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ORIGINAL ARTICLE

Polymorphic expression of glutathione transferases A1, M1, P1 and T1 in epithelial ovarian cancer: a Serbian case-control study

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Summary

Purpose: *Since several studies have proposed that epithelial ovarian cancer should not be considered as a single disease entity and that it results from an accumulation of genetic changes, we aimed to assess the polymorphic expression of major cytosolic glutathione S-transferases (GSTM1, T1, A1 and P1) with respect to ovarian cancer susceptibility and aggressiveness.*

Methods: *This case-control study was conducted on 93 newly diagnosed epithelial ovarian cancer patients and 178 healthy matched controls. The multiplex polymerase chain reaction (PCR) was used to detect homozygous deletions of GSTM1 and GSTT1 genes. Analysis of the single nucleotide polymorphism (SNP) GSTA1 C69T was performed using PCR-restriction fragment length polymorphism (RFLP), while for SNP GSTP1 Ile105Val real-time PCR was used.*

Results: *No significant association to ovarian cancer risk was found for individual GSTM1, GSTA1 and GSTP1 genotypes (p>0.05). However, the carriers of GSTT1-active genotype were at 2-fold higher risk of ovarian cancer development (95%CI: 1.00-4.01, p=0.049), which was even more elevated in the subgroup of patients with positive family history of cancer. Moreover, the frequency of all three GST genotypes that might be associated to ovarian cancer risk (GSTT1-active, GSTA1-active and GSTP1-referent) was significantly higher in patients than in the control group (p=0.042). Even more, patients who were carriers of combination of these three genotypes represented over 64% of the total number of patients within any of the International Federation of Gynecology and Obstetrics (FIGO) stages of ovarian cancer.*

Conclusions: *This study provides supportive evidence that GSTs might affect both susceptibility and progression of ovarian cancer.*

Key words: *glutathione S-transferase, ovarian cancer, polymorphism, risk*

Introduction

Ovarian cancer is the seventh most common cancer in women worldwide and accounts for nearly 4% of all new cancer cases in females [1,2]. Its high death rate is due to the fact that approximately 60% of women are diagnosed at an advanced stage of the disease [3]. Maximal surgical cytoreduction followed by chemotherapy, with initial response rate of 65-80% to first-line chemotherapy, is considered gold standard for these patients [4,5]. Still, in most cases, the disease re-

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lapses and patients acquire resistance to further chemotherapy, leading to 5-year survival rate of 30-50% [3,6].

Although, over the past decade, attempts have been made to develop more effective surgical techniques, as well as chemotherapy with combinations of cytotoxic drugs, the overall cure rate for ovarian cancer remains approximately 30% [7]. Since ovarian cancer is thought to result from an accumulation of genetic changes [8], it is believed that significant changes in long-term survival in patients with ovarian cancer might only be achieved by personalized individual treatment strategies, as well as early detection of the disease [7]. For that reason, identification of inter-individual genetic variations, especially in genes encoding enzymes involved in detoxification of anticancer drugs, seems reasonable.

Glutathione S-transferases (GSTs) are a large family of enzymes responsible for catalyzing the conjugation of xenobiotics, including anticancer drugs with glutathione [9]. Great inter-individual differences exist in GST isoenzyme profile, due to the fact that almost all members of seven classes of cytosolic GSTs exibit genetic polymorphism [9]. As a consequence, complete lack or alteration in GST enzyme activity might affect the capacity for biotransformation in certain individuals, making them more prone to cancer development.

In ovarian cancer, the cytosolic classes M1, T1 and P1 gained most attention as potential risk determinants [10–13], while GSTA1 class, which affects cellular redox balance, has not been evaluated in ovarian cancer as yet.

Apart from the well established role of GSTs in the susceptibility to cancer, they might also affect chemotherapy response. Sawers et al. quite recently demonstrated that GSTP1 has an important role in cisplatin and carboplatin metabolism in ovarian cancer cells and that inter-tumor differences in GSTP1 expression directly influence response to platinum-based chemotherapy in ovarian cancer patients [14]. These results imply that identification of subgroups of patients with ovarian cancer who might benefit from novel first-line therapies, based on their detoxification capacity and the ability to maximize benefits and minimize toxicity of applied antitumor drugs, is of high importance.

For that reason, in this study, polymorphic expression of major cytosolic GST classes, M1, T1, A1 and P1 were analyzed with respect to ovarian cancer susceptibility. Furthermore, the association with ovarian cancer aggressiveness was assessed.

Methods

Study population

This case-control study was conducted on 93 newly diagnosed epithelial ovarian cancer patients (all Caucasian, mean age 59.34 ± 11.22) treated at the Clinic of Gynecology University Teaching Hospital Zemun-Belgrade, Institute of Oncology Clinical Center of Serbia and Oncology Institute of Vojvodina between May 2014 and October 2015. Ovarian cancer (serous, endometrioid, mucinous and clear-cell) was diagnosed according to the criteria of the World Health Organization classification of tumors of female reproductive organs [15] and staged according to the International Federation of Gynecology and Obstetrics (FIGO) staging classification [16]. Response rate was 92% and the most common reason for patients not to participate in the study was personal. The control group comprised 178 women (all Caucasian, mean age 60.65 ± 12.40) who had undergone surgery for benign conditions. Selection criteria for control individuals were no evidence of any personal or family history of ovarian cancer. The basic demographic data and assumed risk factors for ovarian cancer were obtained from the study subjects using a structured questionnaire composed at the Institute of Epidemiology, Faculty of Medicine University in Belgrade, during the time of blood collection. In our study, obese patients were defined as individuals with body mass index (BMI) above 25 and smokers as individuals who reported every day smoking for a minimum of 60-day period prior to their enrollment in the study. Furthermore, participants were asked about the number of cigarettes smoked per day and duration of smoking. All collected data referred to a time period prior to the diagnosis of ovarian cancer for the cases, and a corresponding period for the controls. The study was approved by the Ethics Board of the Faculty of Medicine, University of Belgrade, Serbia, and was performed in accordance with principles of Helsinki declaration. Written informed consent was obtained from all recruited subjects.

DNA isolation and GST genotyping

Genomic DNA was isolated from whole peripheral blood, using DNA kit (Qiagen, Chatsworth CA, USA). DNA concentration was measured on GeneQuant pro (Biochrom, Cambridge, England).

The multiplex PCR was used according to the method by Abdel-Rahman et al. [17] to detect gene deletion polymorphism of *GSTM1* and *GSTT1* genes. Apart from primers used for *GSTM1* (forward 5′-GAACTCCCTGAAAAGCTAAAGC-3′ and reverse 5′-GTTGGGCTCAAATATACGGTGG-3′) and *GSTT1* (forward 5′-TTCCTTACTGGTCCTCACATCTC-3′ and reverse 5′-TCACCGGATCATGGCCAGCA-3′), the method included primers for the *CYP1A1* (forward 5’-GAACTGCCACTTCAGCTGTCT-3’ and reverse 5’-CAGCTGCATTTGGAAGTGCTC-3’) housekeeping gene that served as an internal control for am-

plifiable DNA. The PCR protocol started with the denaturation at 94°C for 4 min, followed by 94°C for 30 s, annealing at 59°C for 30 s, extension at 72°C for 45 s (#cycles: 30), with final extension at 72°C for 5 min. This method detects the presence (at least one allele present, homozygote or heterozygote) or absence (complete deletion of both alleles, homozygote) of genotype.

Analysis of the SNP *GSTA1 C69T* (rs3957356) was performed using PCR-RFLP, using *GSTA1* primers (forward 5'-GCATCAGCTTGCCCTTCA -3' and reverse 5'-AAACGCTGTCACCGTCCTG-3') with Eam1104I (Thermo Fisher Scientific, Massachusetts, USA) restriction enzyme, according to the method by Ping et al. [18]. The PCR protocol included denaturation at 94°C (4 min), followed by 94°C for 20 s, annealing at 58°C for 20 s and extension at 72°C for 40 s (#cycles: 33) with final extension at 72°C for 5 min.

For analyses of SNP *GSTP1 Ile105Val* (rs1695), real-time PCR allelic discrimination was performed on Mastercyclerep realplex (Eppendorf, Hamburg, Germany) using Applied BiosystemsTaqMan® Drug Metabolism Genotyping assay according to the manufacturer's instructions (Life Technologies, Applied Biosystems, Carlsbad, CA, USA, assay ID C_3237198_20).

Researchers that performed genotyping were unaware of the case-control status, and blinded quality control samples were inserted to validate genotyping identification procedures. Concordance for blinded samples was 100%.

Statistics

Statistical analyses were performed using the Statistical Package for Social Sciences software (IBM Statistics SPSS, version 20.0). In descriptive statistics continuous variables were summarized by mean ± standard deviation (x±SD) or median with min-max values. Relative associations between the studied genotypes and ovarian cancer risk were evaluated by multinomial logistic regression to calculate odds ratios (OR) and 95% confidence intervals (CI). Differences in investigated parameters were assessed by using Student's t-test for continuous data with normal distribution and Mann–Whitney rank-sum test for continuous data with non-normal distribution. Finally, x^2 test was used for categorical variables. A p value of ≤ 0.05 was considered as statistically significant.

Results

Patients with epithelial ovarian cancer and respective controls did not differ in terms of age, obesity and smoking (p>0.05). Namely, the mean BMI in patients was 25.94 ± 4.42 in comparison to 26.26 ± 5.01 in the control group (p=0.611). Regarding smoking, median pack-years was 15.75 in the patient group compared to 20 in the control group (p=0.382) (data not shown). All other baseline demographic characteristics of patients with ovarian cancer are shown in Table 1. The majority of patients had 2 births (52%), endometriosis was diagnosed in only 5 patients (5%), while only 3 patients (3%) were under hormone treatment. Fourteen patients (15%) had positive family history of cancer (either ovarian or breast cancer), while, interestingly, one patient had two closest relatives with ovarian and breast cancer (Table 1). When stratified according to FIGO staging classification, 35 patients (38%) had FIGO I, 23 (24%) FIGO II and the remaining 38% FIGO III.

Table 1. Baseline demographic characteristics of patients with ovarian cancer

Characteristics	*Patients, N (%)*
Age (years), mean ±SD	59.34 ± 11.22
Obesity	
BMI < 25	44 (48)
BMI > 25	49 (52)
BMI (kg/m^2), mean ±SD	25.94 ± 4.42
Smoking	
Never	55 (59)
Ever[1]	38 (41)
Pack-years, median (range)[1]	15.75 (0.30 - 66.00)
Number of births	
0	13 (14)
1	24 (26)
2	48 (52)
3	7 (7)
4	1 (1)
Endometriosis	
No	88 (95)
Yes	5 (5)
Family history of cancer	
No	79 (85)
Yes	14 (15)[2]
Ovarian cancer	6 (6)
Breast cancer	9 (10)
Ever user of hormones	
No	90 (97)
Yes	3 (3)
FIGO stage	
I	35 (38)
II	23 (24)
III	35 (38)

[1]At least 60 cigarettes smoked prior to the study onset. [2]One patient had two closest relatives with ovarian and breast cancer. BMI: body mass index, FIGO: International Federation of Gynecology and Obstetrics staging classification

Table 2. *GST* genotypes in relation to the risk of ovarian cancer

GST genotype	*Patients N (%)*	*Controls N (%)*	*OR (95%CI)*[7]	*p value*
GSTM1				
Active[1]	44 (52)	89 (50)	1.00 (reference group)	
Null[2]	41 (48)	89 (50)	0.93 (0.55-1.59)	0.811
GSTT1				
Null[2]	13 (15)	47 (26)	1.00 (reference group)	
Active[1]	72 (85)	131 (74)	2.00 (1.00-4.01)	0.049
GSTA1 (rs 3957356)				
CC+CT (active)[3]	80 (92)	151 (85)	1.00 (reference group)	
TT (low activity)[4]	7 (8)	27 (15)	0.52 (0.22-1.23)	0.162
GSTP1 (rs1695)				
IleIle+IleVal (referent)[5]	78 (92)	150 (84)	1.00 (reference group)	
ValVal (variant)[6]	7 (8)	28 (16)	0.53 (0.21-1.31)	0.169

[1]if at least one active allele present; [2]if no active alleles present; [3]if at least one *C* allele present; [4]Low activity, if both *T* alleles present; [5]Referent, if at least one *Ile* allele present; [6]Variant, if both *Val* alleles present; [7]Odds ratio adjusted to age, BMI and pack-years; CI: confidence interval; *GSTM1* and *GSTT1* genotyping was successful in 85 cases and all recruited controls; *GSTA1 C69T* and *GSTP1 Ile105Val* genotyping was successful in 87 and 85 ovarian cancer cases, respectively, and all recruited controls

Table 3. *GST* genotypes in relation to the risk of ovarian cancer in patients with family history of cancer

GST genotype	*Patients N (%)*	*Controls N (%)*	*OR (95%CI)*[7]	*p value*
GSTM1				
Active[1]	6 (46)	89 (50)	1.00 (reference group)	
Null[2]	7 (54)	89 (50)	1.35 (0.41-4.33)	0.620
GSTT1				
Null[2]	1 (7)	47 (26)	1.00 (reference group)	
Active[1]	13 (93)	131 (74)	5.17 (0.64-41.66)	0.123
GSTA1 (rs 3957356)				
CC+CT (active)[3]	11 (85)	151 (85)	1.00 (reference group)	
TT (low activity)[4]	2 (15)	27 (15)	0.82 (0.25-6.38)	0.769
GSTP1 (rs1695)				
IleIle+IleVal (referent)[5]	10 (83)	150 (84)	1.00 (reference group)	
ValVal (variant)[6]	2 (17)	28 (16)	0.79 (0.23-6.01)	0.843

[1]if at least one active allele present; [2]if no active alleles present; [3]if at least one *C* allele present; [4]if both *T* alleles present; [5]if at least one Ile allele present; [6]Variant, if both Val alleles present; [7]Odds ratio adjusted to age, BMI and pack-years; CI: confidence interval

GST genotypes and ovarian cancer risk

The distribution of *GST* genotypes in ovarian cancer patients is presented in Table 2. No significant association with ovarian cancer risk was found for *GSTM1* genotype (OR=0.93, 95%CI: 0.55-1.59, p=0.811). On the other hand, the frequency of *GSTT1-active* genotype was higher in patients with ovarian cancer than in controls and these individuals were at 2-fold higher risk of ovarian cancer development (95%CI:1.00-4.01, p=0.049).

Results obtained on the *GSTA1 C69T* (rs3957356) SNP polymorphism showed that carriers of at least one *GSTA1-active* allele were more frequent in the patient group, however without statistical significance (p=0.162). Similar results were found with

respect to *GSTP1 Ile105Val* (rs1695) SNP polymorphism. Namely, the frequency of *GSTP1-referent* genotype was higher, while the frequency of *GSTP- variant* genotype was lower in patients with ovarian cancer in comparison to controls (OR=0.53, 95%CI: 0.21-1.31, p=0.169).

Analysis of *GST* genotypes with regard to the risk of ovarian cancer in patients with family history of cancer further confirmed the results obtained on the whole study group, especially with respect to *GSTT1* genotype (Table 3). Namely, patients with familial history of ovarian or breast cancer, who were carriers of *GSTT1-active* genotype were at 5.17-fold increased risk of getting ovarian cancer, however without statistical significance (p=0.123), possibly due to the small sample size.

Combined effect of GST genotypes on ovarian cancer risk

Statistical analysis of the combined effect of *GST* genotypes on ovarian cancer risk showed no significant association for any of *GSTM1*, *GSTT1*, *GSTA1* and *GSTP1* genotypes. Still, it seems that genotypes that might be associated to ovarian cancer risk (*GSTT1-active*, *GSTA1-active* and *GSTP1-referent*) were more frequent in patients than in controls in any of the examined combinations (Table 4). For that reason, the cumulative effect of suggested risk-associated *GST* genotypes was analyzed in patients with ovarian cancer in comparison to the control group (Table 5). Indeed, the results obtained showed that the combination of all three *GST* genotypes that might be associated to ovarian cancer risk (*GSTT1-active*, *GSTA1- active* and *GSTP1-referent*) was present in 72% of the patients which was significantly higher (p=0.042) compared to the control group (54%).

Finally, the distribution of ovarian cancer patients with risk-associated genotypes (*GSTT1-active, GSTA1-active* and *GSTP1-referent*) was analyzed within the total number of patients stratified according to FIGO classification. Interestingly, patients who were carriers of *GSTT1-active/ GSTA1-active/GSTP1-referent* genotype represented over 64 % of the total number of patients within any of FIGO stages of ovarian cancer (64, 78 and 75% within FIGO I, FIGO II and FIGO III, respectively) (Figure 1).

Discussion

To best of our knowledge, this is the first attempt to study the association between the 4 most common *GST* polymorphisms and epithelial ovarian cancer risk in Serbian women. Women with *GSTT1-active* genotype were at increased risk for ovarian cancer genesis in comparison to those with *GSTT1-null* genotype, which was even more evident in the subgroup of patients with positive family history of cancer. Moreover, the frequency of all 3 *GST* genotypes that might be associated to ovarian cancer risk (*GSTT1-active*, *GSTA1-active* and *GSTP1-referent*) was significantly higher in patients than in the control group.

Table 4. Effects of combined *GST* genotypes in relation to the risk of ovarian cancer

GST genotype combination	*Patients N (%)*	*Controls N (%)*	*OR (95%CI)[7]*	*p value*
GSTM1 and GSTT1				
GST-M1[1] active/GSTT1-null[2]	4 (5)	23 (13)	1.00 (reference group)	
GSTM1-null[2]/GSTT1-active[1]	32 (37)	65 (36)	2.92 (0.92-9.32)	0.069
GSTT1 and GSTA1				
GSTT1-null/GSTA1-low activity[3]	1 (1)	8 (5)	1.00 (reference group)	
GSTT1-active/GSTA1-active[4]	67 (78)	112 (63)	5.42 (0.66-44.59)	0.116
GSTT1 and GSTP1				
GSTT1-null/GSTP1-variant[5]	1 (1)	10 (6)	1.00 (reference group)	
GSTT1-active/GSTP1-referent[6]	64 (77)	113 (63)	6.11 (0.74-49.96)	0.091
GSTA1 and GSTP1				
GSTA1-low activity/GSTP1-variant	1 (1)	5 (3)	1.00 (reference group)	
GSTA1-active/GSTP1-referent	72 (85)	128 (72)	2.91 (0.31-2.71)	0.347

[1]Active, if at least one active allele present; [2]Null if no active alleles present; [3]Low activity, if both *T* alleles present; [4]Active, if at least one *C* allele present; [5]Variant, if both *Val* alleles present; [6]Referent, if at least one *Ile* allele present; [7]Odds ratio adjusted to age, BMI and pack-years. CI: confidence interval out of the total number of controls included in the study

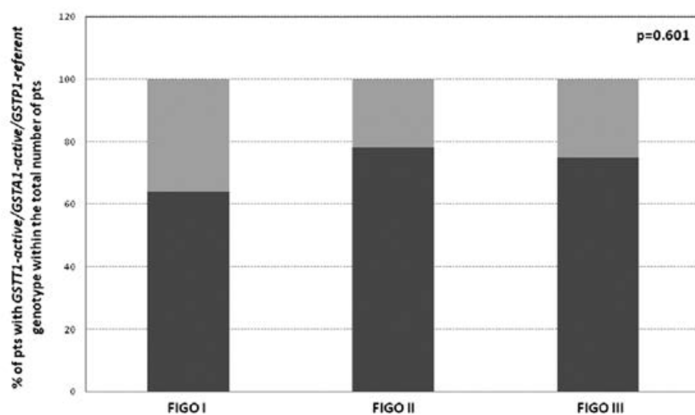

Figure 1. Distribution of patients with risk-associated genotypes (*GSTT1-active, GSTA1-active* and *GSTP1-referent*) within the total number of patients stratified according to FIGO classification.

Over the years, many studies have attempted to elucidate triggering genetic factors in the development of ovarian cancer, most of which were focused on the association between *BRCA1*, *BRCA2* and *p53* genes and the ovarian cancer risk. However, the frequencies of mutations in the mentioned genes were quite low [19,20]. Recently, several studies have shown that polymorphisms of GSTs could also affect the susceptibility to ovarian cancer due to their important role in the modulation of the biological effects of carcinogens. Special emphasis in this regard has been put on *GSTM1* and *GSTT1* deletion polymorphisms, which, in carriers of *GSTM1-null* or *GSTT1-null* genotype, result in complete absence of active enzyme and thus reduce the cell's ability to metabolize toxins [9]. However, the obtained results over the contribution of these GSTs polymorphism to ovarian cancer risk are rather inconsistent [11,21–23] Moreover, the data from several meta-analyses do not provide a strong evidence for causal associations between *GSTM1* and *GSTT1* polymorphisms and risk of ovarian cancer in Caucasians [11,21-23]. Our results with lack of significant contribution of *GSTM1* gene polymorphism to the risk of ovarian cancer are in concordance with the aforementioned studies [11,21-23].

On the other hand, the present study found increased risk to ovarian cancer in carriers of *GSTT1-active* genotype. This result might sound contradictory, having in mind that several environmental carcinogens found in combustion products and tobacco smoke, such as halogenated solvents, are among GSTT1 substrates [9]. However, there is strong evidence that GSTT1 enzyme might also be involved in bioactivation, rather than detoxification of several bifunctional alkylating agents, present in environmental pollution and certain occupational hazards [24]. As a consequence, bioactivation process can yield potent electrophiles that modify DNA and are potentially genotoxic [24,25]. Therefore, our results are in concordance with the study of Sgambato et al. who showed that *GSTT1-null* genotype is associated with decreased risk of cancer [26].

GSTP1 SNP (rs1695), resulting from amino acid substitution from isoleucine (Ile) to valine (Val) [9], can affect both its catalytic and non-catalytic activity [27]. Although the carriers of *GSTP1*Ile105*genotype have a higher catalytic efficiency for standard GST substrate (1-chloro-2,4-dinitrobenzene) than the **Val105* variant [9], the latter seems to confer higher catalytic efficiency in detoxification of polycyclic aromatic hydrocarbon (PAH) diol epoxide, present in tobacco smoke [28]. GSTP1 also participates in the regulation of stress signaling and apoptosis via its non-catalytic activity [29]. Namely, it has been shown that the presence of specific GSTP1 polymorphic variant can influence the degree of interaction between GSTP1 and c-Jun N-terminal kinase (JNK), proapoptotic member of mitogen-activated protein kinase (MAPK) signaling pathway. In that way, the substitution of amino acid isoleucine (Ile) with valine (Val) at position 105 can alter GSTP1-mediated inhibitory effect of JNK activity. Based on our results on increased ovarian cancer risk in *GSTP1*Ile* (*referent*) allele carriers, it might be speculated that the stronger GSTP1:JNK interaction could prevent activation of apoptosis in these women, further affecting the progression of disease. In addition, our data on 6-fold increased ovarian cancer risk in women carriers of combined "risk" genotypes (*GSTT1-active/GSTP1*Ile*) suggest

Table 5. Risk-associated *GST* genotypes in ovarian cancer patients

Risk-associated GST genotypes[1], *N (%)*	*Patients with ovarian cancer N (%)*	*Controls, N (%)*
0	0	2 (1)
1	3 (4)	17 (10)
2	20 (24)	62 (35)
3	60 (72)	97 (54)
		p = 0.042

[1]GSTT1-active and/or GSTA1-active and/or GSTP1-referent

a high probability of their synergetic risk effect on carcinogenesis in these women.

The role of *GSTA1* polymorphism in ovarian cancer risk assessment has not been investigated as yet. This polymorphism is represented by three linked SNPs, resulting in differential expression with lower transcriptional activation of the variant *GSTA1*B* than the common *GSTA1*A* allele [9]. Although we did not find a significant individual association between *GSTA1* polymorphism and ovarian cancer risk, surprisingly, it was a part of the suggested "risk associated GST combination" (*GSTT1-active*, *GSTA1-active* and *GSTP1-referent*), which was present in 72% of all patients. What is more, patients carriers of this combination represented over 64% of the total number of patients within any of FIGO stages, implying increased susceptibility to chemical-induced carcinogenesis in these individuals. Thus, it might be speculated that variations in the expression of GSTs due to genetic polymorphisms probably modulate the process of carcinogenesis and in that way contribute to the individual disease susceptibility.

In addition to their influence on ovarian cancer risk, it is important to note that GST polymorphisms also can affect both the prognosis and the efficacy of chemotherapy in ovarian cancer patients. Namely, Khrunin et al. showed that *GSTP1* Ile105Val* polymorphism was strongly associated with progression-free survival [30]. Moreover, it has been shown that *GSTP1*B* allele is also involved in the development of drug resistance [9] and as suggested in the study of Ghalia et al., GSTP1 levels may be useful for monitoring during chemotherapy [31]. In this field there are recent data on the beneficial effect of Hsp90 inhibitors in reversing cisplatin resistance of human ovarian cancer cell line (SKOV3), which was mediated by modifying the expression of multiple drug resistance related genes, especially *GSTP1, p53, bcl-2, survivin, BRCA1* and *BRCA2* [32].

Some limitations to the present study should be addressed. First of all, for case–control studies, the selection bias is unavoidable. Furthermore, the statistical power of this study is limited due to the relatively small sample size, which is a consequence of inclusion criteria that only patients with epithelial ovarian cancer were included. However, despite the mentioned limitations, this study provides supportive evidence that GSTs might affect both susceptibility and progression of ovarian cancer.

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Conflict of interests

The authors declare no confict of interests.

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