Heterogeneous Expression of Serine Protease Inhibitor Maspin in Ovarian Cancer

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Abstract. *Ovarian cancer (OC) is a disease with poor prognosis, and molecular markers are needed to improve understanding of disease progression and resultant treatment. Only limited data concerning the expression of maspin, a serine protease inhibitor, in ovarian cancer (OC) are available. This study investigates the prognostic value of maspin expression (ME) in various OC cell lines and clinical tissue specimens from OC patients. Patients and Methods: Tumour purified mouse anti-human maspin monoclonal antibody was applied to tissue specimens from 87 OC patients. ME was recorded by an immunoreactive score, which was correlated with grading, stage, histopathological subtypes and overall survival. Additionally ME was evaluated in established ovarian cancer cell lines (HEY, SKOV3, OVCAR3/8) and paclitaxel- and docetaxel-resistant HEY cells by QRT-PCR. Results: There was significant correlation between cytoplasmatic ME and overall survival ($p<0.05$). OC patients with high levels of ME had a median survival of 28 vs. 57 months for those with low levels. Significant differential ME was detected between benign, borderline ovarian lesions and OC, as well as among different tumour gradings. Normal ovarian epithelial cells expressed less maspin than ovarian cancer cells as measured by QRT-PCR. Docetaxel- and paclitaxel-resistant ovarian cell lines showed an even higher level of ME, suggesting an unfavourable role of ME in OC cell lines. Conclusion: Maspin is expressed differentially in OC, and low expression levels of maspin are correlated with a longer survival.*

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Ovarian cancer (OC) accounts for around 4.5% of all female cancers, and around 8,000 new cases are diagnosed in Germany each year (11). Because of the absence of early symptoms, OC is often (>75%) diagnosed at an advanced stage (FIGO III or IV) with a poor prognosis. The five-year survival rate for stage III is 25%, whereas the five-year survival rate for stage I is 70-80% (1). Standard treatment is based on platinum and taxane-containing chemotherapy and cytoreductive surgery (2). There is neither a suitable screening tool nor a biological prognostic marker available for OC. New markers which would allow a prognostic evaluation of the disease in terms of overall survival or prediction of chemotherapy response could be used to optimise therapy in improve the benefit from chemotherapy.

The clinical relevance of the serine protease inhibitor maspin in human cancer has been extensively investigated since its discovery in 1994 (25). In breast, maspin is highly expressed in normal epithelial cells, especially in myoepithelial cells, down-regulated in invasive and metastatic breast carcinoma cells and correlated with an unfavourable prognosis in breast cancer (3). In oral squamous carcinoma, maspin expression (ME) correlates with better prognoses (4). In prostate cancer, loss of ME correlates with higher tumour stages and increasing histological dedifferentiation (5). In contrast, prostate cancer patients who retain ME have a significantly longer recurrence-free survival compared to prostate cancer patients whose ME is lost on tumour progression (6). ME in both breast and prostate epithelial cells may be directly activated by tumour suppressor p53 (7), or inactivated by a negative hormone responsive element recognized by androgen receptor (8). Although there is growing insight in the molecular role of maspin in general and also in OC, there is little specific data concerning the ME in OC.

This study evaluated the value of the cytoplasmic and nuclear expression patterns of maspin in OC. In order to investigate the prognostic value of maspin in OC patients, the correlation between ME and clinical parameters was

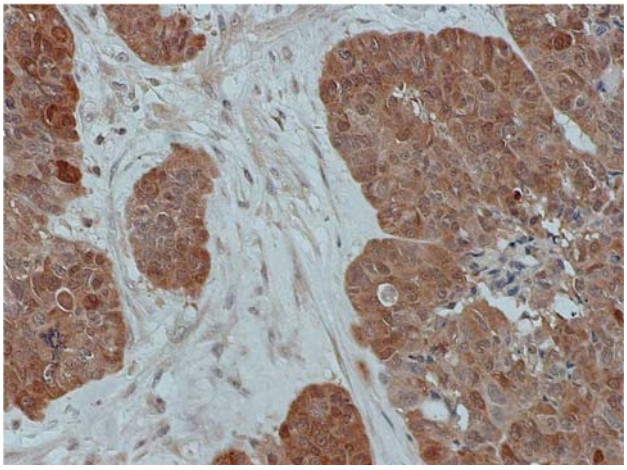

Figure 1. *Serous/papillary ovarian cancer tissue (×200) showing strong cytoplasmic maspin staining (IRS>4) and positive maspin staining in the nucleus (IRS>0).*

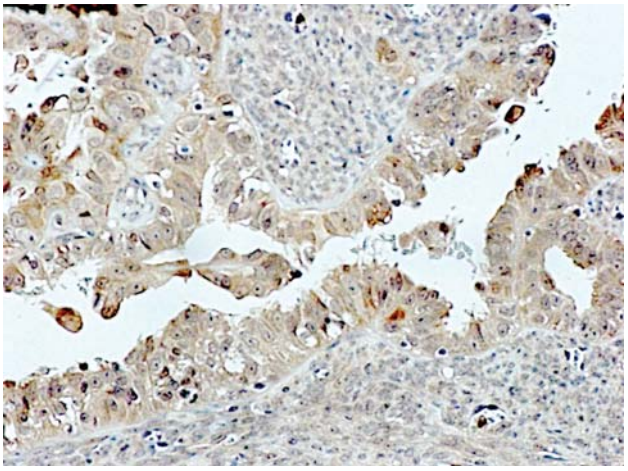

Figure 2. *Adenocarcinoma of the ovary (×200) with no nuclear maspin staining (IRS=0) and moderate cytoplasmic staining (IRS 1-4).*

investigated. Response rates to chemotherapeutic regimens were also analysed in order to judge the predictive value of maspin in the response to standard chemotherapy of OC.

Patients and Methods

Ovarian carcinoma patients. Eighty-seven patients who had undergone surgery between 1993 and 2002 in the Department of Gynecology and Obstetrics, University Hospital in Kiel, Germany, were included in the study: 73 patients were diagnosed with an invasive tumour, 6 patients with a low malignant potential (LMP) tumour and 8 patients with a benign tumour. Mean age of first diagnosis was 62.3 years. Of 79 patients with malignant or LMP tumours, 12 (15.2%) patients were diagnosed with early stage disease (FIGO I/IIa) and 66 (83.5%) patients with advanced stage disease (FIGO IIb/III/IV). The histological subtype was mainly serous (63/79 invasive cancers). Sixty-seven patients received platinum based chemotherapy, with a response rate of 59.7% (40/67), representing a diminished tumour mass or total disappearance of detectable tumour burden after chemotherapy. Eleven patients (16.4%) showed a partial and 29 patients (43.3%) a complete remission.

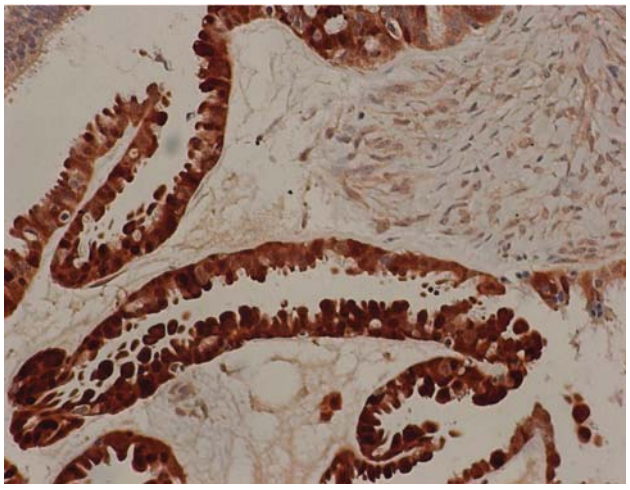

Figure 3. *Ovarian adenocystoma (×200) showing no nuclear colouration (IRS=0) but strongly stained cytoplasm (IRS >4).*

Immunohistochemistry. Paraffin-embedded OC tissue samples, obtained from surgical primary tumor specimens, were sectioned at 4 μm and mounted on slides (Menzel GmbH & CoKG, Braun-schweig, Germany). The slides were deparaffinised using xylene and rehydrated in graded alcohols (100%, 96%, 70%). A microwave-based antigen retrieval with 0.05 M Tris buffer (pH 9.0) for 15 minutes (600 W) was performed. After cooling for 20 minutes, endo-genous peroxidase was blocked with Peroxidase Blocking Reagent (DAKO, Glostrup, Denmark). Thereafter slides were rinsed in washing buffer (pH 7.4). The sections were then incubated overnight at 4˚C with anti-human maspin antibody (Purified Mouse Anti-Human Maspin Monoclonal Antibody, clone: G167-70; Pharmingen International, San Diego, CA, USA), and diluted 1:75 in a wet chamber. Upon a further rinsing step, Dako Real Envision Detection System (DAKO) was used to visualise the antibody binding. After washing with distilled water, slides were counterstained with haematoxylin (Hämalaunsolution, C. Roth GmbH & CoKG, Karlsruhe, Germany) for 10 minutes and placed for 30 minutes under dripping tap water. Negative controls were performed by omitting the primary antibody and normal mammary gland tissue was used as a positive control in every assay. All of the samples were reviewed by a specialised gynaecopathologist. ME was determined by appraising the percentage of stained tumour cells and the staining intensity based on the immunoreactive score according to Remmele and Stegner (9,10). The percentage of positive cells was rated as follows: no positive cells: 0 points, <10% positive cells: 1 point, 10-50% positive cells: 2 points, 51-80% positive cells: 3 points, >80% positive cells: 4 points. The staining intensity was rated as follows: no staining: 0 points, weak intensity: 1 point, moderate intensity: 2 points, strong intensity: 3 points. Points for percentage of positive cells and expression were multiplied, with a possible maximum of 12 points (Figures 1-3).

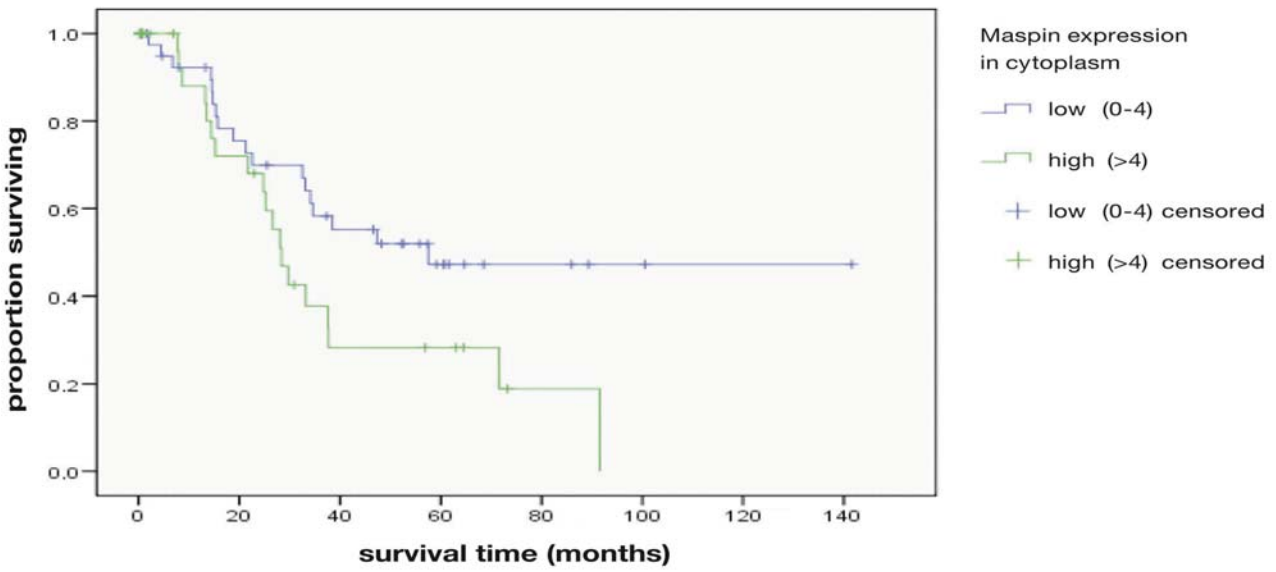

Figure 4. *Overall survival according to cytoplasmic maspin expression identified by Kaplan–Meier model in ovarian cancer patients.*

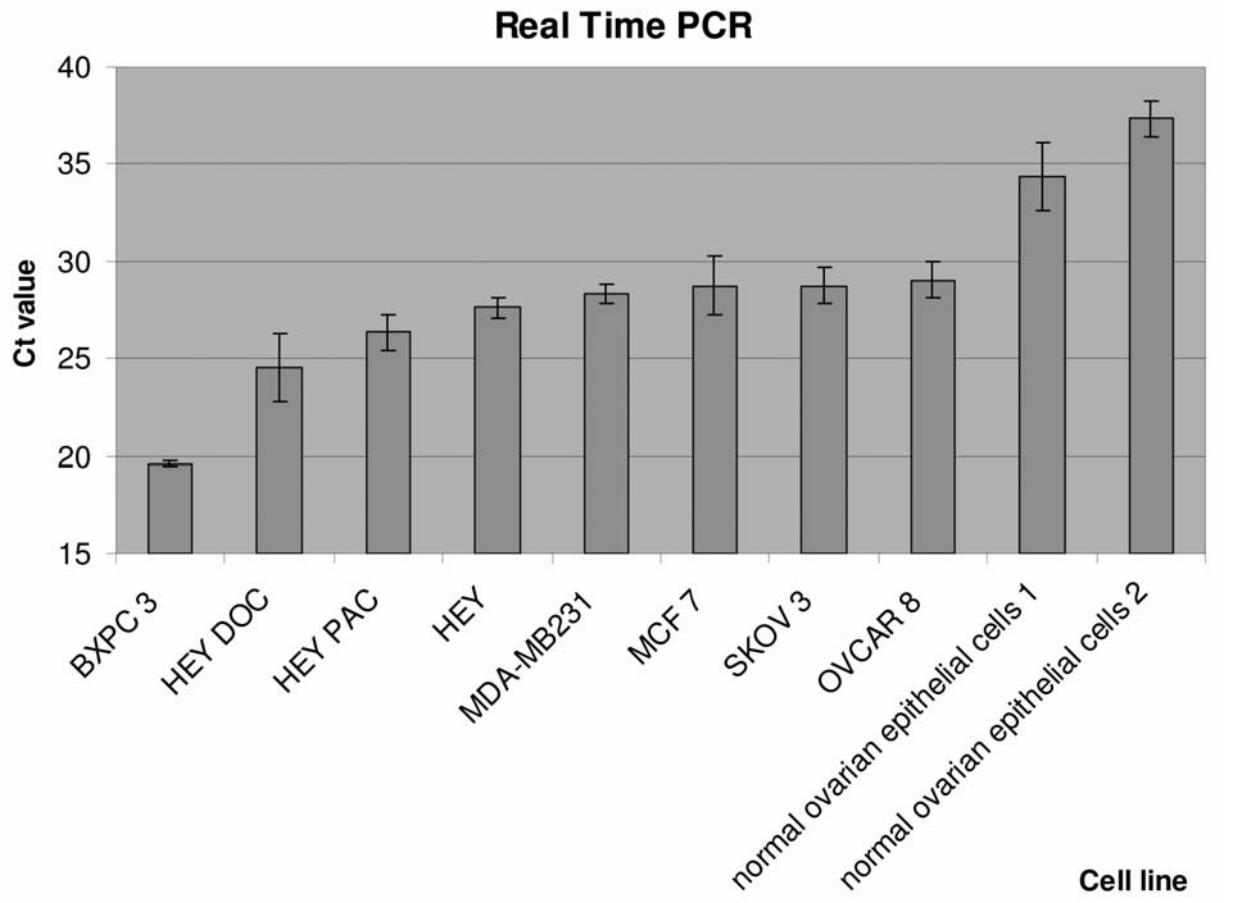

Figure 5. *QRT-PCR Ct values for maspin expression of selected cell lines. Normal ovarian epithelial cells derived from clinical specimens (1 and 2). Cancer cell lines: BXPC3 (pancreatic cancer), HEY, OVCAR8, SKOV3 (ovarian cancer), MDA-MB231, MCF7 (breast cancer), HEY DOC (docetaxel-resistant HEY cells), HEY PAC (paclitaxel-resistant HEY cells).*

Cell culture. The OC cell lines used in this study were OVCAR 3, OVCAR 8, SKOV 3, HEY and HEY cells with selective resistance against docetaxel and paclitaxel as described elsewhere (11). Furthermore, the breast cancer cell lines MDA-MB 231 and MCF 7, the pancreatic cancer cell line B×PC 3 and normal ovarian epithelial cells were used as controls. To obtain RNA for QRT-PCR these cells were cultivated, supplied and propagated *in vitro* by serial passage in RPMI 1640 (Biochrom AG, Berlin, Germany) supplemented with 10% foetal bovine serum and 60 U/ml penicillin and streptomycin (Biochrom AG, Berlin, Germany).

Real-time PCR. Total cellular RNA was isolated using the RNeasy mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Total RNA was converted into cDNA by using Quantiscript Reverse Transcriptase (Qiagen). Each PCR reaction consisted of 2 μl (200 ng) of cDNA added to 12,5 μl of QuantiTect SYBR Green PCR Master Mix (Qiagen, Valencia, CA, USA), 1.25 μl of each gene-specific maspin primer (forward and reverse; QuantiTect Primer Assays; Qiagen) and 8 μl RNase-free water, giving a total volume of 25 μl. The PCR conditions were 95˚C for 15 min, followed by 40 cycles at 94˚C for 15 s, 55˚C for 30 s and 72˚C for 30 s. Real-time PCR was conducted using the i-cycler from BIO RAD, Hercules CA, USA.

In order to evaluate the differences in cell line, raw Ct values for ME were compared. The Ct value delineates the 'threshold cycle' and is the cycle at which a significant increase in fluorescent signal is first detected (12). The more target product is available, the earlier this fluorescence level will be reached and the lower the Ct value will be. Therefore, low Ct values indicate high ME in the cell lines analyzsd (13) (Figure 5).

Statistical analysis. Statistical analyses were performed using SPSS version 15.0 (SPSS Inc, Chicago, IL, USA). The correlation between ME and other variables was evaluated using the Chi-square test. Survival analysis was generated using the Kaplan-Meier model. Cox regression analysis was performed for multivariate analysis and a statistical significance was defined as a probability value <0.05 (Figure 4).

Table I. *Descriptive statistics and histopathological grading of patients included in this study.*

Variable		Number	%
Tumour	Total	87	100
	OVCA	73	83.9
	LMP	6	6.9
	Benign	8	9.2
FIGO stage	Total	79	100
	I	12	15.1
	II	4	5.1
	III	47	59.5
	IV	15	19
	Missing data	1	1.3
Histological subtype	Total	87	100
	Serous	63	72.4
	Endometrioid	9	10.3
	Other malignant	7	8.1
	Benign	8	9.2
Tumour grade	Total	79	100
	1	10	12.7
	2	32	40.5
	3	37	46.8
Platinum based	Total	67	100
Chemotherapy	<6	18	26.9
Relapse-free interval	6-12	11	16.4
(months)	>12	29	43.3
	Missing data	9	13.4
Mean age at time of first diagnosis		62.3 years	

Results

Immunohistochemistry showed that maspin was mostly localized in the cytoplasm. The cytoplasm of 92.4% (73/79) of the malignant tumours (OC+LMP tumours) was maspin positive, whereas nuclear ME was observed in only 30.4% (24/79) of the specimens (Table I). Nine (10.3%) tumours had no maspin staining in either the cytoplasm or the nucleus. Univariate analysis showed no significant correlation between cytoplasmic ME and histological subtype (p=0.4), FIGO stage (p=0.14) and tumour grade (p=0.84). However, a significant correlation was observed between cytoplasmic ME and response to platinum-based chemotherapy (p=0.005). Nuclear ME detected by IHC correlated significantly with tumour grade (p=0.01) but not with FIGO stage (p=0.29), histological subtype (p= 0.34) or response to platinum-based chemotherapy (p=0.73). Furthermore, univariate analysis showed a significant correlation between cytoplasmic ME and survival (p<0.05). OC patients with a high expression of maspin in the cytoplasm had a median survival of 28 month *versus* 57 month for patients with a low expression (Figure 4).

However, multivariate analysis did not verify the results of univariate analysis. The multivariate analysis using the Cox proportional hazards model involved tumour grade, FIGO stage, age, ME and response to platinum-based chemotherapy. Only response to platinum-based chemotherapy showed a significant correlation with survival (p<0,05). Results of real-time PCR show low Ct values for BXPC3 and ovarian cancer cell lines which were made drug resistant for docetaxel and paclitaxel (HEY Doc and HEY PAC) (Table 1). BXPC3 is a pancreatic cancer cell line. A low Ct value indicates a high ME. Univariate analysis demonstrated that OC patients with high ME had a shorter median survival than those with low ME.

Discussion

There are few published data regarding ME in ovarian cancer. Sood *et al*. (16) were the first to study ME in normal and cancerous ovarian cell lines. Like Abd El-Wahed (17), they detected the majority of maspin in the cytoplasm, too. Furthermore, with univariate and multivariate analysis they showed a significant correlation between maspin overexpression and poor survival. The univariate analysis of this study confirmed this correlation, although this result was not supported by multivariate analysis. It was demonstrated that ovarian carcinoma patients with a high ME have a median survival of 28 *versus* 57 months for patients with a low expression (Figure 4). Under multivariate analysis, it was only possible to verify a significant association between survival and response to platinum-based chemotherapy, which is already known (16). The small sample number in this study may be the reason for differences in the results. Both Sood *et al*. and Secord and Lee found no evidence for the association between ME, histological subtype and FIGO stage (16, 18). Sood *et al*. described a significant correlation between ME, tumour grade and ascites, whereas Secord and Lee detected no correlation between ME and tumour grade. In this study, there was no significant correlation between ME and histological subtype, FIGO stage or tumour grade in tumours with ME mainly in the cytoplasm. Only response to platinum-based chemotherapy seemed to be associated with cyto-plasmic ME (Figure 1). However, in tumours with ME mostly in the nucleus there was a significant association between ME and tumour grade but not between ME and histological subtype, FIGO stage or response to platinum-based chemotherapy (Figure 2). Thus, localisation of maspin may play an important role in OC as it does in other tumour entities. In breast cancer, only nuclear maspin staining was significantly associated with good prognostic factors, while cytoplasmic staining was associated with poor prognostic factors. These findings suggest that the presence of maspin in two different compartments of the cell may have different biological and clinical implications. Tsuji *et al*. investigated mucinous borderline tumours in further detail and concluded that cytoplasmic localisation of ME may reflect the presence of intraepithelial carcinoma and stromal microinvasion (19). Microinvasion and angiogenesis are vital for OC progression. The statistical power of this investigation could not support this investigation in tumours of low malignancy or early-stage disease. However, maspin paradoxically promotes invasion and metastasis of OC at higher stages of the disease (20). This phenomenon is still not understood. VEGF expression is very high in various OC subtypes and therefore multiple clinical trials are ongoing investigating antiangiogenic drugs in OC patients. The regulation of angiogenesis within the tumour cell underlies multiple pathways which can be influenced by ME. In this aspect, maspin may play a special paradoxical role in OC. Usually being antiproliferative and antiangiogenic in prostate, lung and breast cancer, ME is positively correlated with VEGFA, C, and D expression and tumour progression in human OC (21). The subcellular localisation of maspin in the nucleus reflects excessive cell proliferation and poor differentiation, whereas cytoplasmic localisation reflects promotion of angiogenesis and therefore possible susceptibility to antiangiogenic drugs. The correlation of cyto-plasmic ME with poor prognosis but good response to platinum-based therapy implies that good response to chemotherapy might not be enough to overcome tumour-induced neoangiogenesis and therefore progression.

Thus, high cytoplasmic ME may be an indicator for the beneficial addition of antivascular therapy in OC in order to increase overall survival. Certainly additional studies are needed in order to profoundly understand this special immunohistological observation in OC. The results of real-time PCR somewhat support this hypothesis (Figure 5). BXPC3 is a pancreatic cancer cell line. The ME in pancreatic cancer is increased (14, 15) therefore a low Ct value is expected. The drug-resistant cell lines also had a low Ct value, which supports the result of the univariate analysis. In OC cell lines, most of the endogenous maspin is cytoplasmic in location. However, wild-type maspin transfected into these cells became localized to the nucleus and was highly effective in blocking the *in vitro* invasive potential of OC cells (16). The taxane-resistant cell lines HEY DOC and HEY PAC did show a higher ME than normal ovarian epithelial cells and slightly higher expression than the non-resistant HEY cell line. The ovarian carcinoma cell lines HEY which were made drug-resistant represent patients who do not respond to chemotherapy and consequently have a worse prognosis. Rose *et al*. showed similar results in 2006 (22). Again, higher ME was linked to unfavourable characteristics of OC cell lines, whereas maspin overexpression in breast cancer is believed to be beneficial (23, 24, 25). Taxane- and platinum-drug resistance is critical to overall survival in OC. It is unknown whether the silencing of ME will be able to overcome this drug resistance.

In conclusion, ME in OC differs from its expression modalities in other tumour entities. Subcellular localisation of ME is an important factor and more detailed studies of ME are needed in order to understand its impact on drug resistance and tumour progression in OC.

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