Relationship between chemotherapy with paclitaxel, cisplatin, vinorelbine and titanocene dichloride and expression of proliferation markers and tumour suppressor gene p53 in human ovarian cancer xenografts in nude mice

H.-C. Kolberg[1], *M.D.*; **C. Villena-Heinsen**[2], *M.D.*; **M.-M. Deml**[2], *M.D.*; **S. Kraemer**[3], *M.D.*; **K. Diedrich**[1], *M.D.*; **M. Friedrich**[1], *M.D.*

[1]*Department of Gynecology and Obstetrics, University Clinic of Schleswig-Holstein, Campus Lübeck*
[2]*Department of Gynecology and Obstetrics, University of the Saarland, Homburg/Saar*
[3]*Department of Mastology, Krankenhaus Gerresheim (Germany)*

Summary

Purpose: In this study the relationship between therapy with paclitaxel, cisplatin, vinorelbine and titanocene dichloride and of the expression of proliferation markers (ki67 and S-phase fraction) and tumour suppressor gene p53 was analyzed using a human ovarian cancer xenograft model.

Methods: Biopsy material from one human ovarian cancer was expanded and transplanted into 102 nude mice. The mice were divided into six groups with different intraperitoneal treatments with paclitaxel, cisplatin, vinorelbine, titanocene dichloride and a control group treated with 0.9% saline solution. After the observation period the tumours were extracted and immunohistochemically stained with monoclonal antibodies against ki67 and p53. The s-phase-fraction was identified by flow cytometry.

Results: There where no statistically significant differences. Regarding the treatment groups, the vinorelbine-group showed the highest percentage (53.3%) and the titanocene dichloride-3x40 mg/kg-group the lowest percentage (7.1%) of ki67-positive specimens, whereas in the control group 35.7% of the specimens were positively stained for ki67. The results for the expression of p53 were similar. The vinorelbine-group had the highest percentage of p53-positive specimens (60%), in both titanocene-groups no specimen showed a positive staining for p53 and in the control group 7.1% of the specimens were positively stained for p53. The mean S-phase-fraction was 14.48% (SD ± 3.98), no statistically significant relation between s-phase-fraction and expression of p53 ($p = 0.883$) or of ki67 ($p = 0.351$) could be shown. The change of tumour volume was independent of the results for ki67, p53 and the S-phase-fraction.

Conclusion: Although, as previously published, a significant difference of tumour volume change occurred between the treatment groups, in this study we could not find a relation between this change of tumour volume and the expression of p53 or ki67. The absolute number of p53- and ki67-positive staining specimens was too small for statistical analysis, therefore the relevance of the differences between the different treatment groups and the control remains unclear. The results for the S-phase-fraction showed no correlation between the change of tumour volume, different treatment protocols or the expression of p53- and ki67. Our findings contribute to the controversy of the influence of chemotherapy on the expression of proliferation markers and p53.

Key words: Nude mice; Ovarian cancer; Chemotherapy; Proliferation markers; p53.

Introduction

Ovarian cancer is the gynaecologic malignancy with the highest mortality. In Germany about 8,300 women are diagnosed with ovarian cancer every year and about 6,000 per year die of the disease. Seventy percent of the tumours are diagnosed in FIGO Stage III or IV. According to the results of the EUROCARE-study the 5-year-survival-rates vary between 23% and 38% in European countries [1]. The therapy of choice is cytoreductive surgery obtaining a R0-situation if possible, followed by systemic platinum-based chemotherapy with the addition of paclitaxel. Because of its nephro- and neurotoxicity cisplatin was replaced by carboplatin. The current standard first-line treatment is a combination of carboplatin and paclitaxel [2, 3].

Although the response rate to carboplatin/paclitaxel-chemotherapy is high, 70% of the patients relapse and are candidates for second-line treatment. If the time between the first-line platinum-based treatment and the diagnosis of the relapse is shorter than 12 months, the tumour is classified as platinum-resistant with an expected response rate to second-line therapy between 10 and 20% and a median survival of 10 to12 months [4]. Zytotoxic agents used in the second-line therapy of platinum-resistant ovarian cancer are docetaxel, etoposid, vinorelbine, paclitaxel (if paclitaxel-naïve), gemcitabine, epi- and doxorubicine and topotecan [5]. The prognosis of these patients is poor, as mentioned above.

The results of second-line chemotherapy in patients with a relapse-free interval longer than 12 months after standard chemotherapy are significantly better. Their tumours are classified as platinum-sensitive; a reinduction with carboplatin/paclitaxel is possible and shows response rates of about 54%. Single agents show

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response rates between 20 and 30%; substances used are paclitaxel, docetaxel, cis- or carboplatin, topotecan, vinorelbine, etoposid, hexamethylamine, ifosphamide and epi- or doxorubicine. The median survival time is about twice as long as in patients with platinum-resistant disease [5].

New drugs with better response rates in platinum-resistant tumours and more detailed information about changes in tumour characteristics during chemotherapy are urgently required.

Human tumour/nude mice-models are well established in preclinical trials about the effects of cytostatics and the relevance of different therapeutical approaches [6-8]. Therefore in this study a human ovarian cancer xenograft model was chosen to obtain information about the effects of cytotoxic agents currently used in the therapy of ovarian cancer (paclitaxel, cisplatin and vinorelbine), the effects of the promising new drug titanocene dichloride and the relation of therapy with these agents and the markers of proliferation and tumour suppression.

Titanocene dichloride is an organometallic compound that has shown significant antiproliferative activity in vitro and in vivo [9-16]. Especially interesting is the fact that for ovarian cancer it has been shown that titanocene dichloride has antitumour activity in cisplatin-resistant tumour cells [12-14]. The drug interferes with DNA, blocks the S/G(2) phase of the cell cycle and shows antiangiogenic properties. In phase I clinical trials renal and liver toxicity were the dose limiting toxicities, whereas no alopecia or myelosuppression have been reported yet [17, 18]. In vitro data suggest a synergy between titanocene dichloride and other chemotherapeutic agents, e.g. 5-FU [19]. Although titanocene dichloride showed promising results in xenografted human renal-cell carcinoma [20], the results of a phase II clinical trial in advanced renal-cell carcinoma were disappointing [21].

The p53 gene is the most frequently mutated gene in human cancer [22]. After genotoxic stress the p53 protein is induced and activates several downstream genes [23]. It interacts directly with other proteins playing a role in DNA repair and can arrest cell cycle progression and induce apoptosis. Ovarian cancer in the FIGO Stages III and IV shows a loss of p53 function in up to 68% [24]. Overexpression of p53 has been proposed as a marker for poor prognosis and recurrence in several studies [25-29] whereas other groups did not find p53 to be an independent prognostic factor [30-34]. Response to platinum-based chemotherapy seems to be less likely in ovarian carcinoma with an overexpression of p53 [35-37].

S-phase-fraction and ki67-expression are markers for the proliferation activity of tissues. A correlation between s-phase and ki67-expression with the survival of patients with ovarian cancer could be shown previously [38, 39]. The monoclonal ki67-antibody is able to detect cell-cycle associated antigens in the nucleus only expressed by cells not in the G0-phase of the cell-cycle [40, 41]. The ki67-proliferation index has been described as a prognostic factor in ovarian cancer and seems to be more specific than histologic grading or FIGO stage [42]. A reduction of ki67-defined cell proliferation has been found in patients with good response to chemotherapy for ovarian cancer [37]. Several studies showed a significant correlation between the overexpression of ki67 and the overexpression of p53 [25, 26, 28].

The S-phase-fraction, measured by flow cytometry, has been proposed to be a predictive factor for the sensitivity to platinum-based chemotherapy in human ovarian cancer xenograft models [43]. In contradiction to previous results, recent studies showed no prognostic significance of the measurement of S-phase-fraction in ovarian cancer [44].

In this experiment with 102 nude mice the relationship between chemotherapy with paclitaxel, cisplatin, vinorelbine and titanocene dichloride and the S-phase-fraction, and the expression of ki67 as markers of proliferation and the expression of p53 was investigated.

Materials and Methods (small print)

Chemotherapeutics: Titanocene dichloride was provided by Medac (Hamburg, Germany), cisplatin was purchased from Rhone-Poulenc (Rorer, France), paclitaxel from Bristol-Myers-Squibb (USA) and vinorelbine from Pierre Fabre (France).

Animals: Six-week-old athymic mice derived from an independent company (Harlan-Winkelmann GmbH, Borchen, Germany) were used for the experiment. The mice were maintained under barrier conditions and given sterilized food (Altromin GmbH, Lage, Germany).

Heterotransplantation of tumour into nude mice: Human tumour tissue was freshly obtained from a patient suffering from advanced epithelial ovarian cancer. The tumour was cut into small fragments of about 20 mm^3 and implanted subcutaneously into both sides of the backs of nude mice. No major difference in tumour growth was observed between either implantation site.

Characteristics of primary tumour: Staging of ovarian cancer was carried out in accordance with the FIGO classification. The primary tumour stage was FIGO IIIc. Histology showed a dedifferentiated serous ovarian adenocarcinoma with tumour stage pT3c pN0 G3. Abdominal hysterectomy with bilateral salpingo-oophorectomy, omentectomy, removal of the pelvic lymph nodes and exploratory peritoneal excisions was performed. Postoperatively the patient received adjuvant chemotherapy.

Treatments: All treatments were administered intraperitoneally. The protocol is shown in Table 1. Paclitaxel was given at a dose of 26 mg/kg, cisplatin at a dose of 4 mg/kg, vinorelbine at a dose of 20 mg/kg and titanocene dichloride at doses of 30 mg/kg and 40 mg/kg (Table 1).

Table 1. — *Chemotherapy regimens.*

Cytotoxic agent	Dose (mg/kg)	Days of administration	Observation period (days)
Titanocene dichloride	3 x 40	1, 3, 5	17
Titanocene dichloride	3 x 30	1, 3, 5	17
Cisplatin	3 x 4	1, 3, 5	17
Paclitaxel	5 x 26	1, 2, 3, 4, 5	17
Vinorelbine	1 x 20	1	17
NaCl 0.9%		1	17

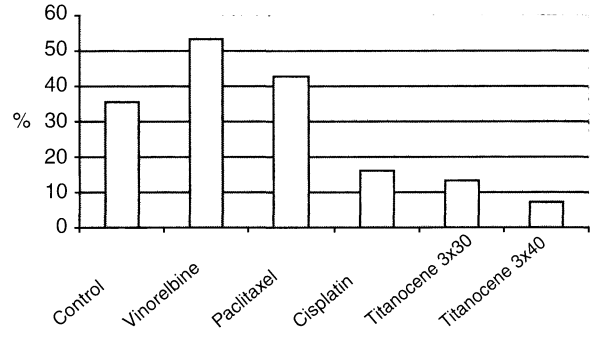

Figure 1. — ki67-positive specimens.

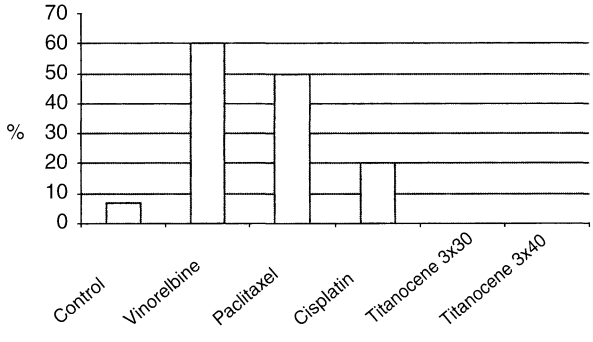

Figure 2. — p53-positive specimens.

Procedures: At the end of the observation period all mice were killed and the tumours were explanted and frozen in fluid nitrogene at a temperature of –220°C. They were cut into frozen sections of 5 µm at a temperature of –20°C and then stored at –80°C. For flow cytometry the frozen sections measured 40 µm and those specimens were stored in fluid nitrogen.

Immunohistochemistry

Before staining the specimens for immunohistochemistry they were fixed in Zamboni solution and washed with PBS. Then the blocking solution (Kaninchennormalserum, Dako, No. X0902) was applied. After removing the blocking solution and washing with PBS the specimens were incubated with the primary monoclonal mouse antihuman antibodies (ki67: Dako, No. M0722 and p53: Oncogene Science, Nr. OP09 and OP09-2) overnight. After washing with PBS the specimens were incubated with the secondary antibody (mouse immunoglobuline, Dako, No. Z0259) for 30 minutes and washed with PBS. Then the specimens were incubated with the peroxidase-anti-peroxidase complex (PAP mouse, Dako, No. P0850) for 30 minutes and afterwards washed with PBS. Then the specimens were incubated with a 3,3'-diaminobenzidine-solution, washed with aqua dest, and stained with haematoxyline. After treatment with alcohol and xylol the specimens were covered with Entellan (Merck).

Flow cytometry

The specimens stored in fluid nitrogene were transformed into a nucleus suspension by the standardized method described by Vindelov *et al.* [45]. For the flow cytometric measurements a FACScan cytometer by Becton & Dickinson was used. After treatment with an Argon laser (λ = 488 nm) fluorescence was measured with a photo multiplier. The cell cycle phase was determined with a special software (Cellfit, Becton & Dickinson). Calibration of the system was performed by diploid chicken erythocytes. The S-phase-fraction was determined by the "RFIT"-method for euploid cells.

Statistics

Statistical analysis was performed by ANOVA (analysis of variance).

Results

The intensity of immunohistochemical staining for ki67 and p53 was generally weak in all specimens. Therefore the specimens were divided into two groups with either positive or negative staining.

The absolute number of ki67-positive specimens was too small in the different groups to perform statistical analysis; a trend towards a correlation between change in tumour volume and expression of ki67 could not be shown. Regarding the treatment groups, the vinorelbine-group showed the highest percentage (53.3%) and the titanocene dichloride-3x40 mg/kg-group the lowest percentage (7.1%) of ki67-positive specimens, whereas in the control group 35.7% of the specimens were positively stained for ki67 (Figure 1). Because of the small absolute numbers of ki67 positively stained specimens in each group statistical analysis could not be performed.

Results for the expression of p53 were similar. The absolute number of p53-positive specimens was too small for a statistical analysis, and no trend towards a correlation between the expression of p53 and change in tumour volume could be shown. The vinorelbine-group had the highest percentage of p53-positive specimens (60%), in both titanocene-groups no specimen showed a positive staining for p53 and in the control group 7.1% of the specimens were positively stained for p53 (Figure 2), also without statistical relevance because of the small absolute numbers.

The mean S-phase-fraction was 14.48% (SD ± 3.98); no statistically significant correlation between S-phase-fraction and expression of p53 ($p = 0.883$) or of ki67 ($p = 0.351$) could be shown. The change of tumour volume was independent of the results for the S-phase-fraction.

Discussion

Xenografting of human tumours to nude mice is an in vivo model for preclinical investigation of cytostatic agents, since human tumours preserve their drug susceptibility and histologic reactivity after transplantation to athymic mice [46, 47]. It could be demonstrated that there is a correlation between the preclinical response data in nude mice and the clinical results with the same drugs [48, 49].

In previous studies it could be shown that titanocene dichloride, vinorelbine, paclitaxel and cisplatin had an antitumour effect on human ovarian carcinoma xenotransplanted to nude mice [50, 51]. To investigate the role of markers of proliferation and the tumour suppressor gene p53 as markers for the activity of antineoplastic

agents we used the same material as in the previous studies, which already showed a reduction in tumour size.

Several studies have been published studies regarding p53 as prognostic marker in ovarian cancer with differing results, postulating either a role of p53 as a marker for a poor prognosis [25-29] or a lacking role as an independent prognostic factor [30-34]. Other investigations could demonstrate a role of p53 as a marker for a lacking sensitivity of ovarian cancer to platinum-based chemotherapy [35-37]. These results are not necessarily contradictory to our findings since we measured p53 after therapy, whereas in the other studies p53 was measured before therapy. The tumours in our study had shown a response to the cytostatic treatment with different cytostatic agents, but there was no correlation between tumour size after therapy and the expression of p53. No statistically significant difference could be detected between the different treatment protocols although the response measured by tumour volume was statistically different between the groups. Thus a role of p53 as a marker for the antineoplastic activity of a cytostatic agent could not be shown in the xenografted nude mice model. These results do not interfere with a possible role of p53 as a prognostic marker for ovarian cancer.

The markers of proliferation ki67 and S-phase-fraction have also been thought to be prognostic markers in ovarian cancer [38, 39] and predictive markers for the response to platinum-based chemotherapy. In a study measuring ki67 at initial surgery and after platinum-based chemotherapy at the time of second-look surgery for ovarian cancer, a reduction of the expression of ki67 could be shown in the responders to chemotherapy [37]. In our study the expression of ki67 did not correlate with the reduction of tumour volume in the xenografted nude mice model. S-phase-fraction has been described to be a predictive marker for the response to platinum-based chemotherapy [43], whereas its role as a prognostic marker in ovarian cancer has been questioned by recent results [44]. We did not find a correlation between S-phase-fraction and tumour volume reduction or the treatment with different cytostatic agents. A role of the markers of proliferation as markers for the activity of chemotherapy could not be shown in this xenografted nude mice model for ovarian cancer.

Conclusion

Although, as previously published, a significant difference of tumour volume change occurred between the treatment groups, in this study we could not find a correlation between this change of tumour volume and the expression of p53 or ki67. The absolute number of p53- and ki67-positive staining specimens was too small for statistical analysis, therefore the relevance of the differences between the different treatment groups and the control remains unclear. The results for the S-phase-fraction showed no correlation between the change of tumour volume, different treatment protocols or p53- and ki67-staining. Our findings contribute to the controversy of the influence of chemotherapy on the expression of proliferation markers and p53. The lacking correlation between the expression of p53, ki67 and S-phase with the change in tumour volume due to chemotherapy in our model should be subject to further investigations about the role of p53, ki67 and S-phase-fraction as markers for the activity of antineoplastic agents.

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Address reprint requests to:
H.C. KOLBERG, M.D.
Leitender Oberarzt
Klinik für Frauenheikunde
und Geburtshilfe
Universitätsklinikum Schleswig-Holstein, Campus Lübeck
Ratzeburger Allee 160
D-23538 Lübeck (Germany)