

## MMP-2 mRNA Expression in Ovarian Cancer Tissues Predicts Patients' Response to Platinum-Taxane Chemotherapy

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**Abstract.** *Background/Aim:* Ovarian cancer is the most frequent cause of death in women among gynecological cancers in Poland. MMP-2 and MMP-9 are frequently dysregulated in cancers and they are considered as potential biomarkers. Our goal was to assess the associations between MMP-2 and MMP-9 mRNA expression, clinicopathological parameters and patients' response to chemotherapy. *Materials and Methods:* We evaluated MMP-2 and MMP-9 mRNA expression in epithelial ovarian cancer (EOC) tissues from 44 untreated patients, four ovarian cancer cell lines, and human skin fibroblasts (HSF). The expression of both MMPs was estimated using qPCR. *Results:* MMP-2 expression was significantly higher ( $p=0.020$ ) in EOCs sensitive to chemotherapy compared to resistant and refractory tumors. The highest MMP-2 expression was found in HSF and MMP-9 expression was the highest in EOCs ( $p<0.001$ ). The expression of neither MMP was significantly associated with patients' overall survival (OS). *Conclusion:* MMP-2 may be engaged in early stages of ovarian carcinogenesis. MMP-2 expression in EOCs may discriminate patients with a favorable response to first line chemotherapy.

Ovarian cancer is still a serious diagnostic and therapeutic problem all over the world. Among gynecological cancers in Europe, ovarian cancer is estimated to be the second most frequently diagnosed cancer with 67,771 new cases (standardized incidence rate 9.5/100,000), and the most often cause of death with 44,576 deaths (standardized mortality

rate 5.1/100,000) in 2018 (1). Because of lack of effective screening tests and early symptoms for most patients, ovarian cancer is diagnosed at an advanced stage, which leads to a short 5-year survival (2). Treatment strategies include cytoreductive surgery and platinum-taxane chemotherapy (3). In spite of good initial response to therapy, within two years most patients relapse and due to inefficient second line chemotherapy most of them die (3, 4). For that reason, there is a need to look for new markers and new targeted therapies.

Matrix metalloproteinases (MMPs) are proteolytic enzymes that are engaged in cancer progression and metastasis (5). MMPs digest extracellular matrix proteins and release active growth factors that facilitate proliferation, tissue invasion and spread of tumor cells. MMPs promote tumor angiogenesis by degradation of capillary basal membranes and detachment of angiogenic cytokines, as VEGF. MMP-2 and MMP-9 are most frequently reported as dysregulated in cancers and they are thought as potential biomarkers. High MMP-2 expression has been found in lung and prostate cancer, where it is associated with poor prognosis. Patients with elevated MMP-9 expression in breast, lung, and prostate cancer show worse survival (5). There are also reports regarding MMP-2 and MMP-9 expression in ovarian cancer but there are still some discrepancies regarding their role in tumorigenesis and their prognostic value. Generally, MMP-9 is more often correlated with poor prognosis compared to MMP-2 in ovarian cancer patients, but most studies regarding both enzymes expressions have been performed on protein level only (6, 7).

The aim of our study was to evaluate MMP-2 and MMP-9 mRNA expressions in epithelial ovarian cancer (EOC) tissues from previously untreated patients and ovarian cancer cell lines, as well as to investigate possible associations with clinicopathological parameters, the response to primary chemotherapy, and patients' survival.

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## Materials and Methods

**Patients' characteristics.** Tumor tissues were obtained during primary surgery before adjuvant therapy from 44 patients with primary EOC (median age=54 years, range=37-85 years), and 5 patients with benign cystadenoma ovarian tumors (median age=65 years, range=48-74 years), who were diagnosed and treated between 2005 and 2010 at the First Department of Oncologic Gynecology and Gynecology, Medical University of Lublin, Poland. EOCs were classified as optimally debulked, when residual disease was not larger than 1 cm (8). Following primary surgery, 35 patients (79.5%) received adjuvant chemotherapy: i) paclitaxel-platinum (n=27), ii) cyclophosphamide-platinum (n=7), or iii) carboplatin alone (n=1), and iv) radiotherapy (n=1). The response of patients to chemotherapy was evaluated according to RECIST criteria version 1.1 (9) and platinum sensitivity of tumors was determined according to Eisenhauer *et al.* (8). Overall survival (OS) of EOC patients was calculated from the date of primary surgery to the date of death or the end of follow-up. The approval for this study was granted by the Research Ethics Committee of the Medical University of Lublin (KE-0254/244/2002 and KE-0254/137/2007).

**Cell culture.** Four ovarian cancer cell lines (TOV-112D, OV-90, OVCAR-3 and Caov-3) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Human skin fibroblasts (HSF) were obtained as a laboratory strain from patients who underwent surgery (approved by Research Ethics Committee of the Medical University of Lublin (KE-0254/298/2015), as described previously (10). All cells were grown as monolayers at 37°C in a humidified air atmosphere with 5% CO<sub>2</sub> in appropriate media with added 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA). TOV-112D and OV-90 cell lines were cultured in a mixture (1:1) of Medium 199 (PAA, Pasching, Austria) and MCDB 105 (Sigma-Aldrich) supplemented with 15% fetal bovine serum (FBS) Gold (PAA). OVCAR-3 cell line was grown in RPMI-1640 medium (Sigma-Aldrich) with added 0.01 mg/ml bovine insulin (Sigma-Aldrich) and 20% FBS Gold (PAA). Caov-3 cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich) modified to contain 1.5 g/l sodium bicarbonate, 0.584 g/l L glutamine, 4.5 g/l glucose, 0.11 g/l sodium pyruvate, and 10% FBS Gold (PAA). HSFs were grown in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% FBS Gold (PAA).

**RNA isolation and quantitative real-time reverse transcription PCR (qPCR).** Total RNA from tissues was isolated using TRI Reagent (Sigma-Aldrich), according to the manufacturer's protocols, and was subsequently digested using DNase I (Fermentas, Vilnius, Lithuania). Total RNA from cultured cells was isolated and DNased using High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany), according to the supplier's instructions. The concentration and purity of RNA was evaluated spectrophotometrically at 260 nm, and 260 nm/280 nm respectively, and samples with a ratio higher than 1.7 were qualified for further analyses. RNA quality was checked electrophoretically on 2% agarose gels stained with ethidium bromide. Reverse transcription reaction was performed using Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Mannheim, Germany) with oligo(dT) primer, according to the manufacturer's manual. Obtained cDNA was used as a template for qPCR reaction using LightCycler® 480 II

Instrument and LightCycler® 480 Probes Master (Roche Diagnostics) in the following steps: initial denaturation for 10 minutes at 95°C followed by 45 cycles of amplification: 10 seconds (s) at 95°C, 30s at 60°C, 10s at 72°C. The reaction was performed in a triplicate using the Universal Probe Library (UPL) probes (Roche Diagnostics) labelled with fluorescein amidite (FAM) and primers sets for target genes: *MMP-2* (UPL#29: 5'-CTTCTGCC-3', For: 5'-TACGACCGCGACAAGAAGTA-3', Rev: 5'-AGTTCCCA CCAACAGTGGAC-3') or *MMP-9* (UPL#6: 5'-CAGAGGAA-3', For: 5'-GAACCAATCTCACCAGACAGG-3', Rev: 5'-GCCACCC GAGTGTAACCATA-3'), in duplex with UPL probe labelled with Yellow 555 and primers set for the reference gene *GAPD* (Universal Probe Library Human GAPD Gene Assay (Roche Diagnostics): Probe: 5'-CTTTTGCCTCGC-3', For: 5'-CTCTGCTCTCCT GTTCGAC-3', Rev: 5'-GCCCAATACGACCAATCC-3'). Primers for target genes were purchased at <http://oligo.pl> (IBB Pan, Warsaw, Poland). cDNA obtained from TOV-112D cells was used as a calibrator. Relative quantity (RQ) values were calculated using an efficiency method with LightCycler 480 software ver. 1.5 SP3 (Roche Diagnostics).

**Statistical analysis.** The normality of *MMP-2* and *MMP-9* expression was analyzed using Kolmogorov-Smirnov test. The differences between groups were analyzed using Kruskal-Wallis and Mann-Whitney tests, as the expression of both genes was non-normally distributed. Spearman's rank correlation test was used to evaluate correlations between analyzed variables. *p*-Value equal to or lower than 0.05 was considered statistically significant. Statistical analysis was performed using Statistica 13.0 (Dell Inc., Tulsa, OK, USA). Cut-off values for the expression of both *MMPs* used for survival analysis were determined with Cut-off Finder version 2.1 (11).

## Results

Table I shows *MMP-2* and *MMP-9* mRNA expression in EOC patients' tumors. *MMP-2* expression was found in all EOCs, while *MMP-9* expression was found in 40 EOCs (90.1%). *MMP-2* expression was the highest in serous EOCs compared to other histological subtypes (*p*=0.037, Kruskal-Wallis test). *MMP-2* expression was significantly higher (*p*=0.020, Mann-Whitney test) in EOCs sensitive to chemotherapy compared to resistant and refractory tumors. *MMP-9* expression was not significantly associated with any of clinicopathological variables (Table I). In the group of benign tumors *MMP-2* expression was present in all cases, while *MMP-9* expression was found in 4 of 5 benign tumors (80%). The highest *MMP-2* expression was found in HSF and it gradually decreased from benign tumors, EOCs, and ovarian cancer cell lines (*p*<0.001, Kruskal-Wallis test, Figure 1). *MMP-9* expression was the highest in EOCs, moderate in OV-90 and benign tumors, low in other ovarian cancer lines, and the lowest in HSF (*p*<0.001, Kruskal-Wallis test, Figure 1). We showed a direct and significant correlation between the expression of *MMP-2* and *MMP-9* in EOCs (*R*=0.384, *p*=0.010, Spearman's rank correlation test).

EOC patients were followed for at least 6 years or until death (median: 52 months, range: 1-117). At the end of the

Table I. *MMP-2 and MMP-9 mRNA expression in epithelial ovarian cancer patients' tissues.*

Variable	N	MMP-2 (RQ)			MMP-9 (RQ)		
		Mean±SEM	Median (min-max)	p-Value	Mean±SEM	Median (min-max)	p-Value
Age							
≤54	23	1.36±0.31	0.90 (0.03-6.69)	0.385 <sup>a</sup>	886.22±282.18	289.10 (0.00-4775.00)	0.155 <sup>a</sup>
>54	21	1.22±0.31	0.36 (0.04-4.25)		466.56±152.17	208.70 (0.00-2536.00)	
Menopausal status							
Pre	15	1.25±0.41	0.90 (0.03-6.69)	0.843 <sup>a</sup>	1070.69±413.87	289.10 (0.00-4775.00)	0.211 <sup>a</sup>
Post	29	1.31±0.26	0.56 (0.03-4.25)		486.92±124.62	208.70 (0.00-2536.00)	
Histology							
Serous	22	1.85±0.04	1.17 (0.07-6.69)	0.037 <sup>b</sup>	765.55±242.73	316.65 (0.00-4770.00)	0.429 <sup>b</sup>
Endometrioid	8	0.78±0.23	0.69 (0.03-2.14)		1023.80±574.01	378.35 (0.00-4775.00)	
Mucinous	4	1.04±0.78	0.41 (0.03-3.32)		758.11±440.62	584.87 (23.70-1839.00)	
Other	10	0.57±0.24	0.36 (0.04-2.62)		211.59±83.37	109.14 (0.00-815.70)	
Tumor grade							
G1/G2	17	1.36±0.33	0.90 (0.03-3.83)	0.284 <sup>a</sup>	660.48±284.28	217.10 (0.00-4775.00)	0.969 <sup>a</sup>
G3	24	0.94±0.22	0.45 (0.03-4.25)		660.59±224.54	248.90 (0.00-4770.00)	
NA	3						
FIGO stage							
I/II	14	1.52±0.38	0.89 (0.03-3.83)	0.554 <sup>a</sup>	636.56±346.06	124.42 (0.00-4775.00)	0.308 <sup>a</sup>
III/IV	30	1.19±0.27	0.55 (0.04-6.69)		708.97±186.02	327.55 (0.00-4770.00)	
Debulking							
Optimal ≤1cm	17	1.36±0.29	1.07 (0.03-3.83)	0.386 <sup>a</sup>	999.81±382.10	217.10 (0.00-4775.00)	0.866 <sup>a</sup>
Suboptimal >1cm	27	1.25±0.31	0.44 (0.04-6.69)		488.30±116.38	309.20 (0.00-2536.00)	
Patient response to treatment							
CR	17	1.45±0.27	1.08 (0.03-3.83)	0.077 <sup>a</sup>	1066.42±375.71	324.10 (0.00-4775.00)	0.347 <sup>a</sup>
PR, SD, and PD	18	0.99±0.30	0.39 (0.04-4.25)		449.41±149.57	235.30 (0.00-2536.00)	
NA	9						
Tumor sensitivity							
Sensitive	25	1.44±0.25	1.08 (0.03-4.25)	0.020 <sup>a</sup>	931.56±272.39	324.10 (0.00-4775.000)	0.324 <sup>a</sup>
Resistant and refractory	10	0.65±0.31	0.30 (0.04-3.32)		292.97±112.55	157.55 (0.00-1115.00)	
NA	9						

RQ: Relative quantity; SEM: standard error of mean; p-Value: <sup>a</sup>Mann-Whitney test, <sup>b</sup>Kruskal-Wallis test; FIGO: International Federation of Gynecology and Obstetrics; CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease.

follow-up 27 patients died (61.4%) and 17 were still alive (38.6%). Survival analysis did not reveal significant association of *MMP-2* and *MMP-9* mRNA expression with patient OS. Among clinicopathological variables, advanced stage (FIGO III and IV), suboptimal debulking, lack of complete response to chemotherapy, and resistance of EOCs to chemotherapy were significantly ( $p<0.05$ ) associated with patients' shorter OS.

## Discussion

Previous studies have demonstrated an unfavorable prognostic value of *MMP-2* and *MMP-9* in patients with ovarian cancer (12-15). In our study, *MMP-2* and *MMP-9* mRNA expression was not significantly associated with OS. Similarly, other reports have not shown a prognostic value of either *MMP* in patients with advanced ovarian cancer (16). This observation can be partially explained by the significantly higher expression of *MMP-2* mRNA in benign tumors compared to

EOC discovered in our study. Although, some authors have reported elevated *MMP-2* expression in ovarian cancers compared to benign adenomas (17-19), other studies have shown higher *MMP-2* expression in benign tumors compared to malignant ones (16, 20, 21). The higher *MMP-2* expression in benign ovarian adenomas and precancerous lesions compared to ovarian cancers may emphasize the importance of this *MMP* in the degradation of basement membranes, the key process in transformation of epithelial cells and early stages of tumorigenesis (20). However, the aforementioned studies did not evaluate mRNA levels in ovarian cancer tissues. Therefore, our finding completes the knowledge regarding *MMP-2* function in ovarian cancer development and could be clinically relevant. Moreover, similarly to work of Cai *et al.* (20), *MMP-2* expression in our study was not related to tumor grade and FIGO stage, which implicates low enzyme importance in stabilized disease. It has been previously described that silencing of *MMP-2* mRNA or inhibiting *MMP-2* enzymatic activity counteracts the adhesion of ovarian cancer cells *in vitro*

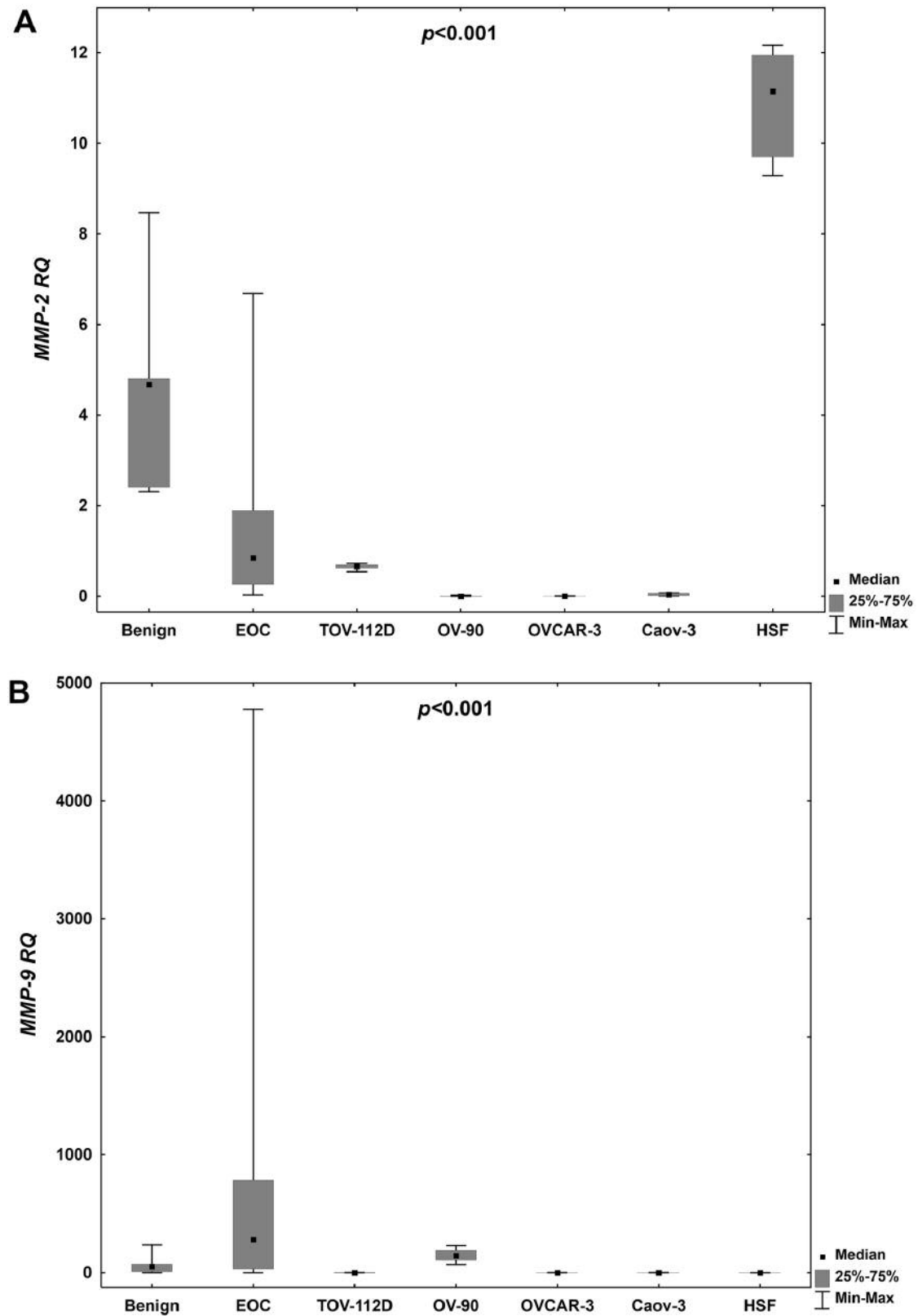


Figure 1. MMP-2 (A) and MMP-9 (B) mRNA expression in benign tumors and epithelial ovarian cancer tissues, ovarian cancer cell lines, and human skin fibroblasts. RQ: Relative quantity; Benign: benign ovarian tumors; EOC: epithelial ovarian cancer; HSF: human skin fibroblasts.

and *in vivo* and slows tumor growth and formation of metastases, while *MMP-2* silencing following peritoneal implantation of tumor cells has only limited effect on their metastatic potential (22). Importantly, here we detected a significantly higher *MMP-2* expression in EOCs sensitive to chemotherapy. One of the cisplatin effects is reduced migration and invasiveness of cancer cells, which has been shown to be connected with decreased *MMP-2* activity (23). In our study, we measured *MMPs* expression in untreated patients, which implies that the platinum drugs used in therapy of EOC patients may be more effective in patients with initially higher expression of *MMP-2*. Significantly higher *MMP-2* expression found in serous EOCs compared to other histological subtypes may implicate the important role of this MMP in the development of serous EOCs. On the other hand, *MMP-2* immunoreactivity and mRNA expression measured by means of semiquantitative RT-PCR was similar in serous *versus* other histological subtypes of EOCs (15, 24). It seems that conducting quantitative real-time PCR to find differences in *MMP-2* mRNA expression between various histological subtypes of ovarian cancer could provide additional, clinically useful information, as demonstrated in our present study.

We found a significant positive correlation between *MMP-2* and *MMP-9* mRNA levels in EOC patients. However, in our study, *MMP-9* expression was not significantly associated with any clinicopathological variables of EOC or patients' survival. Similar findings were reported by other authors (16, 25). Our data, as well as other reports question the prognostic value of both these MMPs in ovarian cancer patients, which may result from additional regulation of MMPs' activity during tumor development by TIMPs and diversified excretion by various cells in tumor mass. Recently, it was discovered that an essential factor of tumor initiation and progression may be related to activated fibroblasts or myofibroblasts originating from epithelial cells through EMT (26). In this study we found the highest *MMP-2* expression and the lowest *MMP-9* expression of all analyzed samples in cultured HSF cells. Our results are in agreement with reports showing that fibroblasts co-cultured with keratinocytes *in vitro* produce only *MMP-2*, and no *MMP-9* (27). In contrast, keratinocytes excrete mainly *MMP-9* and very low levels of *MMP-2*, whereas both types of cells cooperate in the remodeling of ECM by regulation of MMPs and their inhibitor TIMPs expression (27, 28). It has also been shown that ovarian cancer cells could stimulate the release of pro-*MMP-2* and *TIMP-2* by co-culturing with fibroblasts (29). These findings seem to be related to our study, because we detect only relatively low or very low *MMP-2* expression in ovarian cancer cell lines. At the same time, we found *MMP9* mRNA expression in all analyzed ovarian cancer lines, however, in OV-90 its expression level was considerably higher compared to the rest. This suggests that *MMP-9* may be engaged in the

induction or maintenance of the malignant phenotype, because this cell line was established from metastatic cancer cells present in ascites, from patients with advanced stage ovarian cancer (30). It has been previously demonstrated that *MMP-2* is often expressed in normal ovarian lines, while its expression is decreased in ovarian cancer lines (20). It has also been reported that *MMP-2* mRNA expression in OVCAR-3 and Caov-3 lines may be induced by the interaction between laminin and 67-kDa non-integrin laminin receptor, which increase cancer cell invasion (31). These findings suggest that *MMP-2* expression in ovarian cancer cells and therefore their invasiveness may be elevated only after adhesion to ECM (20, 31). Additionally, it has been found that *MMP-2* immunoreactivity is stronger in stromal cells adjacent to epithelial ovarian cancer cells compared to tumor cells or distant stromal cells (24). *In situ* hybridization has also revealed that *MMP-2* mRNA expression is mainly present in fibroblasts in the EOC subepithelial stroma and that this may be implicated in ECM remodeling (32). Moreover, Ekinici *et al.*, have indicated shorter OS in ovarian cancer patients with stromal *MMP-2* expression (33), even though, a meta-analysis showed that higher *MMP-2* expression in ovarian tumor cells, but not in stromal cells, is involved with patients' shorter survival (7). Interestingly, one study has demonstrated that *MMP-2* and *MMP-9* proteins are mainly expressed in ovarian tumor cells, while *MMP-2* and *MMP-9* mRNAs are rather present in stromal part of EOCs (34). These findings underline the important role of complex interactions between normal and neoplastic cells in EOC progression.

In conclusion, the lack of a correlation between *MMP-9* mRNA and patients' OS or clinicopathological variables suggests that this is not a useful biomarker in EOC. Interestingly, the high *MMP-2* expression detected in benign ovarian tumors may indicate its important role in early stages of ovarian carcinogenesis. Higher *MMP-2* expression in EOCs sensitive to platinum-based chemotherapy suggests that this may become a novel predictive tool for personalized chemotherapy, but it requires further investigation.

## Conflicts of Interest

The Authors declare that there are no conflicts of interest.

## Authors' Contributions

Concept and design of the study was performed by WJ, supervision by AS, sample collection by AN, BMK, clinical data collection by AN, RNA isolation by ASB, Cell culture and qPCR by WJ, statistical analysis by MC. Analysis and interpretation of results were performed by WJ and MC, writing of manuscript by WJ and MC, correction of draft by MG, literature review by WJ, MC and MG, funding by MC and AS and critical review by AS and JK.

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