Activated p38-MAPK and Gemcitabine Sensitivity in Recurrent Ovarian Cancer

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Abstract. *Background: Our study aimed to investigate if p38-MAPK determined in primary ovarian cancer can serve as a predictive marker for sensitivity to gemcitabine treatment in recurrent disease. Materials and Methods: Activated (phosphorylated) p38-MAPK was immunohistochemically assessed in paraffin-embedded tumors obtained at primary debulking surgery from 45 patients treated with gemcitabine for platinum-resistant recurrence. The value of activated p38-MAPK in predicting sensitivity to gemcitabine treatment was statistically evaluated. Results: Activated p38-MAPK was demonstrated in all healthy ovaries and all ovarian carcinomas examined. In controls, the median histological score (H-score) for activated p38-MAPK staining was 200, while in ovarian cancer the median H-score was 100. Activity of p38-MAPK in ovarian cancer tissue was not associated with overall response or survival after gemcitabine chemotherapy. Conclusion: P38-MAPK activity, determined by immunohistochemistry in ovarian cancer specimens obtained at primary diagnosis, cannot serve as a predictive marker for sensitivity to gemcitabine treatment in platinum-resistant disease.*

Ovarian cancer remains the gynecological malignancy with the highest mortality (1, 2). Although most patients respond to primary treatment consisting of debulking surgery and a platinum-based chemotherapy, the majority of patients experience a relapse within two years, and over 50% of the patients with initially advanced ovarian cancer develop platinum resistance (3). In this situation, other cytostatic agents, including gemcitabine, are used as salvage treatment. However, crossresistance to most of the available drugs is frequently observed and response rates to gemcitabine are only up to 22% when used as single agent and up to 33% in combination with other chemotherapeutic drugs (4, 5). Gemcitabine (2'-2'-difluorodeoxycytidine) is a nucleoside analog and inhibits DNA synthesis by causing strand breaks after integration into the DNA as a triphosphate (6). It is characterized by various intracellular targets, self-potentiation and relatively low hematological- and non-hematological toxicity. The most studied mechanism of resistance to gemcitabine is a deficiency of the deoxycytidine kinase, resulting in a lower activation status (6). But also a change in the expression or activity of proteins of intracellular signalling pathways which are involved in the mechanisms of action of gemcitabine, such as p38-mitogen-activated protein kinase (p38-MAPK), can cause resistance to gemcitabine (7, 8).

p38-MAPK belongs to a family of evolutionarily highly conserved enzymes, connecting cell surface signals to critical regulatory targets in the cytoplasm and nucleus of cells. Mammals express three subfamilies of MAPKs, extracellular signal-related kinases (ERKs), c-Jun *N*-terminal kinases (JNKs) and p38-MAPK. p38-MAPK is activated by various stress-related stimuli in a four-level cascade through phosphorylation and causes a variety of biological effects including cytokine secretion and apoptosis. However, depending on cell types and conditions, it can, on the other hand, also lead to cell growth and differentiation (9-17). The dynamic balance between the various MAPKs determines whether a cell develops towards proliferation or apoptosis (16, 18, 19).

Habiro *et al.* (7) showed that p38-MAPK plays an important role in gemcitabine-induced apoptosis in human carcinoma cells of the pancreas. Gemcitabine specifically augmented phosphorylation, and thus activity, of p38-MAPK in these cells. A specific inhibitor of p38-MAPK, SB203580, was able to inhibit gemcitabine-induced apoptosis. The present retrospective study aimed to investigate whether the activation status of p38-MAPK, determined immunohistochemically in ovarian cancer samples obtained from primary debulking surgery, can serve as a predictor of gemcitabine sensitivity in recurrent disease.

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Materials and Methods

Patients. Our collective included 45 patients with histologically confirmed epithelial ovarian cancer who received gemcitabine as treatment in second- to fourth-line chemotherapy. All patients were monitored within the outpatient follow-up program at the Department of Obstetrics and Gynecology of Innsbruck University Hospital, and the median observation period for included patients was 33 months. While 64% of the patients received gemcitabine as a monotherapy, gemcitabine was administered in combination with either liposomal doxorubicin, treosulfan or topotecan in 36%. Clinicopathological characteristics are summarized in Table I.

Response evaluation. For response evaluation CA125 levels were analysed according to the criteria of Rustin *et al*. (20). Using the 50% response definition and the 25% progression definition, computed tomography, and sonography were analysed according to the RECIST criteria (21). Response evaluation was performed after three cycles of gemcitabine.

Immunohistochemistry. Staining for activated (dually phosphorylated) p38-MAPK was performed using the Envision method (Dako, Vienna, Austria) on formalin-fixed, paraffin-embedded sections of ovarian carcinomas collected during primary debulking surgery (n=45). As controls, sections from normal surface epithelium of the ovary or the fallopian tube were used. Briefly, sections were deparaffinized and hydrated in the usual manner. For antigen retrieval, sections were put into 10 mmol citrate buffer (pH 6) in a pre-heated waterbath (98°C) for 40 minutes. Automated immunohistochemical procedures were performed using a Dako Autostainer (Dako, Vienna, Austria). Endogenous peroxidase activity was blocked by a 5-minute treatment with Chemmate Peroxidase Blocking Solution (Dako). Slides were incubated for 30 minutes with the primary antibody [phospho-p38 MAPK (Thr180/Tyr182) (12F8) Rabbit mab, Cell Signaling, New England Biolabs GmbH, Frankfurt am Main, Germany], diluted 1:50 in Chemmate Antibody Diluent (Dako). Immunostaining was accomplished using Chemmate Dako Envision™ K5007 for 30 minutes and diaminobenzidine (Dako) for 10 minutes. Slides were counterstained with hematoxylin, dehydrated and mounted with Histokitt (Assistant-Histokitt, Germany). Positive controls consisted of samples of serous ovarian cancer, in which immunoreactivity for the studied antigen was previously demonstrated. For negative controls, the primary antibody was omitted, while all other incubation steps were identical.

Evaluation of immunostaining. All sections were independently evaluated by two experienced histopathologists from the Department of Obstetrics and Gynecology of Innsbruck University Hospital who were blinded to the patients' clinical course.

Results from immunohistochemistry were described in a histological score (H-score) resulting from the number of cells with activated p38-MAPK (0-100% of cells) multiplied by the intensity of staining (1=low, 2=medium and 3=high). Thus, the lowest possible H-score was 0 and the highest possible H-score was 300.

Statistical analysis. Differences in median progression-free survival (PFS) and overall survival (OS) between the gemcitabine monotherapy and combination therapy groups were assessed by Mann-Whitney *U*-test. Evaluation for possible associations between p38-MAPK activity and response to gemcitabine was carried out using the Kruskal-Wallis H test, dividing patients into three groups according to their estimated H-score. Survival analyses were conducted with the Kaplan-Meier method. Patients were dichotomized according to the estimated H-score and the median was used as a cut-off. Differences between groups were determined with the log-rank test. Statistical significance was defined as $p<0.05$. SPSS for Windows 15.0 software (SPSS, Inc., Chicago, USA) was used for all analyses.

Table I. *Clinicopathological characteristics*.

Characteristic	% of patients
Median age (range)	61 (26-79) years
Histological type	
Serous	72.7
Mucinous	11.4
Other	15.9
FIGO stage	
I, II	6.6
III	82.3
IV	11.1
Histopathological grading	
G1	0.0
G2	58.9
G3	41.1
Residual disease	
None	16.7
<2 cm	19.0
>2 cm	64.3
Gemcitabine therapy	
Monotherapy	64.4
Combination therapy	35.6
Gemcitabine administration	
2nd line	37.8
3rd line	26.7
4th line	35.5
Number of cycles of gemcitabine	
< 3 cycles	31.1
3-6 cycles	60.0
>6 cycles	8.9
Median time to start of gemcitabine (Q1-Q3)	18.0 (11.5-29.0) months

FIGO: Fédération Internationale de Gynécologie et d'Obstétrique; G: grade; Q1-Q3: interquartile range.

Results

Response to gemcitabine treatment. In our patient selection, the overall response to gemcitabine therapy was 17% for gemcitabine monotherapy and 19% for gemcitabine in combination with other chemotherapeutic agents. The difference in median PFS and median OS since the start of gemcitabine therapy between the two groups was not statistically significant (Table II).

Immunohistochemistry findings. In ovarian cancer as well as in controls, activated (phosphorylated) p38-MAPK was found in all samples. Staining was present predominantly in the nucleus of the epithelial cells and also in the cytoplasm in some samples, but to a lesser extent. The stroma showed

Table II. *Response to gemcitabine chemotherapy.*

Characteristic	Monotherapy (%)	Combination therapy (%)
Response		
PD	62.1	43.8
SD	20.7	37.5
PR	13.8	12.5
CR	3.4	6.3
PFS (months)		
median (Q1-Q3)	3 (1-4)	4.5 (1-10.7)
OS (months)		
median (Q1-Q3)	9 (5-14.5)	16 (4.5-22.5)

PD: progressive disease; SD: stable disease; PR: partial response; CR: complete response; PFS: progression-free survival; OS: overall survival since start of gemcitabine therapy; Q1-Q3: interquartile range. Differences in PFS (p=0.188) and OS (p=0.362) between gemcitabine monotherapy and combination therapy were not significant (Mann-Whitney U-test).

Table III. *Immunohistochemistry of activated p38-MAPK in ovarian cancer samples and controls.*

	H-score		
Characteristic	<100	101-200	>200
% Samples	51	38	11
% Controls	0	100	0
Median H-score (Q1-Q3) of samples		100 (60-160)	
Median H-score of controls		200	
Response to gemcitabine (% of patients)			
PD (n=25)	52	44	4
SD (n=12)	42	42	16
PR (n=6)	83	0	17
CR (n=2)	0	50	50

H-score: histological score; PD: progressive disease; SD: stable disease; PR: partial response; CR: complete response; n: number of patients. Kruskal-Wallis H test: p=0.927.

almost no staining. In control sections (Figure 1A and B), 100% of epithelial cells of the fallopian tube and 100% of cells of the ovarian surface epithelium showed moderate activation of p38-MAPK, giving a median H-score of 200. In ovarian cancer tissue (Figure 1C and D), the proportion of cells with phosphorylated p38-MAPK ranged from 20%-90%. The intensity of staining in p38-MAPK-positive cells was low, moderate or high, resulting in an H-score ranging from 20-270 and a median H-score of 100 (Table III).

p38-MAPK activity and response to gemcitabine. There was no association between p38-MAPK activity in ovarian cancer samples obtained from primary debulking surgery and response to gemcitabine in relapsed disease (Table III). Moreover, neither PFS nor OS since the start of gemcitabine therapy showed any association with the activation status of p38-MAPK (Figure 2). Furthermore, no associations were revealed between the activation status of p38-MAPK in ovarian cancer tissue specimens and patient age, FIGO stage, tumor grade, histological type or residual disease.

Discussion

In the situation of platinum resistance, when the majority of ovarian carcinomas exhibit cross resistance to most available cytotoxic agents, choosing the right chemotherapy remains a great challenge. In this palliative setting, gemcitabine is a well-studied and well-tolerated therapeutic option (4, 22). Resistance to gemcitabine may result from failure of the apoptotic pathways that are activated in response to chemotherapy.

It was shown that gemcitabine specifically activates p38-MAPK in pancreatic cancer cells, leading to apoptosis (7). An interesting observation was that cancer cells which exhibited greater p38-MAPK activity showed higher sensitivity to gemcitabine than did those with low p38-MAPK activity (7).

The aim of our study was to determine the activation status of p38-MAPK by immunohistochemistry in ovarian cancer samples obtained at the time of primary debulking surgery and to investigate whether phosphorylated p38-MAPK can serve as a predictor for gemcitabine sensitivity.

In control samples as well as in ovarian cancer specimens, activated p38-MAPK was present predominantly in the nucleus and less in the cytoplasm of cells. This is explained by the fact that activated p38-MAPK not only leads to phosphorylation of a variety of cytosolic substrates, but is also translocated to the nucleus where it activates a number of transcription factors and DNA-binding proteins, resulting in a variety of biological effects, including apoptosis (12, 14, 16, 23, 24). In controls, 100% of epithelial cells showed moderate intensity of staining for activated p38-MAPK, thus giving an H-score of 200. In ovarian cancer tissue samples, the percentage of cancerous cells with phosphorylated p38-MAPK ranged from 20%-90% with mostly moderate but also weak or high staining intensity, giving an H-score range from 20-270 with a median of 100. This stands in contrast to the results of Wong *et al*. (25), who examined MAPK expression in a culture model for ovarian carcinogenesis and found that p38-MAPK expression was markedly elevated during neoplastic progression. In addition, gastric cancer has been shown to have increased p38-MAPK activity as compared to adjacent normal mucosa (26). Potentiation of the p38-MAPK pathway in oncogenically transformed cells renders them more susceptible for genotoxic stress than nontransformed cells (7, 27, 28). On the other hand, Iyoda *et al*. (29) reported less p38-MAPK activity in hepatocellular

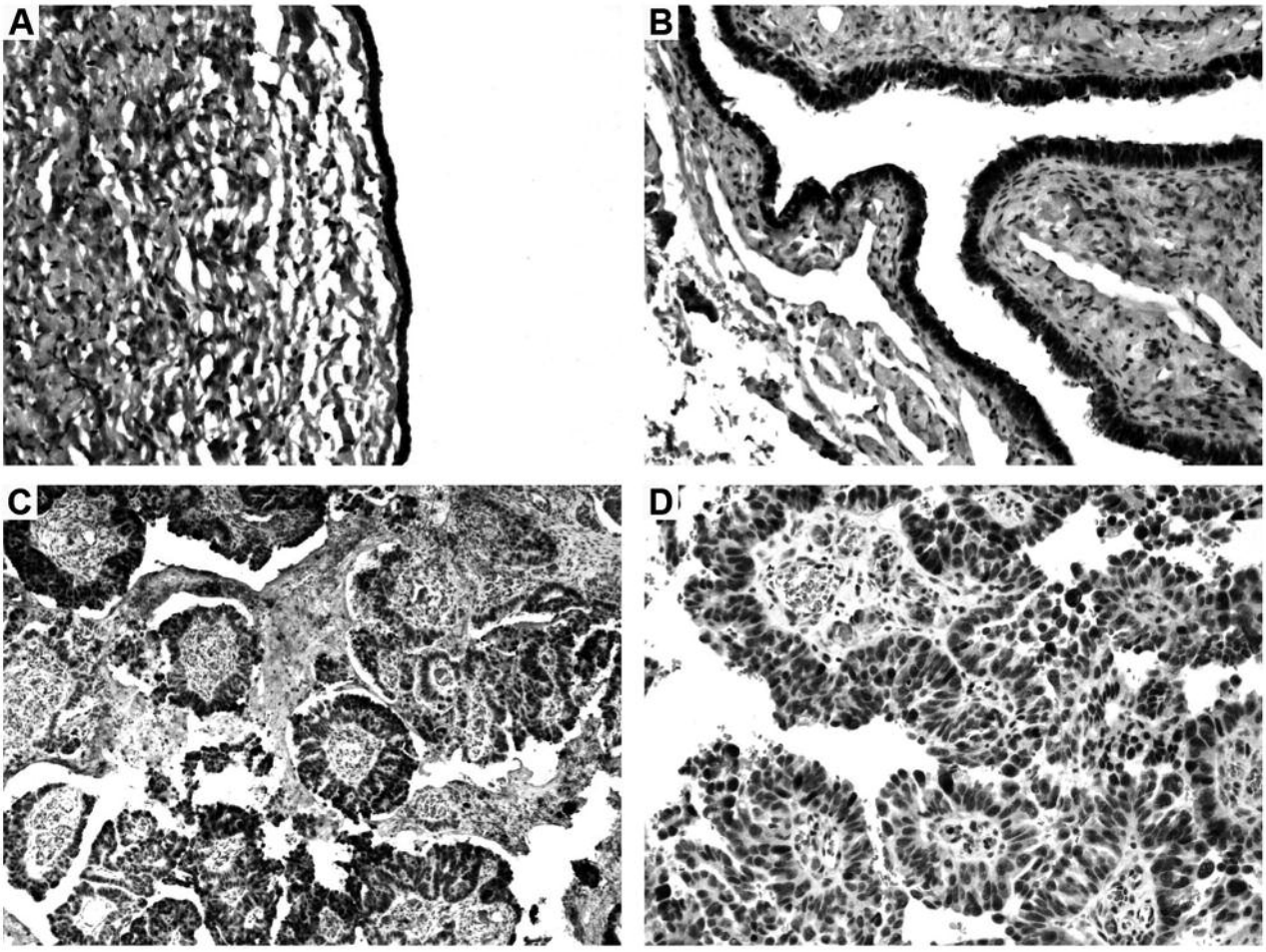

Figure 1. *Immunohistochemistry of ovarian carcinoma samples and controls. Activated p38-MAPK was found in 100% of cells of normal surface epithelium of the ovary (A) and in 100% of normal epithelial cells of the fallopian tube (B) with moderate intensity of staining (H-score: 200). In ovarian cancer samples obtained from primary debulking surgery (C, D), activated p38-MAPK was present in 20% -90% of cancerous cells with mostly moderate, but also high and low intensity of staining (H-score: 20-270). In all controls and ovarian cancer samples, p38-MAPK activity was found predominantly in the nucleus and less in the cytoplasm of epithelial cells, while the stroma showed almost no activated p38-MAPK (original magnification ×40-100).*

carcinoma *versus* normal liver tissue specimens. The loss of p38-MAPK activation in mutant cells has been associated with increased tumorigenesis and defects in growth arrest (16, 30-33). Furthermore, a markedly shorter duration of p38-MAPK activation in chemoresistant cancer cells seems to play a crucial role in enhanced cell survival (28, 34).

Our study found no association between p38-MAPK activation status and FIGO stage, tumor grading, residual disease or patient age. This agrees in part with the data published by Givant-Horwitz *et al.* (35), who examined cells from 64 fresh frozen pleural or peritoneal effusions from serous ovarian cancer patients and found no association between phosphorylated p38-MAPK and FIGO stage or tumor grade. However, in their series, phosphorylated p38-MAPK correlated with younger patient age.

Response rates and survival data for gemcitabine treatment in our patient selection are consistent with the data reported by others (4, 5, 22, 36). Our study found that there was no association between p38-MAPK activity in ovarian cancer samples from primary debulking surgery and sensitivity to gemcitabine chemotherapy, progression-free survival or overall survival since the start of gemcitabine therapy. To our knowledge, there are no other reports on p38-MAPK activity in ovarian cancer samples and its predictive value for sensitivity for gemcitabine treatment in the literature. A possible explanation for the lack of correlation between p38-MAPK activity in ovarian cancer samples and response to gemcitabine or survival data in our patient selection could be the long period between sample collection and gemcitabine administration. Since genetic instabilities evolve in the course

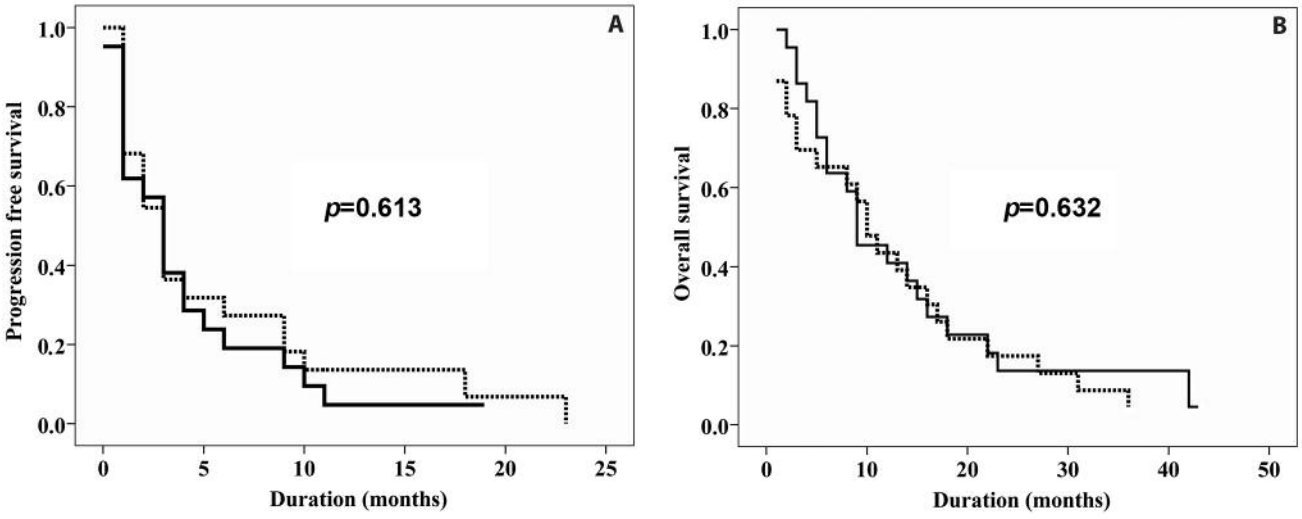

Figure 2. *Association between histological score (H-score) for phosphorylated p38-MAPK, immunohistochemically determined in samples from primary debulking surgery and progression-free survival (A) and overall survival (B) since the start of gemcitabine therapy. Dashed line: patients with an H-score below the median of 100 (n=23), solid line: patients with an H-score >100 (n=22).*

of cancer progression and exposure to various cytotoxic drugs, the activity of p38-MAPK in samples obtained during primary surgery may be markedly different from that seen in recurrent tumors at the time gemcitabine treatment was initiated.

Conclusion

Although we found no associations between the activation status of p38-MAPK in tissue samples of ovarian cancer patients obtained from primary debulking surgery and response to gemcitabine in recurrent disease, we cannot exclude the possibility that p38-MAPK could represent a valuable marker for predicting gemcitabine responsiveness. Since cancerous cells exhibit genetic instability that is further enhanced by various chemotherapeutics, ovarian cancer samples collected directly before gemcitabine administration would be necessary in order to obtain a valid picture of the actual activation status of p38-MAPK.

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