

Single nucleotide polymorphisms in glutathione S-transferase P1 and M1 genes and overall survival of patients with ovarian serous cystadenocarcinoma treated with chemotherapy

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Abstract. The effects of platinum-based drugs are controlled by genes that are involved in DNA detoxification, including glutathione S-transferase (GST)P1 and GSTM1, which have been associated with increased benefits in the chemotherapeutic treatment of patients with ovarian cancer. The present study assessed the effect of single nucleotide polymorphisms in GST genes on the overall survival (OS) of patients with ovarian serous cystadenocarcinoma that were treated with chemotherapy. A total of 95 patients received treatment with a carboplatin-based or alternative chemotherapy. Polymorphisms in the patients were genotyped using the following methods: Pyrosequencing, to identify GSTP1 Ile105Val; a relative quantification method, to identify the copy number variation in GSTM1; and polymerase chain reaction followed by gel electrophoresis, to identify the null vs. non-null genotypes of GSTM1. The association between genotypes and OS of patients was assessed using Kaplan-Meier survival curves and Cox proportional hazards regression analysis. The OS of patients treated with paclitaxel + carboplatin-based chemotherapy was significantly increased, compared with patients treated with alternative forms of chemotherapy ($P=0.035$). The OS of patients did not differ significantly between different GSTP1 genotypes (log-rank test, $P=0.17$). Cox proportional hazards regression analysis revealed that, since the start of the treatment, there was not a significant association between the GSTP1 isoleucine allele and the OS for heterozygous carriers of the isoleucine allele [hazards ratio (HR), 1.78; 95% confidence interval (CI), 0.77-4.12; $P=0.18$] and no homozygous carriers of the valine allele had been detected (HR, 0.00). There was no significant difference between GSTM1 genotypes, according to

Kaplan-Meier survival analysis (log-rank test, $P=0.83$). Patients that possessed ≤ 1 copy of GSTM1 exhibited no decrease in OS (HR, 0.96; 95% CI, 0.37-2.51; $P=0.94$), compared with patients that possessed two copies of GSTM1 (HR, 0.71; 95% CI, 0.22-2.28; $P=0.56$). Overall, the present results suggest that there are no associations between polymorphisms in the GSTP1 and GSTM1 genes and the OS of patients with ovarian cancer following administration of adjuvant chemotherapy.

Introduction

Ovarian cancer is the sixth most common type of cancer diagnosed in women worldwide, accounting for ~4% of all types of female cancer, and is the second most common gynecological cancer, with ~75% of patients presenting with late-stage disease (1-3). Patients with ovarian cancer are generally treated with first-line chemotherapy, including paclitaxel and platinum-based drugs (4). Cisplatin, the first-generation platinum-based drug, was gradually replaced by carboplatin and oxaliplatin, which are second- and third-generation platinum-based drugs, respectively (5,6). Cisplatin was replaced due to liver and kidney toxicity, in addition to side effects such as mucositis, neutropenia and alopecia. Since the genetic profiles of patients with cancer have been associated with drug metabolism, patients may receive targeted chemotherapy, which avoids unnecessary toxicity (7). Pharmacogenetics is important in cancer chemotherapy, and the prognosis of patients with cancer may be explained according to their genetic background (8). The effects of platinum-based drugs are controlled by genes involved in detoxification, including glutathione S-transferase (GST)P1 and GSTM1 (9).

GST belongs to the multifunctional poly-2 protein family, and is primarily responsible for catalyzing the interaction between reduced glutathione hormone and electrophilic substances (10). GST is important in protecting tissues from damage caused by oxidative stress (11). The GSTM1 gene has two variants, and encodes an enzyme that participates in the metabolism of carcinogens (12).

The influence of genetic polymorphisms in the different response to platinum-based chemotherapy has been observed in certain respiratory and digestive types of cancer, including

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non-small cell lung and colorectal cancer (13-17). However, to the best of our knowledge, genetic polymorphisms in patients with ovarian cancer who have been treated with paclitaxel and platinum-based therapies have not been characterized thus far. Therefore, the present study aimed to investigate the association between genetic polymorphisms in drug metabolism and the overall survival (OS) of patients with ovarian cancer treated with paclitaxel and carboplatin-based chemotherapy. The present study evaluated specific polymorphic genes, namely GSTP1 Ile105Val and GSTM1 null, in order to assess the association between the phenotype pattern and the OS of chemotherapy-treated patients with ovarian serous cystadenocarcinoma.

Materials and methods

Patients and tissue samples. Tissue samples from 95 patients with ovarian serous cystadenocarcinoma (median age, 53.6±11.5 years) were obtained from Linyi People's Hospital (Linyi, China). All the patients were diagnosed and treated at the Linyi People's Hospital between August 2005 and July 2013. The tissue samples were obtained during diagnostic or therapeutic surgery. The patients were followed-up once every 2-4 months for 2 years and then every 3-6 months in the subsequent 3 years. Patients required follow-up once a year from 5 years onwards. The median follow-up of patients that were alive at the end of the present study was 25.6±14.3 months (range, 3.0-92.0 months). All the patients provided written informed consent for the use of tissues samples and participation in the study. The present study was approved by the ethics committee at Linyi People's Hospital.

Data from 104 patients was collected for the present study, of which 9 patients were excluded. Patients that lacked information regarding cancer stage or start dates of chemotherapeutic treatment were excluded from the analysis. In addition, patients with a survival time of <1 month were excluded from the study. Information regarding the chemotherapeutic treatment regimen was available for all the patients included in the study. If the patients that possessed stage II-IV cancer, who were treated with 2 cycles of paclitaxel and carboplatin-based neoadjuvant chemotherapy (TP regimen) and 4-6 cycles of chemotherapy post-surgery, were allergic to paclitaxel, their levels of cancer antigen 125 were elevated following 1-2 chemotherapy cycles or computed tomography revealed that the volume of the tumor was not reduced, then the patients would be administered a different chemotherapy regimen, namely gemcitabine (1,000 mg/m², on days 1 and 8; treatment cycle, 21-28 days), ifosfamide (1,700 mg/m², on days 1-3; treatment cycle, 21-28 days) or topotecan (0.75 mg/m², on days 1-5; treatment cycle, 21-28 days). In total, 95 patients that received adjuvant or palliative chemotherapy, of which 56 patients received TP regimen and 39 patients received alternative chemotherapy, were eligible for analysis. The patients that received the TP regimen were intravenously administered with 135-175 mg/m² paclitaxel and 300-400 mg/m² carboplatin on days 1 and 2, respectively. The treatment cycle was 21-28 days. The characteristics of the patients are presented in Table I.

Genotyping of GST. The tumor tissues were obtained from Department of Pathology, Linyi People's Hospital, where the pathologists used the following procedure: Following the

Table I. Characteristics of 95 patients with ovarian serous cystadenocarcinoma.

Characteristic	Value
Age, years	
Median	53.6
Range	20.0-80.0
Follow-up time, months	
Median	25.6
Range	3.0-92.0
Chemotherapy, n (%)	
Paclitaxel + carboplatin	56 (58.9)
Other	39 (41.1)
Tumor stage, n (%)	
II	11 (11.6)
III	63 (66.3)
IV	21 (22.1)

surgery, the tumor specimens were immediately fixed with 10% formalin (Xilong Chemical Co., Ltd., Shantou, China) for 24 h. Subsequent to rinsing with running tap water, the tissues were dehydrated with 70, 80 and 95% ethanol, followed by 100% ethanol, which was changed 3 times. Subsequent to clearing with xylene (twice), the tissues were immersed in paraffin 3 times (Leica, Wetzlar, Germany). The paraffin-embedded tissue blocks were cut into 10-μm thick sections, from which genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA). The germline mutations of GSTP1 and GSTM1 were analyzed in all patients using pyrosequencing. A total of four variants [three single nucleotide polymorphisms (SNPs) and one gene deletion] were assessed using polymerase chain reaction (PCR) and pyrosequencing, as previously described (18).

The specific sequence primers (Shanghai Shengong Biotechnology, Shanghai, China) used were as follows: GSTM1, forward, 5'-CGCCATCTTGTGCTACATTGC-3'; reverse, 5'-CACAAATTCTGGATTGTAGCAGA-3'; and P (the reverse primer resulting in a 237 bp fragment), 5'-GGC CTCCTCCTTGGCTGG-3'; GSTP1 Ile105Val, forward, 5'-AATGACGGCGTGGAGGAC-3'; reverse, 5'-GGTCAG CCCAAGCCACCT-3'; and P (the reverse primer resulting in a 155 bp fragment), 5'-AGGACCTCCGCTGCAAAT-3'. The PCR cycle conditions were as follows: Denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 45 sec, 60°C for 30 sec and 72°C for 30 sec. DNA amplification was performed using Taq DNA Polymerase Master Mix RED (Biomol GmbH, Hamburg, Germany). For quality control purposes and to verify the results, 10% of samples were re-analyzed and 100% concordance was indicated (19). Negative control samples were included in each amplification series. The presence of one or both GSTM1 alleles, as identified by the presence of a 237 bp fragment or complete deletion (null genotype), was analyzed by electrophoresis on a 1.2% agarose gel using DL500 as a DNA marker (Takara Bio, Dalian, China).

Table II. HR of the overall survival of 95 patients with ovarian serous cystadenocarcinoma, according to their GSTP1 and GSTM1 polymorphisms.

Genetic polymorphism	Patients, n	Mortalities, n	P-value ^a	HR	95% CI	P-value ^b
GSTP1						
A/A	37	11	-	1.00	-	-
A/G	58	11	0.17	1.78	0.77-4.12	0.18
G/G	0	0	0.00	0.00	0.00-0.00	0.00
GSTM1						
Homozygous (2 copies)	27	8	-	1.00	-	-
Heterozygous (1 copy)	20	4	-	0.96	0.37-2.51	0.94
Homozygous (0 copies)	48	10	0.83	0.71	0.22-2.28	0.56

^aP-value was calculated using the log-rank test. ^bP-value compares GSTP (A/A) and GSTP (A/G). Therefore, P>0.05 indicates no significant difference in the Cox proportional hazards regression analysis. HR=1.00 indicates the internal reference. Missing data are indicated by -. HR, hazards ratio; GST, glutathione S-transferase; CI, confidence interval.

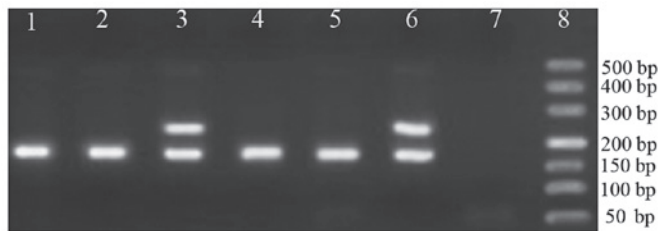


Figure 1. Patient genotyping using multiplex-polymerase chain reaction. Lanes 1, 2, 4 and 5 show samples from patients exhibiting the GSTM4 gene (null genotype), whereas lanes 3 and 6 contain samples obtained from patients exhibiting the GSTM1 and GSTM4 genes (non-null genotype). Lane 7, negative control. Lane 8, DL500 DNA Marker. GST, glutathione S-transferase.

Electrophoresis was performed for 30 min at 120 V. The gel image was captured using BDAdigital (Analytik Jena AG, Jena, Germany). The absence of amplifiable GSTM1 (in the presence of the GSTM4 co-amplified control) indicated a null genotype.

GSTM1 gene expression analysis by reverse transcription-quantitative PCR (RT-qPCR). Relative complementary DNA quantitation for GSTM1 and an internal reference gene (β -actin) was performed on formalin-fixed, paraffin-embedded surgical specimens from 95 patients. Following standard tissue sample deparaffinization using xylene and ethanol (20), samples were lysed in a tris-chloride, ethylenediaminetetraacetic acid, sodium dodecyl sulphate and proteinase K-containing buffer. RNA was then extracted with phenol-chloroform-isoamyl alcohol followed by precipitation with isopropanol in the presence of glycogen and sodium acetate. RNA was resuspended in diethyl pyrocarbonate water (Ambion, Inc.; Thermo Fisher Scientific) and treated with DNaseI (Ambion, Inc.; Thermo Fisher Scientific) to avoid DNA contamination. Complementary DNA was synthesized using the Moloney Murine Leukemia Virus retrotranscriptase enzyme. Template cDNA was added to Taqman Universal MasterMix (Applied Biosystems; Thermo Fisher Scientific, Inc.) in a 12.5- μ l reaction with specific primers and probes for each gene. The primer and probe sets

were designed using PrimerExpress 2.0 Software (Applied Biosystems; Thermo Fisher Scientific, Inc.) and the RefSeq sequences (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene>). The sequences of the primers and probes used were as follows: GSTM1 forward, 5'-CCCAGAGCA ACGCCATCT-3'; reverse, 5'-TCCACACGAATCTTCTCC TCTTC-3'; probe, (FAM)-5'-CTACATTGCCCGCAAGCA CAACCTG-3'-(TAMRA). β -actin (internal reference gene) forward, 5'-TGAGCGCGGCTACAGCTT-3'; reverse, 5'-TCC TTAATGTACACGCACGATTT-3'; probe, (FAM)-5'-ACC ACCACGGCCGAGCGG-3'-(TAMRA). Quantification of gene expression was carried out using the LineGene K system (Bioer Technology Co., Ltd.).

Relative gene expression quantification was calculated according to the comparative cycle threshold (Cq) method using β -actin as an endogenous control. Final results were determined as follows: $2^{-\Delta\Delta Cq}$, where ΔCq values of the sample are determined by subtracting the Cq value of the target gene from the value of the β -actin gene.

Statistical analysis. SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. P<0.05 was considered to indicate a statistically significant difference. All statistical tests were two-sided with a significance level of $\alpha=0.05$. Genotype data were categorized as follows: Homozygous carriers of the wild-type allele for GSTP1 polymorphism (Val105Val); carriers of the heterozygous allele or possessing one gene copy (GSTP1 Ile105Val); and carriers of the homozygous variant allele or possessing no copy (GSTP1 Ile105Ile). Patients with >1 copy of GSTM1 were considered to exhibit the non-null genotype, whereas patients possessing no copies of GSTM1 were considered to exhibit the null genotype. The OS of patients was calculated as the time between the start of the chemotherapeutic treatment and the date of the last follow-up or the date when the patient succumbed to disease. Kaplan-Meier survival function analysis was used to evaluate the association between GST genotypes and the OS of patients. The log-rank test was used to calculate the difference between OS and genotype. Cox proportional hazards regression analysis was used to calculate

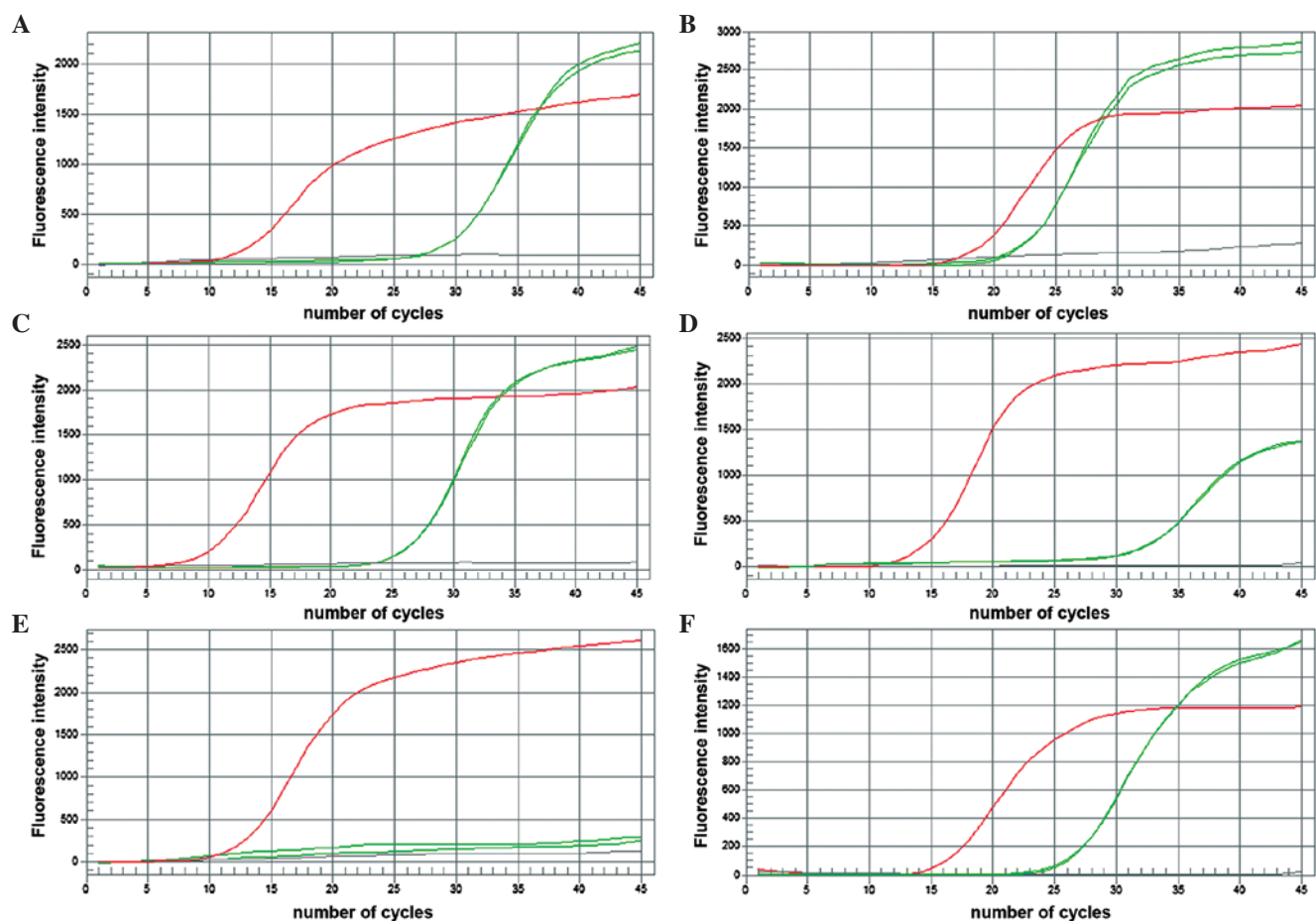


Figure 2. Amplification curves obtained during multiplex-polymerase chain reaction. (A) Sample containing one copy of the GSTM1 gene. The red line represents the positive reference amplification curve for GSTM1, the green line represents the amplification curve for GSTM1 and the black line represents the amplification curve for the negative reference for GSTM1. (B) Amplification curve for the internal reference gene, β -actin for the sample containing one copy of the GSTM1 gene. The red line represents the amplification curve for the positive reference for β -actin, the green line represents the amplification curve for β -actin and the black line represents the amplification curve for the negative reference for β -actin. (C) Amplification curves for samples containing two copies of the GSTM1 gene. (D) Amplification curve for the internal reference gene, β -actin, for the sample containing two copies of the GSTM1 gene. (E) Amplification curves for samples exhibiting the null phenotype. (F) Amplification curve for the internal reference gene, β -actin, for the sample exhibiting the null phenotype. GST, glutathione S-transferase.

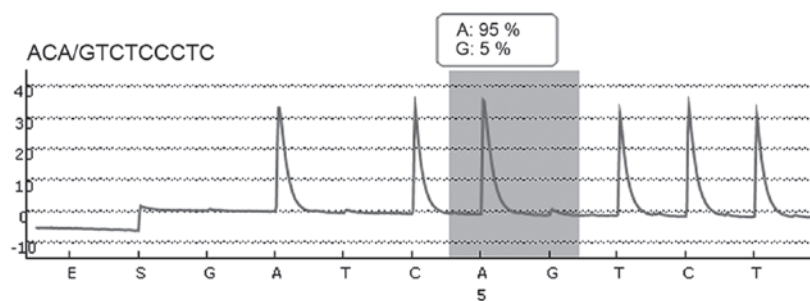


Figure 3. Genotype sequence of homozygous glutathione S-transferase P1 Ile105Val, ACA/GTCT (A/A). E, enzyme mixture (DNA polymerase, ATP sulfurylase, luciferase, apyrase); S, substrate (5-phosphoryl sulfate, fluorescein).

multivariable adjusted hazard ratios (HRs) and their corresponding 95% confidence interval (CI).

Results

The frequency of GSTM1 positive and negative individuals was determined by genotyping using multiplex-PCR as

shown in Fig. 1. Relative GSTM1 gene expression was also quantified (Fig. 2).

OS of patients with ovarian serous cystadenocarcinoma. Patients that received TP or an alternative form of chemotherapy were followed-up for a median time of 25.6 ± 14.3 months. Kaplan-Meier survival analysis demonstrated that the OS of

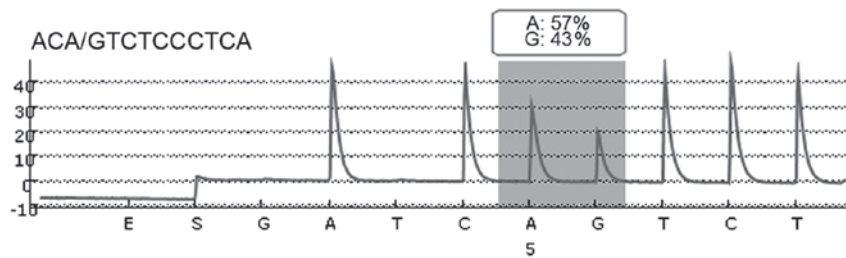


Figure 4. Genotype sequence of heterozygous carriers of glutathione S-transferase P1 Ile105Val, ACA/GTCT (A/G). E, enzyme mixture (DNA polymerase, ATP sulfurylase, luciferase, apyrase); S, substrate (5-phosphoryl sulfate, fluorescein).

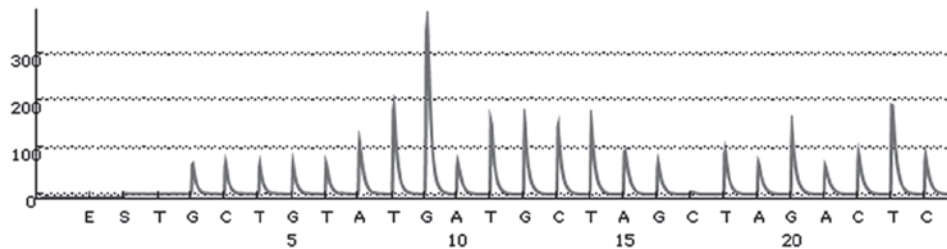


Figure 5. Genotype sequence of glutathione S-transferase M1 non-null, TTGGCCTTAGC/TAGGACTTTC. E, enzyme mixture (DNA polymerase, ATP sulfurylase, luciferase, apyrase); S, substrate (5-phosphoryl sulfate, fluorescein).

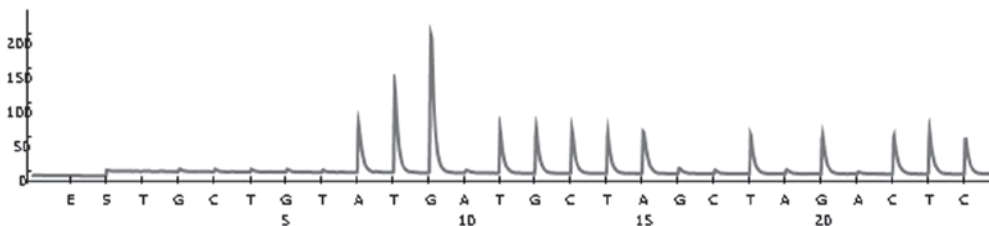


Figure 6. Genotype sequence of glutathione S-transferase M1 null, ATTGGGGTGCTATGCTC. E, enzyme mixture (DNA polymerase, ATP sulfurylase, luciferase, apyrase); S, substrate (5-phosphoryl sulfate, fluorescein).

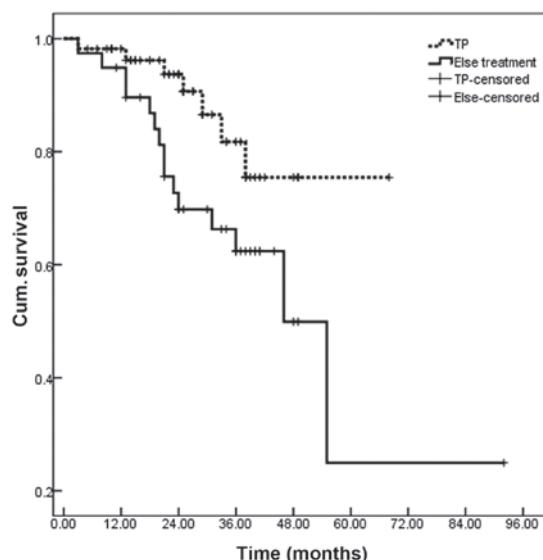


Figure 7. Overall survival of patients with ovarian serous cystadenocarcinoma that were administered with paclitaxel + carboplatin-based chemotherapy or an alternative chemotherapeutic drug (long-rank test, $P=0.035$). TP, paclitaxel + carboplatin-based chemotherapy; Else, non-TP chemotherapy; Cum., cumulative.

patients did not differ significantly between GSTP1 genotypes (log-rank test, $P=0.17$). Cox proportional hazards regression analysis revealed that, since the start of treatment, there was not a significant association between the GSTP1 isoleucine allele and OS for heterozygous carriers of the isoleucine allele (HR, 1.78; 95% CI, 0.77-4.12; $P=0.18$) and no homozygous carriers of the valine allele had been detected (HR, 0.00).

Kaplan-Meier survival analysis also revealed that the OS did not differ significantly between GSTM1 genotypes (log-rank test, $P=0.83$). Compared with carriers of two copies of GSTM1, patients with ≤ 1 copies of GSTM1 exhibited no decrease in the risk of mortality following chemotherapy [HR, 0.96, 95% CI, 0.37-2.51 ($P=0.94$) for patients with one copy of GSTM1 vs. HR, 0.71, 95% CI, 0.22-2.28 ($P=0.56$) for patients with no copies of GSTM1, respectively] (Table II; Figs. 3-6).

Overall, there were no associations between polymorphisms in GSTP1 and GSTM1 and the OS of patients following adjuvant chemotherapy. However, the OS of patients administered with TP was significantly increased, compared with patients that received other type of chemotherapy ($P=0.035$; Fig. 7).

Discussion

Platinum-based chemotherapy drugs are commonly used to treat certain solid tumors, including non-small cell lung and colorectal cancer, in order to induce the formation of DNA adducts, which contributes to the death of tumor cells (21). However, DNA repair and drug metabolism may hinder the prognosis of patients that are administered platinum-based chemotherapy (22,23).

Variations in the GSTP1 and GSTM1 genes have been extensively studied, due to their capacity to modulate the drug response in patients with cancer (24). GSTM1 deficiency has been reported to increase the risk of developing head-neck tumors, squamous cell carcinoma, and lung, colorectal, bladder and breast cancer (25-27).

The present study aimed to evaluate the association between genetic polymorphisms in the GSTP1 and GSTM1 genes and the OS of patients with ovarian serous cystadenocarcinoma treated with chemotherapy. GSTP1 is a member of the GST superfamily, and is important in the defense function of cells (28). GSTP1 Ile105Val polymorphism leads to a decreased ability in cell defense, thus increasing the sensitivity of an individual to platinum-based chemotherapy (29,30). However, the results of the Cox proportional hazards regression in the present study revealed no significant association between the GSTP1 Ile105Val isoleucine allele, and no homozygous carriers of the valine allele were detected. None of the polymorphisms in the GSTP1 and GSTM1 genes that were evaluated in the present study were observed to be significantly associated with the OS of patients that received chemotherapy TP regimen or an alternative chemotherapeutic drug. Similarly, a previous study conducted on 65 patients with colorectal cancer that received first-line chemotherapy of oxaliplatin did not identify an association between GSTP1 genotype and survival (16). However, a dose-dependent association between the number of GSTP1 valine alleles and survival in certain patients with cancer that received second-line oxaliplatin treatment was reported by a previous study, although no association between GSTM1 genotype and survival was observed (31). The present results revealed that there was not a significant association between the GSTP1 isoleucine allele and the risk of mortality for heterozygous carriers of the isoleucine allele (HR, 1.78; 95% CI, 0.77-4.12; $P=0.18$) and homozygous carriers of the valine allele (HR, 0.00; no homozygous carriers of the valine allele were detected) using Cox proportional hazards regression analysis.

Townsend and Tew (32) reported that GSTM1 is important in the detoxification of various carcinogens, and is associated with the metabolism of various chemotherapeutic agents. The results of the present study revealed that the OS of patients did not vary significantly according to their GSTM1 genotype. Patients with ≤ 1 copies of GSTM1 exhibited no decreased risk of mortality following chemotherapy, compared with patients with two copies of GSTM1.

In conclusion, the present study demonstrates that there is a decreased risk of mortality for chemotherapy-treated patients that have a reduced copy number of the GSTM1 allele. In addition, patients that were GSTP1 heterozygous carriers of the valine allele (A/G) had an increased risk of mortality, which is contrary to the results of other studies (33,34).

Tumor characteristics, including classification and stage, may have resulted in the inconsistent results observed. Additional studies with larger sample sizes are required to confirm the results of the present study.

Acknowledgements

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