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Topoisomerase 1A, HER/2*neu* and Ki67 expression in paired primary and relapse ovarian cancer tissue samples

P. Surowiak[1,2,3], V. Materna[1], I. Kaplenko[4], M. Spaczynski[4], M. Dietel[1], H. Lage[1] and M. Zabel[2,5]

[1]Charité Campus Mitte, Institute of Pathology, Berlin, Germany, [2]Chair and Department of Histology and Embryology, University School of Medicine, Wroclaw, Poland, [3]Lower Silesian Centre of Oncology, Wroclaw, Poland, [4]Chair and Department of Obstetrics and Gynaecology, University School of Medicine, Poznan, Poland and [5]Chair and Department of Histology and Embryology, University School of Medicine, Poznán, Poland

Summary. In the present study we examined prognostic value of immunohistochemical estimation of topoisomerase 1A (TOP 1A) and HER-2/*neu* expression in ovarian cancers treated with platinum-based drugs but not with topotecan and the relation between expression of these proteins on the one hand and intensity of proliferation (Ki67) on the other. The analyses were performed on 73 samples of ovarian carcinoma originating from 43 first-look laparotomies (FLL) and, in 30 cases, from secondary cytoreductions (SCR)(after chemotherapy) from the same patients. In paraffin sections immunohistochemical reactions were performed using antibodies directed to HER-2/*neu*, TOP 1A and Ki67. Kaplan-Meier's analysis disclosed a shorter overall survival time in cases with augmented expression of TOP 1A at FLL and with higher expression of Ki67 at SCR. A shorter progression-free time was detected in cases with higher proportion of Ki67 positive cells at FLL. No relationship could be disclosed between HER-2/*neu* expression and the studied clinicopathological parameters. The studies confirmed high value of Ki67 estimation. The augmented expression of TOP 1A was demonstrated to represent an unfavourable prognostic factor. Thus, in cases with elevated expression of TOP 1A application of topotecan-based therapeutic schemes should be considered.

Key words: Ovarian cancer, Topoisomerase 1A, HER-2/*neu*, Ki67

Offprint request to: Dr Hermann Lage, Charité Campus Mitte, Institute of Pathology, Schumannstr, 20/21, D-10117, Berlin, Germany. e-mail: hermann.lage@charite.de

Introduction

Ovarian cancer ranks as the fifth in incidence among women and first in overall mortality among gynaecological cancers, although it accounts for only 4% of all cancer diagnoses. The high mortality rate is usually ascribed to late diagnosis of this tumor, which lacks early symptoms. 75% of ovarian carcinomas are diagnosed in the stage III and IV, and these patients have a 5-year survival rate as poor as 20% (Holschneider and Berek, 2000). But even in late stages of the disease, the courses are highly variable. In advanced ovarian carcinoma, platinum-based combination chemotherapy including paclitaxel or topotecan represents the main pharmacological treatment option (Averette et al., 1993). One of the principal causes of therapeutic failures in cases of chemotherapy involves the phenomenon of resistance to cytostatic drugs (Lage, 2003).

Topoisomerases (TOP) are nuclear enzymes, which transiently break und unwind DNA in the process of DNA replication and transcription. They are also involved in many cellular activities including chromosome condensation, DNA recombination, DNA segregation during mitosis, and DNA repair (Wang, 2002). DNA topoisomerase I (TOP 1) is targeted by camptothecins, such as topotecan and irrotecan (Takimoto et al., 1998). Holden et al. (1997) described expression of topoisomerase 1A in ovarian cancers. So far, no relationship was described between expression of TOP 1 in ovarian cancers and prognosis in patients treated with drugs other than campothecins.

The HER-2/*neu* proto-oncogene encodes a transmembrane receptor tyrosine kinase, homologous of the epidermal growth factor receptor. HER-2/*neu* amplification and/or overexpression occur in 10-15% of ovarian carcinomas (Maihle et al., 2002). Several studies have shown that HER-2/*neu* overexpression is associated

with poor prognosis and lower survival rates (Hogdall et al., 2003, Camilleri-Broet et al., 2004). A relationship was demonstrated between overexpression of HER-2/*neu* and resistance to cisplatin in *in vitro* studies (Marth et al., 1997). Hengstler et al. (1999) showed that chemotherapy (cisplatin and cyclophosphamide) significantly prolonged survival of ovarian cancer patients with low expression of HER-2/*neu*. In cases of overexpression of the discussed protein, cytostatic drugs failed to prolong the survival time. Until now, the mechanism of HER-2/*neu* effect on the phenomenon of resistance to cisplatin remains to be elucidated.

Establishing of role of TOP 1A and HER-2/*neu* expression in ovarian cancer may promote development of individualized chemotherapy, selection of resistant and/or sensitive cases to cisplatin and topotecan. The potential for prediction and, thus, for a more effective therapeutic approach will improve results of treatment in ovarian cancer.

The present study aimed at examining prognostic value of immunohistochemical detection of TOP 1A and HER-2/*neu* expression in ovarian cancers in patients treated with platinum-based drugs and at establishing a relationship between the discussed antigens on the one hand and intensity of proliferation (Ki67) on the other.

Materials and methods

Patients

Immunohistochemical examination was performed retrospectively on tissue samples taken for routine diagnostic purposes. Forty three patients operated in 1999-2002 on account of ovarian carcinoma in the Department of Gynaecology and Obstetrics, University Medical School in Poznan, Poland were qualified for study. The cases were selected based on availability of tissue and were not stratified for known preoperative or pathological prognostic factors. The study was approved by an Institutional Review Board (IRB) and the patients gave their informed consent before their inclusion into the study. Following the first-look laparotomy (FLL), all the patients were subjected to chemotherapy using platinum drug-based schemes (Table 1). Thirty six patients from the same group were subjected also to the secondary cytoreduction (second-look laparotomy – SCR). In 7 cases no SCR was performed due to advancement of the disease. In 6 cases no tumor cells were detected in the material originating from the SCR. The patients were monitored by periodic medical check-ups, CA-125 serum levels, ultrasonographic and radiological examinations. During the follow-up period, 22 patients (51%) had a recurrent disease and 13 patients (30%) died of the disease. The mean (median) progression-free survival time was 16.9 months (range 0 to 52 months), while the mean (median) overall-free survival time was 24.6 months (range 6 to 52 months). Follow-up was performed in December 2004.

Fragments sampled from studied tumors 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 the tumors was assessed according to the International Federation of Gynaecology and Obstetrics (UICC, 1997). Tumors were graded according to the Silverberg grading system (Shimizu et al., 1998).

Immunohistochemistry

The immunohistochemical reactions were performed on sections from 43 primary laparotomies and 30 secondary cytoreductions (where 6 from 36 cases cancer cells were not found). Formalin-fixed paraffin embedded tissue was freshly cut (4 µm). The sections were mounted on Superfrost slides (Menzel Gläser, Germany), dewaxed wtih xylene, and gradually hydrated. Activity of endogenous peroxidase was blocked by 30 min incubation in 1% H_2O_2. The sections were boiled for 15 min in a microwave oven, in Antigen Retrieval Solution (DakoCytomation, Denmark) at 250W. Then, immunohistochemical reactions were performed using the following antibodies: (1) monoclonal (clone 1D6) mouse antibodies against topoisomerase 1A (Novocastra, UK) at dilution 1:100;

Table 1. Patient and tumor characteristics.

CHARACTERISTICS	No. (%)[3]
All patients	43 (100)
Age (mean 51.0)[1]	
≤50	20 (47)
50-60	16 (37)
>60	7 (16)
Grade[1]	
1	7 (16)
2	18 (42)
3	18 (42)
FIGO[1]	
I	1 (2)
II	1 (2)
III	41 (95)
Histology[1]	
Serous	37 (86)
Endometrioid	3 (7)
Other	3 (7)
Clinical response[2]	
Complete response	16 (37)
Stable disease	5 (12)
Progressive disease	22 (51)
Chemotherapy (in total)	
Cisplatin/Paclitaxel	31 (72)
Cisplatin/Cyclophosphamide/Adriblastin	6 (14)
Cisplatin/Cyclophosphamide/Paclitaxel	3 (7)
Cisplatin/Cyclophosphamide/Paclitaxel/Adriblastin	2 (5)
Carboplatin/Paclitaxel	1 (2)

[1]: Data are given for the first operation/diagnosis implemented. [2]: According to RECIST (Response Evaluation Criteria in Solid Tumours) (Therasse et al., 2000). [3]: Differences in the sum to 100 % in groups are due to rounding.

(2) polyclonal rabbit antibodies against HER-2/*neu* (DakoCytomation, Denmark) at dilution 1:350; (3) monoclonal (clone MIB-1) mouse antibodies against Ki67 (DakoCytomation, Denmark) at dilution 1:50. The antibodies were diluted in the Antibody Diluent, Background Reducing (DakoCytomation, Denmark). Tested sections were incubated with antibodies for 1 h at room temperature. Subsequent incubations involved biotinylated antibodies (15 min, room temperature) and streptavidin-biotinylated peroxidase complex (15 min, room temperature) (LSAB+, HRP, DakoCytomation, Denmark). NovaRed (Vector Laboratories, UK) was used as a chromogen (10 min, at room temperature). All the sections were counterstained with Meyer's hematoxylin. In each case, control reactions were included, in which specific antibody was substituted by the Primary Negative Control (DakoCytomation, Denmark).

Evaluation of reaction intensity

In the case of TOP 1A, intensity of the immunohistochemical reaction was appraised using the semi-quantitative IRS scale, in which the score reflected both intensity of the reaction and proportion of positive cells. The final score represented the product of points given for individual characters and ranged between 0 and 12 (Remmele and Stegner, 1987). For evaluation of HER-2 reactivity, the DakoCytomation scoring system was used (0 = negative; + = partially membranous; ++ = complete membranous, weak; +++ = complete membranous, strong). In the case of Ki67, proportion of cells giving positive reaction was scored. Intensity of reaction was evaluated independently by two pathologists. In cases of divergencies, the evaluation was repeated using a double-headed microscope.

Statistical analysis

Statistical analysis of the results took advantage of Statistica 98 PL software (Statsoft, Poland). The employed tests included Mann-Whitney U test and ANOVA rank test of Kruskal-Wallis. Kaplan-Meier's statistics and log-rank tests were performed using SPSS software (release 10.0; SPSS Inc., Chicago, IL, USA) to estimate significance of differences in survival times. The length of progression-free survival was defined as the time between the primary surgical treatment and diagnosis of a recurrent tumor or death. Since we have not found any significant relationships between studied clinicopathological parameters (age, histology, grade, CA-125 at FLL level) and overall survival and progression free time of studied patients with an univariate analysis (P>0.05), we have not performed a multivariate analysis. As 95% of the studied patients were in FIGO stage III, we have not investigated relationships between stage and survival data.

We have also performed Kaplan-Meier's statistics and log-rank tests on two subgroups of studied patients: on 41 FIGO III patients and on 35 FIGO III patients receiving after surgery cisplatin and paclitaxel.

Results

In the case of TOP 1A membranous reaction of variable intensity was obtained in individual cases (Fig. 1A). Mean intensity of TOP 1A at FLL was 3.41±3.08 SD (min. 0, max. 12), while at SCR TOP 1A mean intensity amounted to 4.33±3.65 SD (min. 0, max. 12). In the case of HER-2/*neu*, membranous reactions of variable intensity were obtained in individual cases (Fig. 1B). Mean intensity of the reactions in preparations originating from FLL amounted to 1.5±1.07 SD (min. 0, max. 3), and in samples originating from SCR it was 1.67±1.06 SD (min. 0, max. 3). Ki67 has also a nuclear reaction of a variable intensity in individual cases (Fig. 1C). Mean proportion of cells expressing Ki67 at FLL was 55.18±24.42 SD (min. 3, max. 90), while that of cells expressing Ki67 at SCR amounted to 17.57±21.83 SD (min. 0, max. 90).

At the first stage of statistical analysis the U test of Mann-Whitney was employed to compare expression of TOP 1A, HER-2/*neu* and proportion of Ki67-positive cells at FLL and SCR. In cases of TOP 1A and HER-2/*neu*, no significant differences were disclosed in intensity of expression between material originating from FLL and that sampled at SCR (P=0.54, P=0.39, respectively). In the case of Ki67, a significantly lower intensity of proliferation was detected in SCR material as compared to FLL samples (P<0.001).

In turn, relationships between pairs of all studied variables were examined. Using Spearman's rank correlation, a positive correlation was detected only in Ki67 between FLL and SCR samples (R=0.43, P=0.017) (Table 2).

Using ANOVA rank test of Kruskal-Wallis, relationships were examined between intensity of expression of TOP 1A, HER-2/*neu* and proportion of

Table 2. P values for the Spearman's rank correlation between the examined pairs of variables.

	TOP 1A at FLL	HER-2/*neu* at FLL	Ki67 at FLL	TOP 1A at SCR	HER-2/*neu* at SCR	Ki67 at SCR
TOP 1A at FLL	-	0.7161	0.4173	0.2544	0.7543	0.9151
HER-2/*neu* at FLL	0.7161	-	0.0599	0.6273	0.0752	0.7333
Ki67 at FLL	0.4173	0.0599	-	0.8453	0.2101	0.0170
TOP 1A at SCR	0.2544	0.6273	0.8453	-	0.9113	0.0709
HER-2/*neu* at SCR	0.7543	0.0752	0.2101	0.9113	-	0.6968
Ki67 at SCR	0.9151	0.7333	0.0170	0.0709	0.6968	-

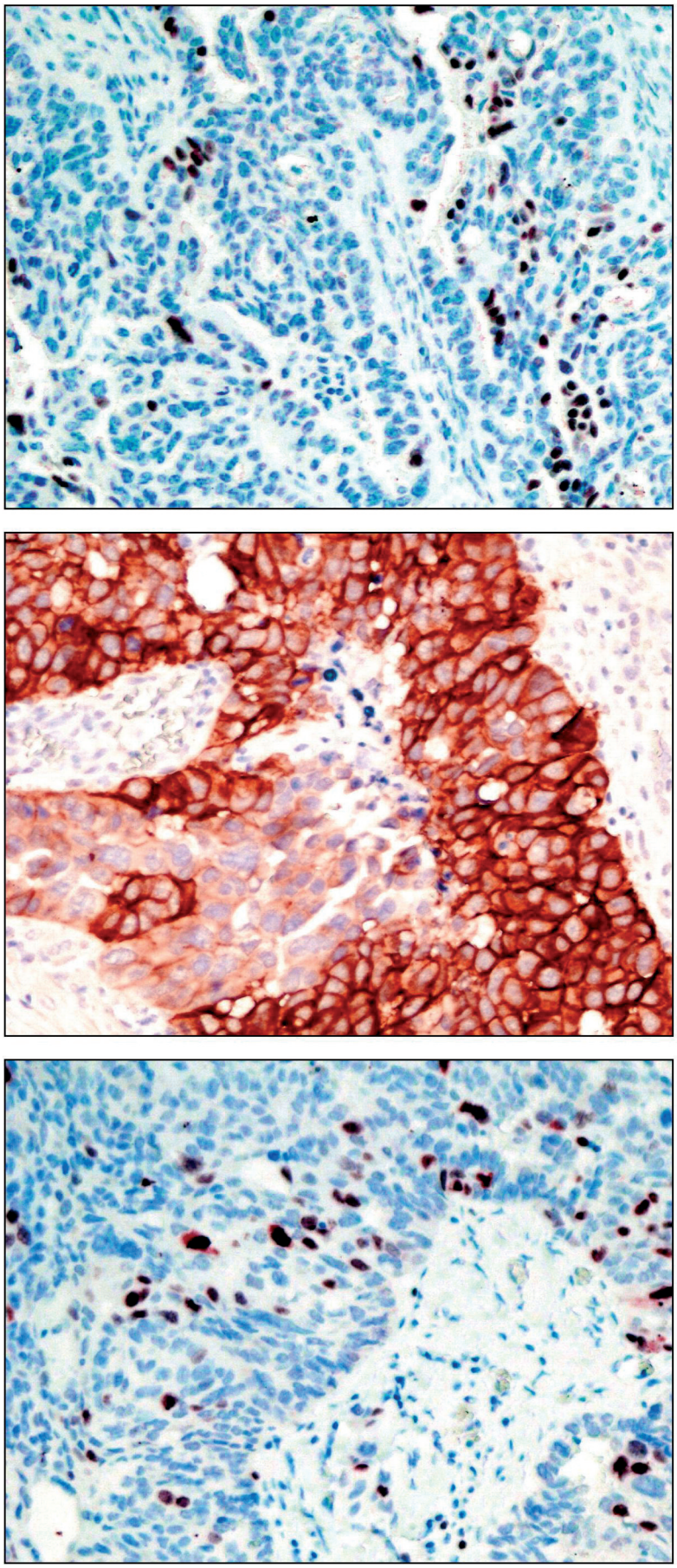

Fig. 1. Immunohistochemical localization of: **A.** Topoisomerase 1A – the red reaction product is localised in cell nuclei of ovarian cancer cells (x200, hematoxylin); **B.** HER-2/*neu* – the red reaction product is localised in cell membranes of ovarian cancer cells (x200, hematoxylin); **C.** Ki67 – the red reaction product is localised in cell nuclei of ovarian cancer cells (x200, hematoxylin).

cells manifesting expression of Ki67 on the one hand and histological type of the tumor, grade, deaths of the patients, manifestation of relapses, responses to the therapy on the other. No relationship could be documented between studied variables and histological type of the tumor, grade and response to the therapy (Table 3). Deaths were particularly frequent in the group of patients manifesting higher percentage of cells with expression of Ki67 at SCR (P=0.0144) (Table 3). Relapses were particularly frequent in cases with higher percentages of Ki67 expression at FLL and SCR (P=0.02, P=0.03, respectively) (Table 3).

In Kaplan-Meier's analysis on the entire group, overall survival time and progression-free time were compared between the following groups: (1) Cases with TOP 1A expression of 0-2 (lower) and cases with the expression of 3-12 (higher) at FLL and SCR. Such separation of the material corresponded to numerical force of the material (n=22 and 21, n=15 and 15, respectively); if the group was separated into cases with the higher than average expression and lower than average expression, the groups with higher expression showed a much lower numerical force (n=16 at FLL and n=12 at SCR, respectively) compared to groups of lower expression (n=27 and n=18, respectively); (2) Cases with HER-2/*neu* expression of 0-1 (designed as negative in DakoCytomation scoring system) and those with the expression of 2-3 (overexpression) at FLL and SCR; (3) Cases showing less than 50% Ki67 positive cells (i.e., with expression lower than average) and those with expression above 50% (i.e., above the average) at FLL and cases showing less than 10% or more than 10% Ki67-positive cells at SCR, respectively.

The analysis demonstrated that significantly shorter overall survival time characterized cases with higher expression of TOP 1A at FLL (P=0.01) (Fig. 2A) and higher proportion of cells with Ki67 expression at SCR (P=0.009) (Fig. 2F). A significantly shorter progression-free time was demonstrated by cases with higher percentage of Ki67 positive cells at FLL (P=0.03) (Fig.

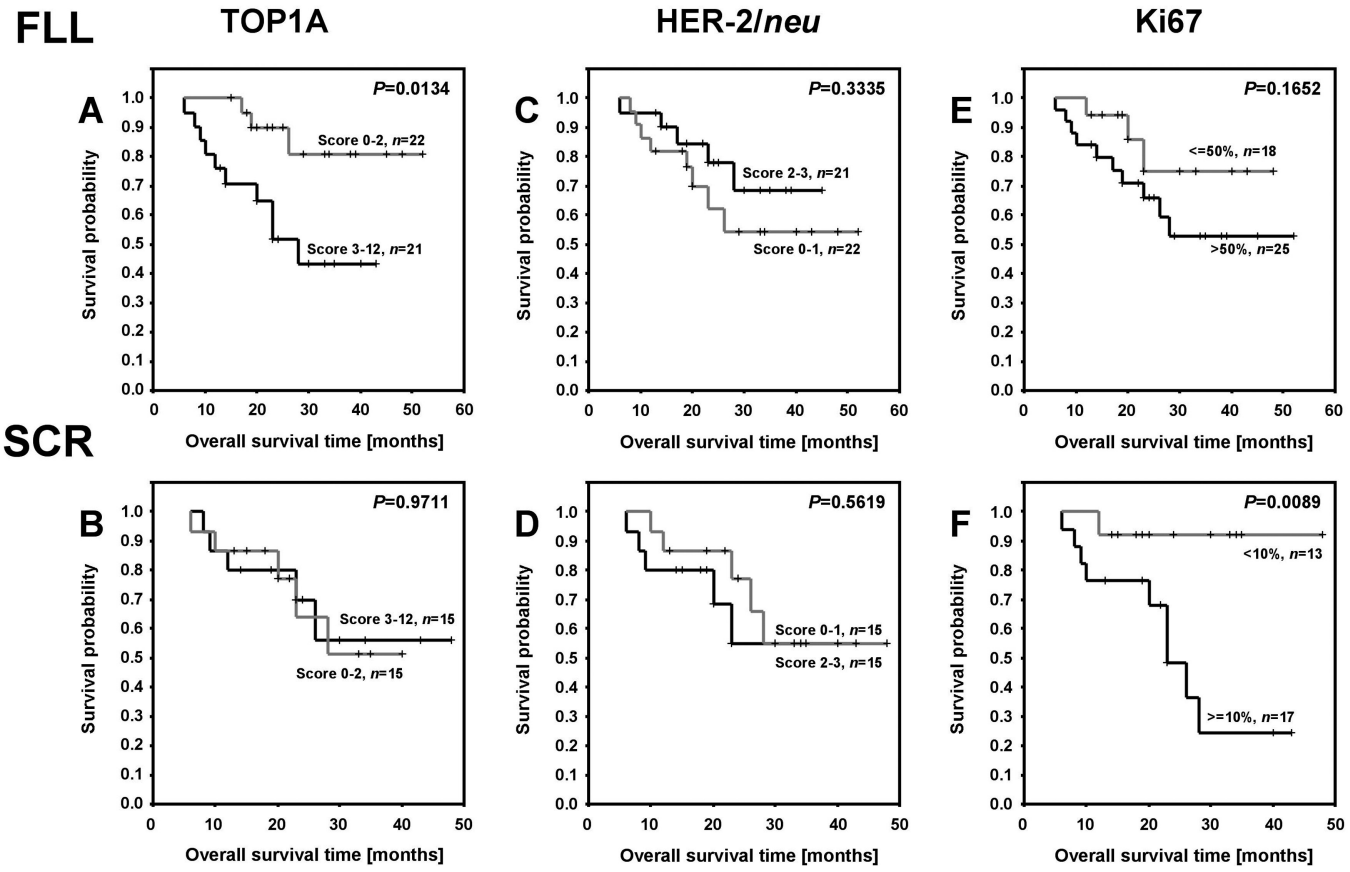

Fig. 2. Kaplan-Meier curves for overall survival and expression of studied antigens in studied group of 43 ovarian cancer patients. **A.** Patients with lower TOP 1A at FLL expression have an increased overall survival time. **B.** No significant differences in overall survival between patients with lower and higher TOP 1A expression at SCR. **C.** No significant differences in overall survival time between patients with lower and higher HER-2/*neu* expression at FLL. **D.** No significant differences in overall survival time between patients with lower and higher HER-2/*neu* expression at SCR. **E.** No significant differences in overall survival time between patients with lower and higher Ki67 at FLL expression. **F.** Patients with lower Ki67 expression at SCR have an increased overall survival time.

2E). No significant relationships could be detected for HER-2/*neu* (Figs. 2, 3).

In the subgroup of FIGO III patients we have demonstrated that significantly shorter overall survival time characterized cases with higher expression of TOP 1A at FLL (P=0.026) (Table 4), significantly shorter progression-free survival time characterized cases with higher percentage of Ki67 positive cells at FLL (P=0.03) (Table 4) and significantly shorter overall survival time characterized cases with higher percentage of Ki67 positive cells at SCR (P=0.01) (Table 4).

In the subgroup of FIGO III patients treated with

Table 3. Relationships between expression of TOP 1A, HER-2/*neu* and Ki67 on the one hand and principal clinical variables in studied patients on the other (ANOVA rank test of Kruskall-Wallis).

	HISTOLOGICAL TYPE[a]	GRADE[a]	DEATH	RELAPSES	RESPONSE
TOP 1A at FLL	0.2482	0.1245	0.2240	0.4521	0.2999
TOP 1A at SCR	-	-	0.8229	0.1537	0.2005
HER-2/*neu* at FLL	0.1977	0.4142	0.3611	0.8456	0.0942
HER-2/*neu* at SCR	-	-	0.9084	0.809	0.4408
Ki67 at FLL	0.9499	0.2356	0.2795	0.0242	0.0788
Ki67 at SCR	-	-	0.0144	0.0346	0.1060

[a]: The relationships between studied parameters at SCR on the one hand and histological type or grade on the other were not examined.

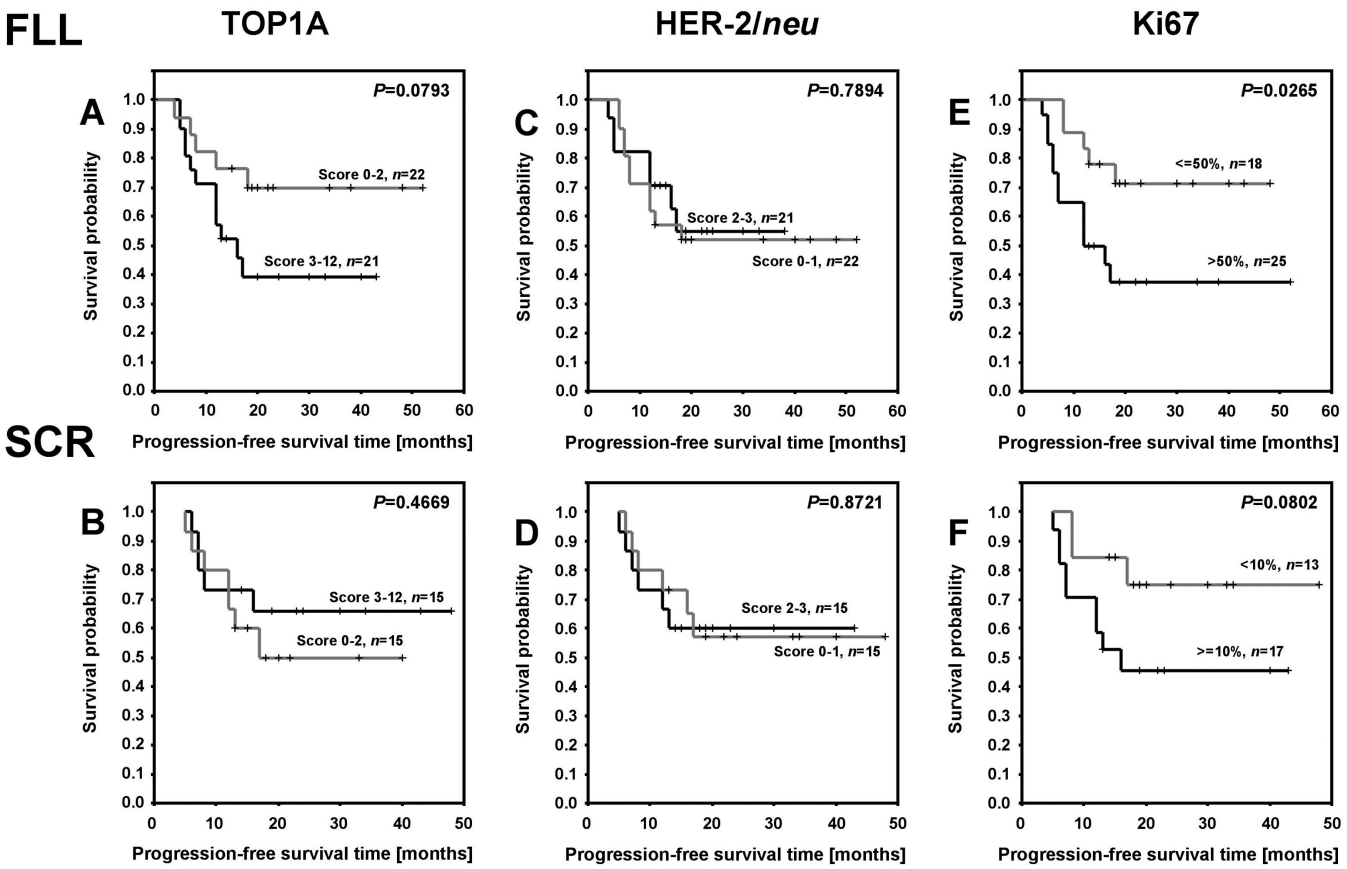

Fig. 3. Kaplan-Meier curves for progression-free survival and expression of studied antigens in studied group of 43 ovarian cancer patients. **A.** No significant differences in progression-free time between patients with lower and higher TOP 1A expression at FLL. **B.** No significant differences in progression-free time between patients with lower and higher TOP 1A expression at SCR. **C.** No significant differences in progression-free time between patients with lower and higher HER-2/*neu* expression at FLL. **D**. No significant differences in progression-free time between patients with lower and higher HER-2/*neu* expression at SCR. **E.** Patients with lower Ki67 expression at FLL have an increased progression-free time. **F.** No significant differences in progression-free time between patients with lower and higher Ki67 expression at SCR.

Table 4. Relationships between overall survival time (OS) and progression-free survival (PFS) and expression of topoisomerase 1A (TOP 1A), HER-2/*neu* and Ki67 in subgroups of FIGO III patients and FIGO III patients treated with platinum-based drugs and paclitaxel.

	FIGO stage III patients				FIGO stage III patients treated with cisplatin or paclitaxel			
	FLL, n=41		SCR, n=29		FLL, n=35		SCR, n=24	
TOP 1A	Score 0-2	n=21	Score 0-2	n=15	Score 0-2	n=20	Score 0-2	n=12
	Score 3-12	n=20	Score 3-12	n=14	Score 3-12	n=15	Score 3-12	n=12
OS	P=0.0255*		P=0.7247		P=0.3246		P=0.9155	
PFS	P=0.0920		P=0.3014		P=0.4386		P=0.3945	
HER-2/*neu*	Score 0-1	n=20	Score 0-1	n=15	Score 0-1	n=18	Score 0-1	n=11
	Score 2-3	n=21	Score 2-3	n=14	Score 2-3	n=17	Score 2-3	n=13
OS	P=0.4330		P=0.7887		P=0.3198		P=0.2056	
PFS	P=0.9843		P=0.8994		P=0.7639		P=0.4678	
Ki67	≤ 50%	n=17	<10%	n=13	≤ 50%	n=16	<10%	n=12
	>50%	n=24	≥ 10%	n=16	>50%	n=19	≥ 10%	n=12
OS	P=0.2508		P=0.0137*		P=0.3551		P=0.0201#	
PFS	P=0.0307*		P=0.1172		P=0.0797*		P=0.3135	

*: patients with smaller expression have better prognosis. #: all the patients with smaller expression are alive

cisplatin and paclitaxel the analysis has only demonstrated that significantly shorter overall survival time characterized cases with higher percentage of Ki67 positive cells at SCR (P=0.02) (Table 4).

Discussion

Due to their location, ovarian cancers are frequently diagnosed at an advanced stage of the disease. Therefore, apart from surgical approaches, chemotherapy constitutes an important component of the therapy while therapeutic failures reflect resistance of neoplastic cells to cytostatic drugs (Holschneider and Berek, 2000). In the disease most of therapeutic modalities are based on cis- or carboplatin in conjunction with paclitaxel or topotecan (Averette et al., 1993).

Augmented expression of TOP 1A has been described in various types of tumors, including ovarian cancers (Husain et al., 1994; McLeod et al., 1994; Lynch et al., 2001; Takimoto et al., 1998). The protein provides target for the group of anti-neoplastic drugs termed campothecins (topotecan and irrotecan) (Takimoto et al., 1998). Expression of TOP 1A has been documented as a significant predictive index in campothecin-based therapy (Rodriguez-Galindo et al., 2000; Materna et al., 2004), although no relationship has been disclosed until now between manifestation of the protein and prognosis in patients treated with other cytostatic drugs. In our study, we have shown that cases with augmented expression of TOP 1A demonstrated a significantly shorter overall survival time. It should be noted that topotecan has not been used in the studied group of patients. The observations indicate that in cases with augmented expression of TOP 1A application of topotecan should be considered, which might favourably change prognosis in the group of patients. To our knowledge, this study demonstrates for the first time significant unfavourable prognostic value of TOP 1A expression in cancers treated with other than campothecins drugs.

Data on prognostic and predictive value of HER-2/*neu* expression in ovarian cancers are divergent. Camilleri-Broet et al. (2004) (in a group of 117 patients), Hengstler et al. (1999) (in a group of 77 patients) and Hogdal et al. (2003) (in a group of 181 patients with ovarian cancer) demonstrated unfavourable prognostic significance HER-2/*neu* overexpression. Protopapas et al. (2004) (in a group of 100 patients) and Skirnisdottir et al. (2001) (in a group of 106 patients) detected no relationship between HER-2/*neu* expression and patient survival time. The cases with HER-2/*neu* overexpression used to be linked to lower sensitivity to cisplatin-based chemotherapy (Marth et al., 1997; Hengstler et al., 1999). In our studies, performed on the group of 43 patients treated with platinum-based schemes, no relationship could be disclosed between expression of HER-2/*neu* and overall survival time or progression-free time. Also, no differences have been demonstrated in intensity of expression of the protein before and after chemotherapy or in association with Ki67. Considering that all the examined patients have been treated with platinum-based drugs, one can conclude that expression of HER-2/*neu* in ovarian cancer does not reflect resistance to platinum-based drugs.

Ki67 is a recognized exponent of cell proliferation and its expression well reflects proliferation dynamics in various types of tumors and its augmented expression is linked to worse prognosis (Scholzen and Gerdes, 2000). In this study, we have noted that cases with higher proportion of Ki67-positive cells at FLL manifest a significantly shorter progression-free time, while in the case of Ki67 at SCR – overall survival time is significantly shorter. Higher intensity of proliferation of neoplastic cells in samples originating from SCR points to their low sensitivity to the applied chemotherapy. Cases with relapse of the tumor have been showing a significantly higher proportion of cells with Ki67

expression at FLL and SCR, while in cases terminated by death, a significantly higher proliferation has been noted in the SCR material. Also, a significantly lower proportion of cells with Ki67 expression has been documented at SCR as compared to FLL, which reflects the inhibitory effect of the applied pharmacological treatment on the tumor growth dynamics.

In summary, we confirmed that Ki67 represents significant exponent of dynamics of ovarian carcinomas. We also found, that cases with higher expression of TOP 1A exhibit a more aggressive clinical course. In such cases, therapeutic schemes should be employed which are based on campothecins. Further studies on other patients with ovarian carcinoma or patients with other cancer entities are needed to validate this finding.

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