating resistance to chemotherapy-induced apoptosis (38). Nevertheless, the significance of different Bcl-x_L expression levels in the context of affecting chemoresistance and the prognostic relevance in cancer patients are still ambiguous (38-41).

In this study, we examined the protein expression of the mismatch repair components hMLH1 and hMSH2, the apoptotic cascade enzyme caspase-3, the anti-apoptotic Bcl-x_L, and the tumor suppressor genes p53 and p21, and the p53-related p63 protein in ovarian cancer patients. Analyses were performed to evaluate the prognostic significance of each of these proteins alone or in combination for patient's survival.

Materials and methods

Patients and histology. Immunohistochemical examination was performed retrospectively on tissue samples taken for routine diagnostic purposes. Forty-three patients operated in 1999-2002 due to ovarian carcinoma in the Department of Obstetrics and Gynecology (Poznan University of Medical Sciences, Poland) were studied. The study was approved by an Institutional Review Board (IRB) and the patients gave their informed consent before their inclusion into the study. The cases were selected based on availability of tissue and were not stratified for known preoperative or pathological prognostic factors. Following the first-look laparotomy all the patients were subjected to chemotherapy using platinum-based schemes. Only the FIGO I and FIGO II patients

Table I. Patient and tumor characteristics.

Characteristics	No. (%) ^b
All patients	43 (100)
First-look laparotomy (FLL)	43 (100)
Secondary cytoreduction (SCR)	36 (84)
No tumor cells at time of SCR	6 (14)
Age (mean 51.0) ^a	
≤50	20 (47)
>50	23 (53)
Grade ^a	
1	7 (16)
2	18 (42)
3	18 (42)
FIGO ^a	
I	1 (2)
II	1 (2)
III	41 (95)
Histology ^a	
Serosum	37 (86)
Others	6 (14)
Clinical response ^c	
Complete response	16 (37)
Stable disease	5 (12)
Progressive disease	22 (51)
Chemotherapy (in total)	
Cisplatin/paclitaxel	31 (72)
Cisplatin/cyclophosphamide/adriablastin	6 (14)
Cisplatin/cyclophosphamide/paclitaxel	3 (7)
Cisplatin/cyclophosphamide/paclitaxel/adriablastin	2 (5)
Carboplatin/paclitaxel	1 (2)

^aData are given to first surgical treatment/diagnosis implemented.

^bDifferences in the sum to 100% in groups are due to rounding.

^cClinical response to first-line chemotherapy was defined according to the criteria suggested by RECIST (Response Evaluation Criteria in Solid Tumors) (42).

achieved optimal cytoreduction after initial surgery. Thirty-six patients from the same group were subjected also to the second-look operation. In seven cases no second-look procedure was performed due to advancement of the disease. In six cases no tumor cells were detected in the material originating from the secondary cytoreduction (Table I). The patients were monitored by periodic medical check-ups, CA-125 serum levels, ultrasonographic and radiological examinations. Tissue samples were fixed in 10% buffered formalin and, then, embedded in paraffin. In each case, hematoxylin and eosin stained preparations were subjected to histopathological evaluation by two pathologists. The stage of

Table II. Procedure for expression evaluation using IRS (immunoreactive score) (45).

Percentage of positive cells	Points	Intensity of reaction	Points
No positive cells	0	No reaction	0
<10% positive cells	1	Weak color reaction	1
10-50% positive cells	2	Moderate intensity	2
51-80% positive cells	3	Intense reaction	3
>80% positive cells	4		

the tumors was assessed according to the International Federation of Gynecology and Obstetrics (43). Tumors were graded according to the Silverberg grading system (44).

Immunohistochemistry. Formalin-fixed paraffin-embedded tissue was freshly cut (4 μ m). The sections were mounted on Superfrost slides (Menzel Glaeser, Braunschweig, Germany), dewaxed with xylene, and gradually rehydrated. Activity of endogenous peroxidase was blocked by 30 min incubation in 1% H₂O₂. Detection of p53, p63, p21, hMLH1, hMSH2 and Bcl-x_L expression was preceded by 10 min exposure to boiling Antigen Retrieval Solution (DakoCytomation, Glostrup, Denmark), in the case of p63 Antigen Retrieval Solution High pH, in a microwave oven.

Immunohistochemical reactions were performed using the following antibodies: anti-p53 mAb (mouse), clone DO-7 (DakoCytomation), dilution 1:200; anti-p63 mAb (mouse), clone 4A4 (DakoCytomation), dilution 1:100; anti-p21 mAb (mouse), clone SX118 (DakoCytomation), dilution 1:100; anti-hMLH1 mAb (mouse), clone G168-15 (BD Bioscience Pharmingen, San Diego, CA, USA), dilution 1:100; anti-hMSH2 mAb (mouse), clone GB12 (BD Bioscience Pharmingen, San Diego, CA, USA), dilution 1:100; anti-Bcl-x_L polyclonal Ab (rabbit) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), dilution 1:100; anti-caspase-3 (active form) mAb, clone Ab-4 (Oncogene Research Products, Darmstadt, Germany), dilution 1:400.

Tested sections were incubated with the antibodies in Antibody Diluent, Background Reducing (DakoCytomation) at RT for 1 h. Subsequently, incubations were performed with biotinylated antibodies (15 min, RT) and with streptavidin-biotinylated peroxidase complex (15 min, RT) (LSAB⁺, HRP, DakoCytomation). NovaRed (Vector Laboratories, Peterborough, UK) was used as a chromogen (10 min, RT). All the sections were counterstained with Meyer's hematoxylin.

Negative controls were included in which specific antibody was substituted by the Primary Mouse Negative Control (DakoCytomation). In the case of p63, control reactions were performed on 6 sections of healthy human breast (from the Archive of Department of Histology and Embryology, Wroclaw Medical University, Poland).

Intensity of the immunohistochemical reactions was appraised using a semi-quantitative immunoreactive score, in which intensity of the reaction and percentage of positive cells were estimated (Table II). Final result represented a product of scores given for individual traits and ranged between 0 and 12 (45). Intensity of immunohistochemical reactions

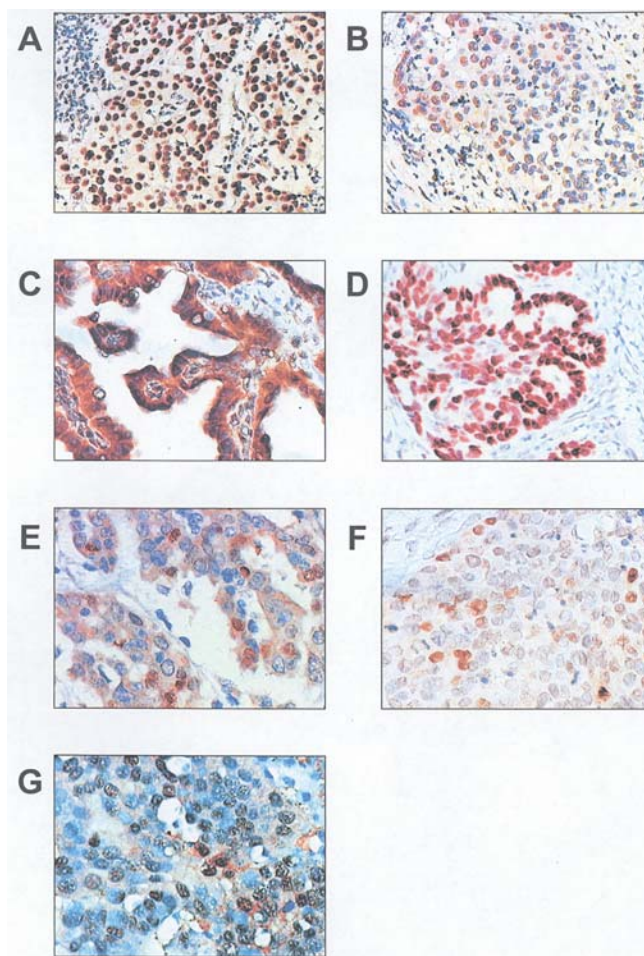


Figure 1. Immunohistochemical localization of analyzed proteins in ovarian carcinoma specimens. (A), hMLH1 expression (x200); (B), hMSH2 expression (x200); (C), Bcl-x_L expression (x400); (D), p53 expression (x200); (E), p63 expression (x400); (F), p21 expression (x400); (G), caspase-3 (active form) expression (x400) (color reaction is localized in cells of apoptotic morphology); each with hematoxylin counterstaining.

was evaluated independently by two pathologists. In cases of divergencies, the evaluation was repeated using double-headed microscope.

Statistical analysis. Kaplan-Meier statistics and log-rank tests were performed using SPSS software (release 12.0.1; SPSS Inc., Chicago, IL, USA) to estimate the significance of differences in survival times. The length of progression-free survival was defined as the time from the primary surgical treatment until the time of diagnosis of a recurrent tumor or death. The χ^2 -test was used to investigate the association between gene expression and other findings. Cox proportional hazard regression model was used for multivariate analysis of survival. All P-values are given for two-sided tests. P-values of <0.05 were considered to be statistically significant.

Results

Ovarian cancer tissues from 43 patients excised at the time point of first-look laparotomy (FLL) and 30 tissue samples from secondary cytoreduction (SCR) procedures of the same patients collective were analyzed by immunohistochemistry (Fig. 1). The clinical characteristics of examined patients are

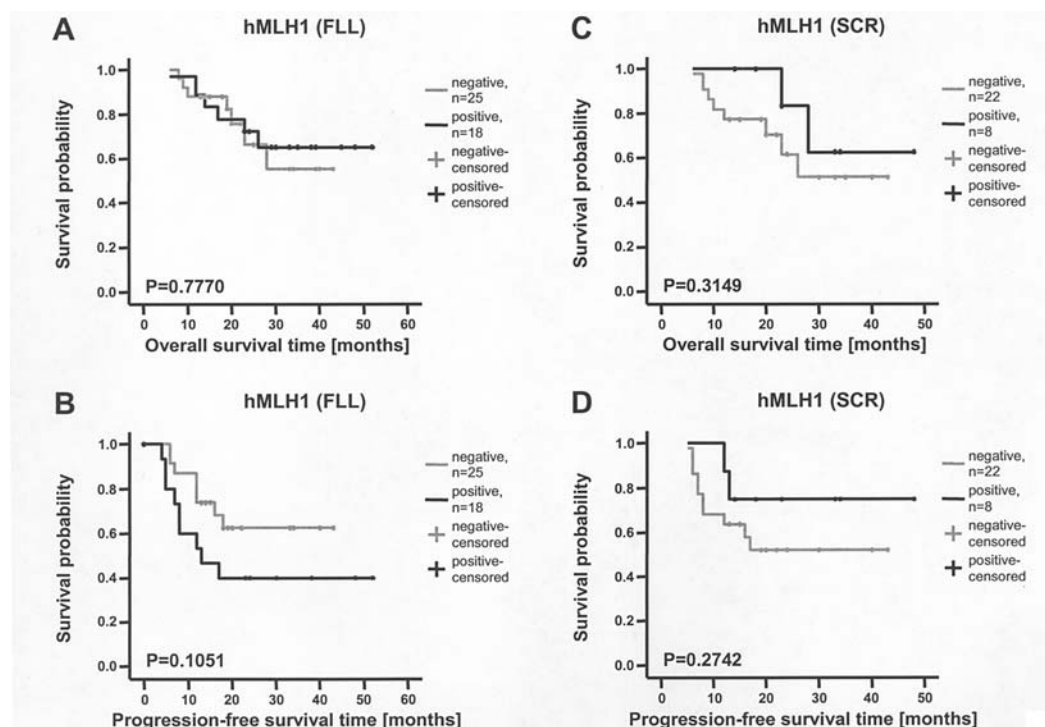


Figure 2. Kaplan-Meier curves for survival and expression of hMLH1 in studied group of 43 ovarian cancer patients. No significant differences in overall survival (A) and progression-free survival (B) at FLL between hMLH1 positive and negative patients; no significant differences in overall survival (C) and progression-free survival (D) at SCR between hMLH1 positive and negative patients.

listed in Table I. Due to the homogeneous set of patients referring to FIGO stage, histology, and applied chemotherapy, we abstained from correlating these parameters with expression differences of the assayed proteins.

Mismatch repair proteins hMLH1 and hMSH2. The immunostaining for the mismatch repair proteins hMLH1 and hMSH2 was evaluated by an immunoreactive score (IRS) from 0 to 12 of the nuclear staining (Table II). hMLH1 protein expression

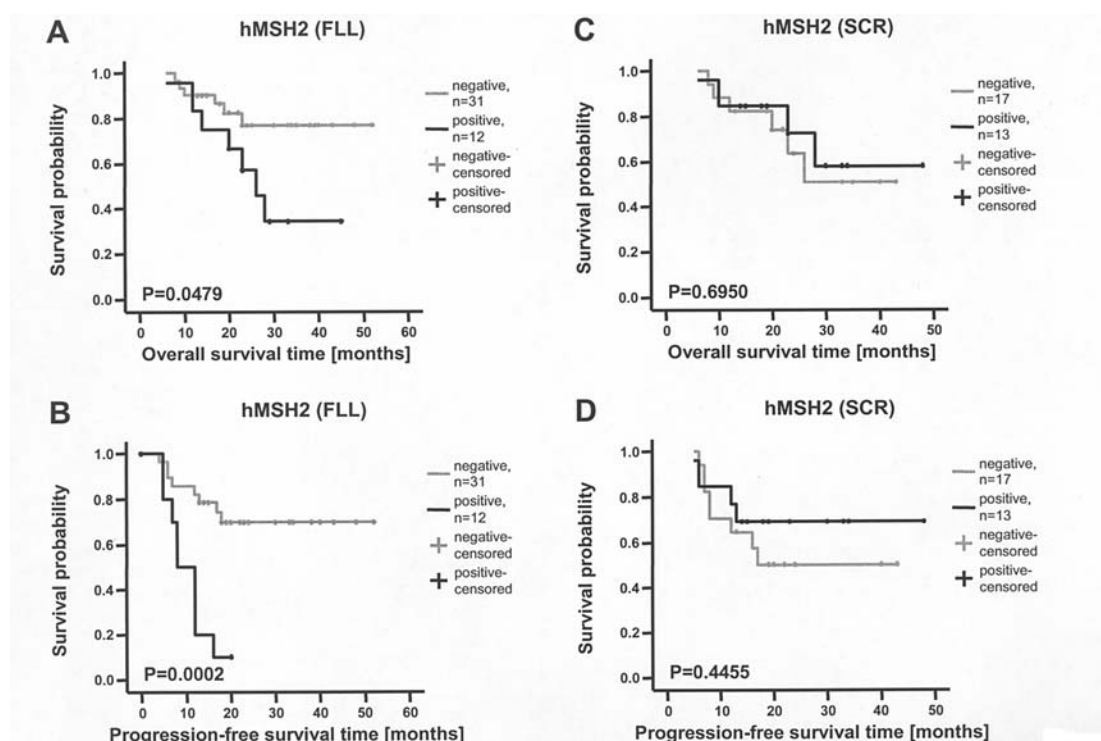


Figure 3. Kaplan-Meier curves for survival and expression of hMSH2 in studied group of 43 ovarian cancer patients. Patients with tumors negative for hMSH2 at FLL have a significant increased overall survival (A) and progression-free survival time (B) as compared to positive cases; no significant differences in overall survival (C) and progression-free survival (D) at SCR between hMSH2 positive and negative patients.

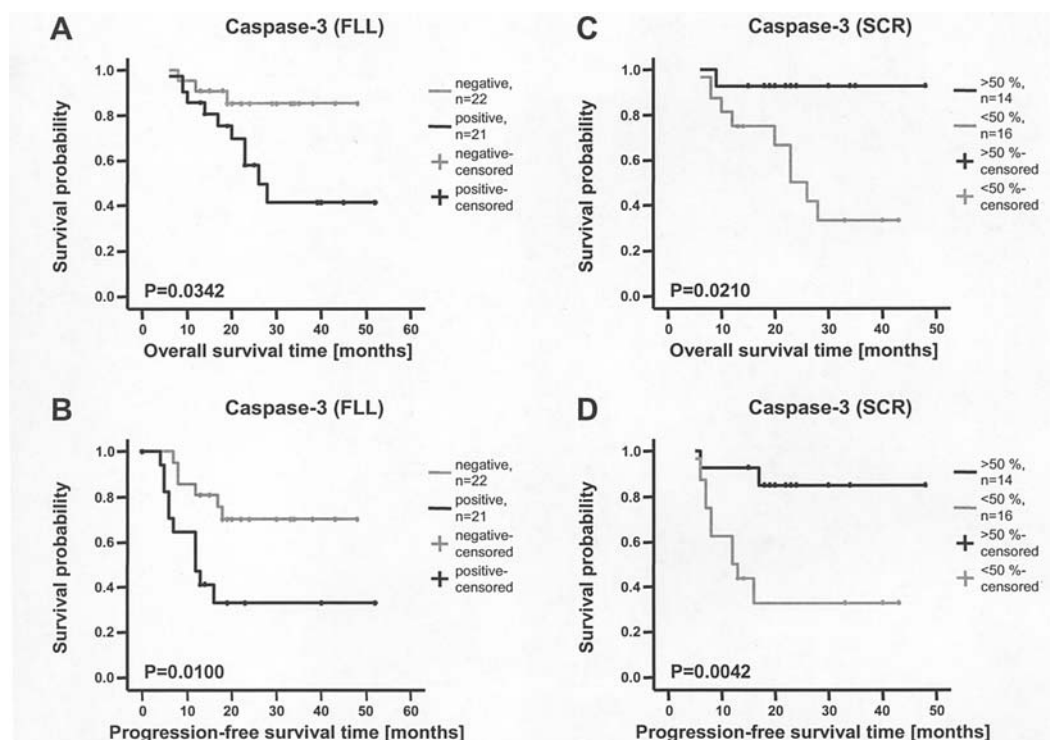


Figure 4. Kaplan-Meier curves for survival and staining of biologically active caspase-3 in studied group of 43 ovarian cancer patients. Positive expression of active caspase-3 before chemotherapy (FLL) significantly shortened overall survival (A) and progression-free survival (B); significant longer overall survival (C) and progression-free survival (D) was observed, when active caspase-3 was expressed in >50% of tumor cells after applying chemotherapy (SCR).

was positive in 42% of FLL and in 27% of SCR tissues. For hMSH2 protein, 28% of FLL and 43% of SCR samples showed a positive staining. There was no significant difference in the mean expression of both proteins in FLL and SCR tissues. Because more than half of the tissue samples showed a negative staining for the examined proteins, the patient collective was divided into two groups (negative or positive) for survival analysis. Examining hMLH1 protein expression, there was no difference either in the overall or in the progression-free survival time (Fig. 2). There was no difference either in the hMLH1 expression between patients who were alive or not. However, patients with negative hMSH2 displayed an advantage in both overall and progression-free survival, when assessed by tissues obtained in FLL (Fig. 3A and B; Table III). Additionally, 81% of patients without hMSH2 staining were alive, in contrast to 42% of surviving patients who were positive for hMSH2 (χ^2 -test, $P=0.013$). SCR material was not predictive in this issue (Fig. 3C and D).

Caspase-3. Caspase-3 expression was evaluated by the percentage of tumor cells showing a positive staining representing the active form of this protein. FLL tissue samples (49%) were positive for caspase-3, and 14 of these 21 patients expressed caspase-3 in only up to 5% of the tumor cells. There was a clear survival advantage in terms of overall and progression-free survival for patients with negative caspase-3 expression before chemotherapy (FLL tissues; Fig. 4A and B). Examining SCR tissue samples, 47% of patients expressed caspase-3 in >50% of tumor cells and only 3 patients (10%) showed no expression of this protein. Patients with expression of caspase-3 in >50% of tumor cells following chemotherapy

(SCR tissues; Fig. 4C and D) had a better prognosis for both overall and progression-free survival (Table III). Because of the tremendous difference in caspase-3 expression levels in tissues before and after chemotherapy (FLL versus SCR) no common cut off point could be used to evaluate the prognostic significance of this protein.

Bcl-x_L. The expression of the cellular oncoprotein Bcl-x_L was estimated using IRS (Table II). Patients were divided into two groups with no/low (IRS 0-6) and high (IRS 7-12) Bcl-x_L expression in the excised tumor tissues. In FLL tissues, 26% of samples showed no expression of Bcl-x_L, whereas all patients expressed this protein at least with IRS 3 in tissues obtained by SCR. The median of Bcl-x_L expression was 4 for FLL and 9 for SCR cancer tissues. Using FLL tissues, patients with a higher Bcl-x_L expression had clearly a shorter time of progression-free survival (Fig. 5B). In terms of overall survival, we observed also an advantage for patients with no/lower expression of Bcl-x_L (Fig. 5A), but this difference was not significant ($P=0.059$; Table III). There was no significance of Bcl-x_L expression for the prediction of clinical outcome, when SCR tissues were used (Fig. 5C and D). Additionally, we could not find any correlations between the expression level of the anti-apoptotic Bcl-x_L and expression of activated caspase-3, either in FLL or in SCR tissues.

Tumor suppressors p53, p63 and p21^(WAF1/CIP1). Expression analyses evaluated by IRS were performed for p53, p63 and p21. As expected, all three proteins displayed a nuclear staining in ovarian cancer tissues (Fig. 1D-F). Among FLL

Table III. Univariate overall and recurrence-free survival analysis.

Factor	n	Survival (%) ^a	OS ^b P-value	Recurrence-free (%) ^a	PFS ^b P-value
hMLH1					
FLL	43		0.7770		0.1051
Negative	25	72		71	
Positive	18	67		50	
SCR	30		0.3149		0.2742
Negative	22	64		55	
Positive	8	75		75	
hMSH2					
FLL	43		0.0479		0.0002
Negative	31	81		74	
Positive	12	42		25	
SCR	30		0.6950		0.4455
Negative	17	65		53	
Positive	13	69		69	
Caspase-3					
FLL	43		0.0342		0.0100
Negative	22	86		73	
Positive	21	52		48	
SCR	30		0.0210		0.0042
<50%	16	44		37	
>50%	14	93		86	
Bcl-x_L					
FLL	43		0.0590		0.0012
IRS 0-6	27	81		78	
IRS 7-12	16	50		31	
SCR	30		0.3987		0.7608
IRS 0-6	9	78		56	
IRS 7-12	21	62		62	
p53					
FLL	43		0.0603		0.0321
IRS 0-4	23	83		74	
IRS 5-12	20	55		45	
SCR	30		0.7109		0.7630
IRS 0-4	11	73		64	
IRS 5-12	19	63		58	
p63					
FLL	43		0.1306		0.4118
Negative	21	81		67	
Positive	22	59		56	
SCR	30		0.6061		0.1396
Negative	12	50		42	
Positive	18	78		72	
p21^(WAF1/CIP1)					
FLL	43		0.0006		0.0002
IRS 0-2	23	48		35	
IRS 3-12	20	95		90	
SCR	30		0.1319		0.0422
IRS 0-2	24	59		50	
IRS 3-12	6	100		100	

^aAt the end-point of observation time (max. 52 months). ^bKaplan-Meier analysis, log-rank test. IRS, immunoreactive score; FLL, first-look laparotomy; SCR, secondary cytoreduction; OS, overall survival; PFS, progression-free survival.

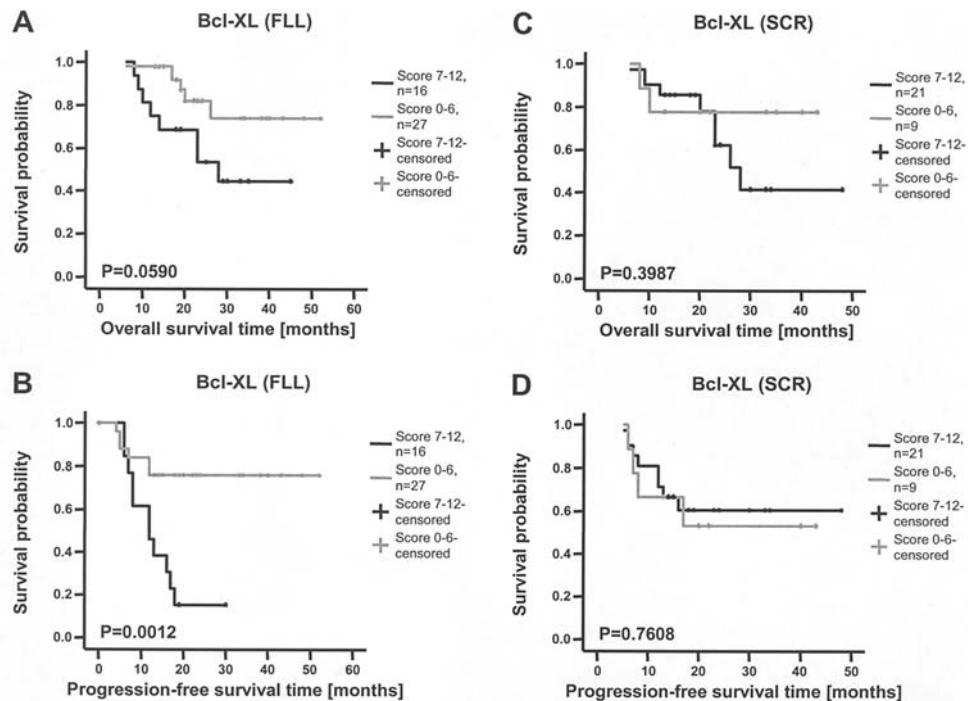


Figure 5. Kaplan-Meier curves for survival and expression of Bcl-x_L in studied group of 43 ovarian cancer patients. No significant differences in overall survival (A), but significantly prolonged progression-free survival (B) for patients with low (score 0-6) Bcl-x_L-specific staining at FLL compared to higher Bcl-x_L expression (score 7-12); no significant differences in overall survival (C) and progression-free survival (D) between patients with low (score 0-6) and high (score 7-12) Bcl-x_L-specific staining at SCR.

tissues, each 4 samples (9%) were negative for p53 and p21, respectively. The median expression was 4 for p53 and 2 for p21. Regarding tissues from SCR procedures, only one of the samples was negative for p53, and 11 tumors (37%) showed

no staining for p21 protein. In these tissues, the median protein expression was 6 for p53 and 1 for p21. FLL tissues [21/43 (49%)] and 12/30 (40%) SCR tissues showed no p63 protein expression, whereas the highest score measured was 6

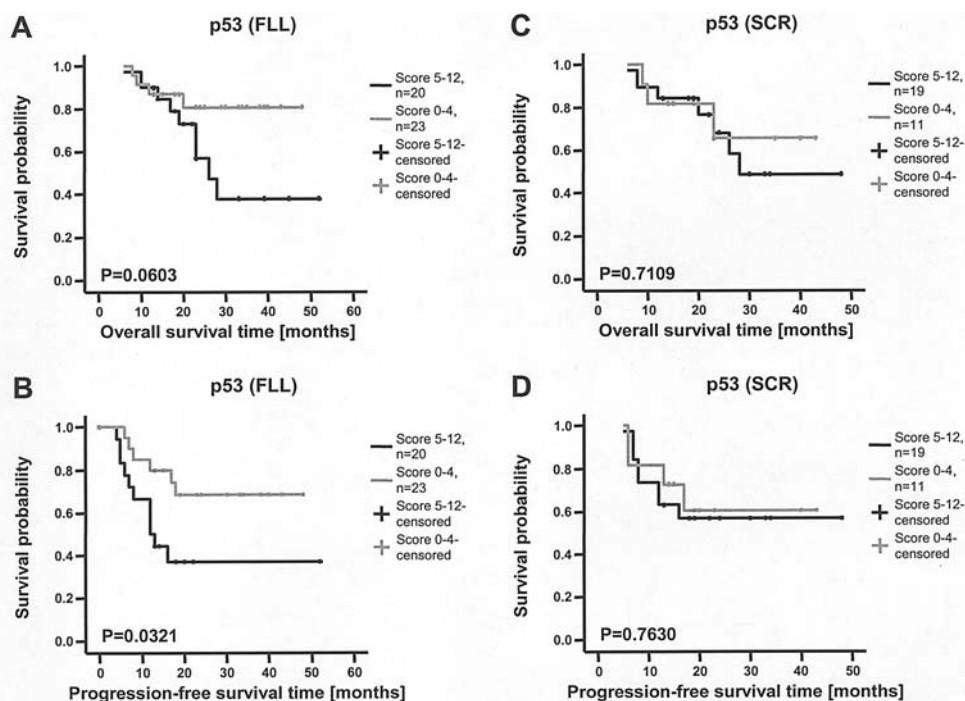


Figure 6. Kaplan-Meier curves for survival and expression of p53 in studied group of 43 ovarian cancer patients. No significant differences in overall survival (A), but significantly prolonged progression-free survival (B) for patients with low (score 0-4) p53-specific staining at FLL compared to higher p53 expression (score 5-12); no significant differences in overall survival (C) and progression-free survival (D) between patients with low (score 0-4) and high (score 5-12) p53-specific staining at SCR.

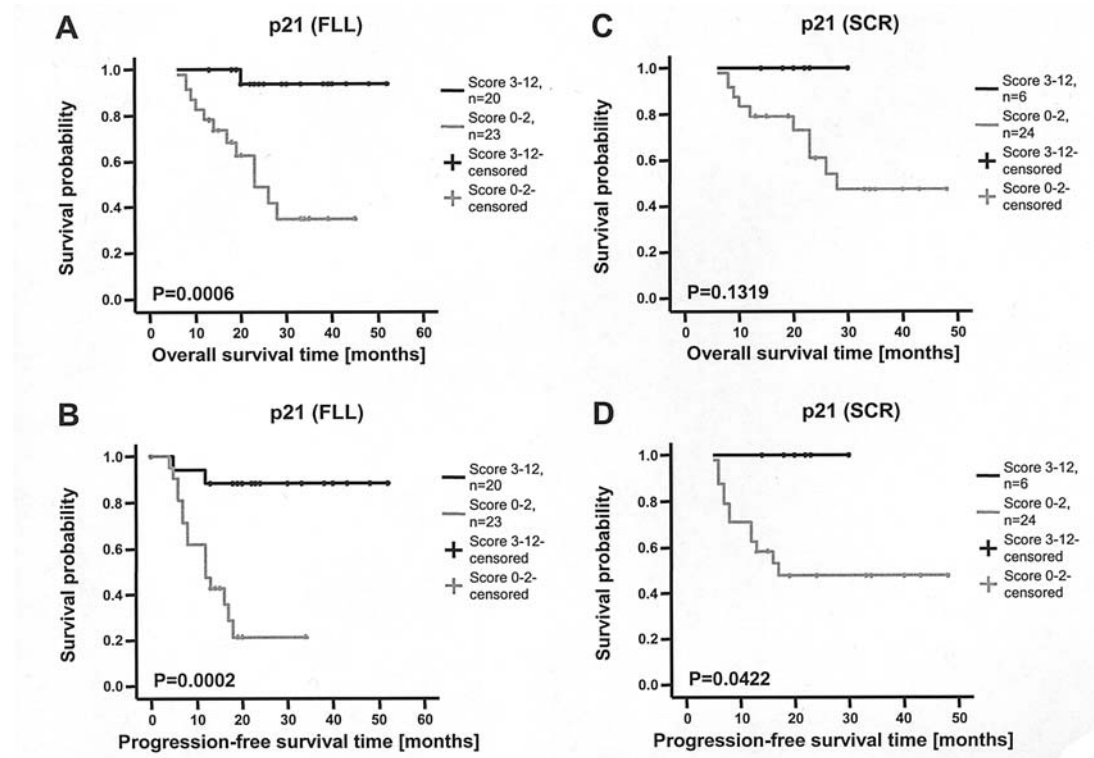


Figure 7. Kaplan-Meier curves for survival and expression of p21 in studied group of 43 ovarian cancer patients. Patients suffering from tumors with high p21-specific staining intensity (score 3-12) at FLL have a high significant increased overall survival (A) and progression-free survival time (B) as compared to patients with tumors showing a low p21-specific staining (score 0-2); no significant differences in overall survival (C) between low (score 0-2) and high (score 3-12) p21-specific staining at SCR, but all 6 patients with high p21 expression were alive until the end of observation time; patients suffering from tumors with high p21-specific staining intensity (score 3-12) at SCR have a significant increased progression-free time (D) as compared to patients with tumors showing a low p21-specific staining (score 0-2).

and 8 in FLL and SCR tissues, respectively. Using tissues from FLL procedures, patients with lower expression of p53 protein (IRS 0-4) presented an advantage in progression-free survival (Fig. 6B). Although a strong tendency was also seen in the context of overall survival for patients with lower p53 expression in Kaplan-Meier diagrams, the significance level was not achieved ($P=0.060$; Fig. 6A; Table III). Analysis of p53 expression in SCR tissues was not predictive for clinical outcome (Fig. 6C and D). In both FLL and SCR tissues, evaluation of p21 expression provided conspicuous results. There was a marked advantage in overall and progression-free survival for patients with higher expression of p21 (Fig. 7). Despite the fact that all patients with higher p21 expression were still alive and progression-free at the end of observation time, the significance level for overall survival analysis was missed due to the small number of cases in this group ($P=0.132$; Table III). No correlation was observed for p53 and p21 expression in FLL and SCR tissues. There was no association of p53 expression level in FLL tissues with the p21 expression level in SCR tissues after chemotherapy. No statistically relevant survival advantage could be observed for patients with or without p63 protein expression (Fig. 8). Nevertheless, it should be recognized, that the percentage of surviving patients and patients with recurrence-free survival until the end of observation time was higher in patients with negative p63 staining in FLL tissues and for patients with positive staining in SCR tissues (Table III).

Multivariate analysis. Although our set of patients was relatively small in number, we performed explorative multivariate analyses for all immunohistologically analyzed factors in FLL and SCR tissues (Table IV). Because age of patients and grading were not statistically significant for survival in univariate or in multivariate analysis (data not shown), we abstained from including these factors in our model. Furthermore, nearly all cancer specimens diagnosed were in stage FIGO III, thus, staging could be not included in the analysis. Expression of caspase-3 and p21 were shown to be independent prognostic factors for overall and progression-free survival before applying chemotherapy. Additionally, caspase-3 expression was also predictive for overall and progression-free survival after administering chemotherapy. Interestingly, also hMLH1 and hMSH2 expression were independent prognostic markers for overall survival in SCR tissues, although hMLH1 was not significant in univariate analysis.

Discussion

Despite the advances in therapy of ovarian cancer, this cancer entity is still a disease with poor prognosis. To obtain further information on the behavior of cancer cells under cytostatic drug treatment, we analyzed the protein expression of a set of genes in ovarian cancer patients by immunohistochemistry before and after platinum-based chemotherapy. The

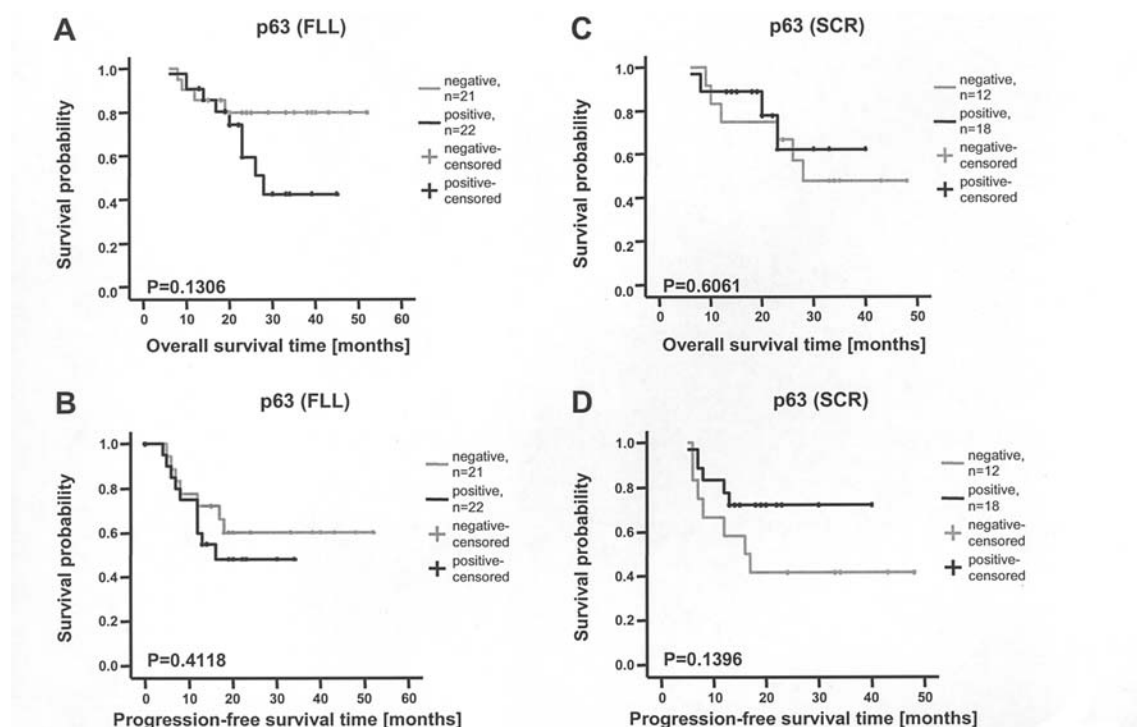


Figure 8. Kaplan-Meier curves for survival and expression of p63 in studied group of 43 ovarian cancer patients. No significant differences in overall survival (A) and progression-free survival (B) at FLL between p63 positive and negative patients; no significant differences in overall survival (C) and progression-free survival (D) at SCR between p63 positive and negative patients.

obtained data were correlated with clinical outcome with the intention of identifying prognostic markers, and possibly finding new objectives for improving ovarian cancer therapy.

Loss of DNA MMR is known to be an important mechanism for the development of platinum resistance in human cancer cell lines (46-48). The first studies examining the most important MMR proteins, hMLH1 and hMSH2,

Table IV. Multivariate overall and progression-free survival analysis.

	Overall survival			Progression-free survival		
	HR	95% CI	P-value	HR	95% CI	P-value
FLL						
hMLH1	0.682	0.213-2.187	0.520	2.082	0.752-5.764	0.158
hMSH2	2.285	0.483-10.808	0.297	3.507	0.911-13.495	0.068
Caspase-3	9.402	1.383-63.919	0.022	7.946	1.294-48.802	0.025
Bcl-x _L	0.709	0.179-2.810	0.624	0.806	0.215-3.018	0.749
p53	0.364	0.041-3.232	0.365	0.765	0.153-3.816	0.744
p63	0.241	0.029-2.002	0.188	0.200	0.036-1.128	0.068
p21	0.018	0.001-0.274	0.004	0.048	0.005-0.424	0.006
SCR						
hMLH1	0.019	0.001-0.387	0.010	0.098	0.009-1.093	0.059
hMSH2	12.117	1.110-132.318	0.041	4.683	0.675-32.507	0.118
Caspase-3	0.043	0.002-0.774	0.033	0.117	0.017-0.808	0.030
Bcl-x _L	12.907	0.909-183.288	0.059	1.536	0.252-9.347	0.641
p53	0.439	0.049-3.924	0.462	0.974	0.180-5.281	0.976
p63	1.056	0.245-4.548	0.941	0.578	0.168-1.990	0.385
p21	0.000	0.000-n.v.	0.978	0.000	0.000-n.v.	0.977

HR, hazard ratio; CI, confidence interval of hazard ratio; FLL, first-look laparotomy; SCR, secondary cytoreduction; n.v., no value given by SPSS statistical program.

resulted in contradictory conclusions (4-7), so that further analyses are needed to clarify the clinical importance of these factors. In this study, we analyzed ovarian cancer patients for their expression of hMLH1 and hMSH2 using immunohistochemistry. In contrast to previous reported results in ovarian cancer patients (n=54) (6), we could not detect any significant difference in the mean expression before and after chemotherapy for either protein. But it has to be considered, that >50% of analyzed tissue samples reported here showed no expression of hMLH1 and hMSH2, respectively. Nonetheless, in agreement with Samimi *et al* (6), we did not find any influence of hMLH1 expression on patient's survival in univariate analysis. Moreover, hMLH1 was shown to be an independent prognostic marker for overall survival using multivariate analysis indicating an influence on clinical outcome of ovarian cancer patients. An increased amount of hMSH2 protein was reported to be an independent prognostic indicator of survival in ovarian carcinoma patients (n=102) by multivariate analysis (5), although there was no significant difference in hMSH2 protein staining between patients that were alive and those who were not. Another study also described significantly lower hMSH2 protein level in ovarian cancer patients not responding to the applied chemotherapy by Western blot analysis of ascites (n=9) and solid tumors (n=10) (4). In the present study, we found that a positive hMSH2 expression before chemotherapy is an unfavorable prognostic factor in overall and progression-free survival. It has been annotated previously, that the MMR system may contribute to cisplatin toxicity by alternative mechanisms, as hMSH2 may bind to cisplatin-DNA-adducts, shielding them from repair and allowing them to persist without triggering apoptosis as a consequence (49). In this context, tumor cells with higher hMSH2 expression level without apoptosis or repair inducing stress (here: before chemotherapy), could use this way to survive after onset of chemotherapeutic treatment resulting in a worse clinical outcome.

Activated caspase-3 is regarded to be the key protein in triggering apoptosis (30), and was reported to cleave p21^(WAF1/CIP1). The cleaved p21 fragment is no longer able to arrest the cells in G1 phase, so that the cells undergo apoptosis after intense DNA damage (50). Hence, we supposed that the expression of activated caspase-3 could be a good marker for the assessment of chemotherapeutic effectiveness. As expected, there was a clear survival advantage for patients showing a higher percentage of apoptotic cancer cells after applying chemotherapy, in expressing higher amounts of activated caspase-3. We showed that a positive staining for active caspase-3 in cancer tissues before chemotherapy is a negative prognostic factor for ovarian cancer patients. Overexpression of caspase-3 without an apoptotic stimulus could refer to abnormal regulative pathways or may be due to mutations in caspase-3 resulting in decrease or loss of function. Prior analyses of human cancer cell lines showed that overexpression of anti-apoptotic genes can affect the activity of caspase-3 (33,51). However, we did not observe a correlation between Bcl-x_L and caspase-3 expression.

The anti-apoptotic protein Bcl-x_L was reported to show higher expression level in ovarian carcinoma tissues compared with normal ovaries (41,52,53) and has frequently been examined in the context of chemoresistance (38,54).

Nevertheless, very little is known about the prognostic relevance of Bcl-x_L in ovarian cancer patients. Using the IRS (45), we observed a longer progression-free survival time in patients with no or lower Bcl-x_L protein expression in univariate analysis, which is consistent with a previously described study on an ovarian carcinoma collective with a smaller number of patients (n=28) using a different scoring system (54). As in that study, we could not find a statistically significant difference in overall survival, although the survival time in patients with no or lower Bcl-x_L was longer. We found also concordance with the fact, that Bcl-x_L was expressed at higher level in patients after platinum-based chemotherapy. However, Bcl-x_L expression was not an independent prognostic marker in multivariate analysis. Further analyses with a more numerous set of ovarian cancer patients should be performed to clarify these data.

Mutations of p53 and/or p53 protein overexpression have been detected in 20-84% of ovarian cancers, and p53 alteration rate was reported to be higher in advanced than in early stages of the disease (24,55). Expression of p53 has been studied extensively by immunohistochemistry, using archival formalin-fixed, paraffin-embedded tissues. These tissues would show reduced immunoreactivity of many proteins due to the fixation process (55). In our study with paraffin-embedded tissues, we observed a positive staining in 39/43 (91%) of cases, which is above the average percentage, but comparable to the previously determined 74% positive p53 staining with the same antibody in snap-frozen ovarian cancer tissues (55). It was reported previously, that paclitaxel-induced apoptosis is not dependent on p53 status, whereas cisplatin-induced apoptosis requires wild-type p53 (10-12,56). Most of the patients in our study were administered a platinum- and paclitaxel-based chemotherapy, so that in theory the combination of both should have been able to induce apoptosis in all cancer cells independent of the existing p53 status. Nevertheless, we found in univariate analysis of cancer tissues before chemotherapy, that a higher expression of p53, which is probably mutated (9), represents an unfavorable prognosis factor in ovarian cancer patients.

Unlike p53, p63 is not a tumor suppressor gene and has been reported to be frequently amplified and/or overexpressed in various cancer entities, e.g., lung cancer and squamous cell carcinomas of head and neck (57). Down-regulation of p63 was observed in bladder and breast cancers associated with loss of differentiation and enhanced invasiveness, respectively (58,59). Because the p63 gene has two promoters and occurs in various isoforms with different expression patterns and functions (57), it is very difficult to clarify the importance of p63 for clinical outcome and chemoresistance. In this study we used an anti-p63 antibody which detects four of the six isoforms of p63. We could not observe any significant difference in survival time for ovarian cancer patients expressing p63 or not. Similar findings were previously published by others (60) using a different antibody detecting all known isoforms. But, an impact of p63 expression cannot be excluded, because the variety of isoforms which may possess opposite functions, thus more detailed analyses should be implemented.

p21^(WAF1/CIP1) is a potent inhibitor of cyclin-dependent kinases, which are necessary for cell cycle progression, and

functions as a central checkpoint in cell cycle control. Although p21 can be induced by p53 during DNA damage induced cell cycle arrest and apoptosis, there is sufficient evidence that p21 can be up-regulated independently of p53 (61). In this study, we could not see any correlation between p21 and p53 expression, which is consistent with previous trials (62). Therefore, apart from the p53-dependent induction of p21 further independent mechanisms of apoptosis induction seem to be involved. We also found p21 protein expression to be a meaningful prognostic marker in ovarian carcinoma patients especially when tissues before chemotherapy were exerted for analysis. These results are concordant with previous studies on ovarian carcinoma patients, which analyzed lower ($\leq 10\%$ positive) and higher ($>10\%$ positive) expressions of p21 in the context of overall and progression-free survival (22,63), whereas studies comparing patients with negative and positive staining for p21 did not show any influence on patient's survival (19,62).

In conclusion, this study shows the importance of analyzed MMR and apoptosis-related proteins to clinical outcome. Except p63, all examined proteins were predictive to some extent in univariate and/or multivariate analysis. In particular, protein expression of active caspase-3 and p21 can be used as prognostic markers for disease progression and effectiveness of applied chemotherapy.

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