Association of single nucleotide polymorphisms in the MVP gene with platinum resistance and survival in patients with epithelial ovarian cancer

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Abstract. The human major vault protein (MVP) has been linked to the development of multidrug resistance in cancer cells, and overexpression of MVP has been observed in ovarian cancer tissues. The aim of the present study was to investigate the association between single nucleotide polymorphisms (SNPs) in the MVP gene and the tumor response to platinum-based chemotherapy and survival of patients affected by epithelial ovarian cancer (EOC), in addition to confirm whether tetra-primer amplification-refractory mutation system (ARMS)-polymerase chain reaction (PCR) is an accurate genotyping method. For this purpose, two polymorphisms in the MVP gene, namely reference SNP (rs)1057451 and rs4788186, were selected from the data obtained by the International haplotype map (HapMap) Project regarding Chinese Han population, and were evaluated by tetra-primer ARMS-PCR. Upon validation by DNA sequencing, the association of these polymorphisms with platinum resistance, progression-free survival (PFS) and overall survival (OS) in patients with EOC was assessed. The results of tetra-primer ARMS-PCR were in agreement with those derived from DNA sequencing. No significant differences were observed between platinum-sensitive and platinum-resistant cohorts in terms of allele and genotype distribution of these two polymorphisms in the MVP gene, which were not associated with PFS or OS. However, a trend toward prolonged PFS was observed in patients carrying the heterozygous AG allele at the rs4788186 locus. These results suggest that rs1057451 and rs4788186 variants in the MVP gene are not associated with favorable therapeutic response to platinum or longer survival in Chinese Han patients affected by EOC. In addition, the data of the present study confirm that tetra-primer ARMS-PCR is a trustworthy and economical genotyping method.

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Key words: epithelial ovarian cancer, MVP, single nucleotide polymorphism, platinum resistance, survival, tetra-primer ARMS-PCR

Introduction

Ovarian cancer remains the leading cause of mortality due to gynecological cancer in the world (1). At present, platinum-based chemotherapy following surgery represents the standard therapy for those cases of ovarian cancer at advanced and high-risk early stages (2-5). A total of 70% of patients with ovarian cancer demonstrate a response to cytoreductive surgery followed by platinum-based chemotherapy (6); however, the majority of these patients will experience relapse. Thus, subsequent treatments are moderate in curative effect and typically short in duration (7).

Tumor cells that are resistant to a particular chemotherapeutic drug are also often observed to be not sensitive to a variety of structurally and functionally unrelated chemotherapeutic drugs (8). This phenomenon is known as multidrug resistance (MDR) (9). The mechanisms of MDR remain unclear, but it may be attributed to increased drug efflux (10-12), enhanced DNA damage repair (13,14), resistance to apoptosis (15,16), self-renewing tumor stem cells (17) and tumor microenvironment (18-20). Since 1995, when major vault protein (MVP) was identified to be identical to lung resistance-related protein (21), numerous studies have investigated the role of MVP in MDR (22-24).

MVP is the main component of vault, which contains two additional proteins known as vault poly-(adenosine diphosphate-ribose) polymerase and telomerase-associated protein 1, in addition to several small untranslated vault RNAs (25-28). The amino acid sequence of MVP is highly conserved among eukaryotic cells (21). MVP is present in normal tissues, including bronchus, digestive tract and macrophages, and in malignant cells, including acute myeloid leukemia, ovarian cancer and colon carcinoma (29). The high degree of conservation and ubiquitous expression of MVP suggest that this protein exerts crucial cellular functions (29). Increasing

evidence from previous studies has demonstrated that high messenger (m)RNA and protein levels of MVP are associated with resistance to antineoplastic agents and reduced survival in certain types of cancer, including ovarian cancer (22,30-32).

Single nucleotide polymorphisms (SNPs) are the most common type of genetic variation among the human population, and are often associated with inter-individual diversity in various malignancies regarding the patient's susceptibility to disease, drug response, toxicity and survival (33-36). However, to the best of our knowledge, the role of MVP SNPs in platinum resistance and prognosis of patients with ovarian cancer has not been reported thus far. Therefore, in order to assess whether SNPs in the MVP gene were associated with platinum resistance and survival in epithelial ovarian cancer (EOC), two polymorphisms were selected from the genotype data of Chinese Han population derived from the phase II International (haplotype map) HapMap Project (date of access to the database, August 14, 2014). The specific MVP genotypes were subsequently identified in the patients with EOC recruited for the present study, and the associations between these genotypes and the response to platinum-based regimens, progression-free survival (PFS) and overall survival (OS) exhibited by these patients were analyzed. Furthermore, the results of the present study confirmed the feasibility of tetra-primer amplification-refractory mutation system (ARMS)-polymerase chain reaction (PCR) as a genotyping tool.

Materials and methods

Patients and clinical data. A total of 116 Chinese Han female patients with EOC and Karnofsky performance status score ≥70 were recruited between June 2005 and February 2012, and treated at the Department of Oncology of The First Affiliated Hospital of Liaoning Medical University (Jinzhou, China). Written informed consent was obtained from all the patients included in the study, which was approved by the Ethics Review Committee of The First Affiliated Hospital of Liaoning Medical University. Following surgery, the patients were intravenously treated with taxol (135 mg/m^2 d1) or taxetere (75 mg/m^2 d1) and cisplatin (30 mg d2-4) or carboplatin (AUC 4-6 d2) at three or four weeks intervals for at least 3 cycles.

Follow-up examinations. Follow-up examinations were performed every 3 months or when patients presented with symptoms of progression and consulted a doctor. The examinations included pelvic examination, determination of the levels of cancer antigen (CA)125 in serum and pelvic computed tomography (CT) scanning. In addition, liver ultrasonography, thoracic or abdominal CT scanning and brain magnetic resonance imaging were conducted when necessary. Patients who exhibited persistent or progressive disease during the treatment or recurred within 6 months of completion of the platinum-based chemotherapy were defined as platinum-resistant (37). By contrast, patients who exhibited disease progression later than 6 months upon completion of the platinum-based therapy were considered to be platinum-sensitive. PFS was calculated as the duration, in months, from the date of histological diagnosis to the first sign of recurrence detected by physical examination, CA125 evaluation or radiographic inspection. OS was calculated as the duration, in months, from the date of histological diagnosis to mortality or last follow-up.

DNA extraction. Prior to the initiation of chemotherapy, genomic DNA was obtained from peripheral venous blood using TIANamp Blood DNA Kit (catalog no., DP318; Tiangen Biotech Co., Ltd., Beijing, China), according to the manufacturer's protocol. The purity and concentration of the extracted DNA were assessed by spectrophotometry using the BioPhotometer Plus (Eppendorf, Hamburg, Germany). The method yielded DNA of relatively high concentration (median = 39.3 μg/ml, range = 18.4-60.5 μg/ml) and purity (median absorbance $(A)_{260}/A_{280}$ ratio = 1.81, range = 1.46-2.37). The extracted DNA was stored at -80˚C until further use.

Genotyping. The tagging SNPs reference SNP (rs)1057451, rs4788186 and rs2288043 were selected from the genotype data of Chinese Han population derived from the International HapMap Project (HapMap Data Release 24/phase II Nov08, on National Center for Biotechnology Information B36 assembly, database SNP b126) to capture the maximum variation based on $r^2 \geq 0.8$ and minor allele frequency ≥0.05. Genotyping of the selected SNPs was performed by the cost-effective method of tetra-primer ARMS-PCR, as proposed by Ye *et al* (38). PCR was conducted in a total volume of 20 μl, which contained 1 μl template DNA, 0.5 μl each of the four primers (the concentration of the working solution was 10 μM; primers were designed by Primer Premier 5.0, Premier Biosoft International, Palo Alto, CA, USA), 10 μl 2XTaq PCR MasterMix (containing 0.1 U/μl Taq polymerase, 500 μM each deoxynucleotide, 20 mM Tris-HCl pH 8.3, 100 mM KCl, 3 mM $MgCl_2$ and other stabilizers and enhancers; catalog no., KT203; Tiangen Biotech Co., Ltd.) and 7 μl double-distilled (dd)H_2O. Table I indicates the primer sets used for the amplification of the three aforementioned polymorphisms. The reaction was performed on 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) under the following conditions: a denaturation step at 95˚C for 5 min, followed by 30 cycles of 95˚C for 30 sec, 30 sec at the corresponding annealing temperature (as described in Table I) and 30 sec at 72˚C, and a final extension at 72˚C for 10 min. All PCR products were added to 2% agarose gel (catalog no., 5260; Takara Biotechnology Co., Ltd., Dalian, China) which was stained with 1 μl/10 ml DuRed (catalog no., 009-500; Fanbo Biochemicals Co. Ltd., Beijing, China). DL1,000 DNA marker (catalog no., 3591Q; Takara Biotechnology Co., Ltd.) was also added into the well as a reference for the targeted DNA bands. The products and marker were defined by agarose gel electrophoresis with the PowerPac™ 3000 system (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and subsequently visualized using the 2500R Gel Imaging System (Tanon Science and Technology Co., Ltd., Shanghai, China).

Genotyping validation. To validate the accuracy of the results obtained by tetra-primer ARMS-PCR analysis, a number of representative samples of each genotype were selected, and conventional PCR was conducted in a total volume of 20 μl, which contained 2 μl template DNA, 1 μl each outer primer, 10 μl 2XTaq PCR MasterMix and 6 μl ddH_2O. The reaction was performed on a 2720 Thermal Cycler (Applied Biosystems) with a denaturation step at 95˚C for 5 min, 30 cycles of 95˚C for 30 sec, the corresponding annealing temperature (described in Table I) for 30 sec, and 72˚C for 30 sec, followed by a final extension at 72˚C for 10 min. The PCR products were then sequenced by the Sanger method [reagents included the following: BigDye®

Table I. PCR primers and conditions.

Polymorphism	PCR primer, 5'-3'	Ta, ˚C	Amplicon size, bp	Sequencing primer, 5'-3'
rs1057451				
F inner primer (G allele)	ATTGATGAAGATCAGGGaTG	54	288 (G allele)	TTCCACTTGTCCTCCCTC
R inner primer (T allele)	CAGGAACCAGGCTTCAaA		420 (T allele)	
F outer primer	ATTGAGGGCACTTAACACTAC		671 (outer primers)	
R outer primer	GACTCAGGAATTGCCAACA			
rs4788186				
F inner primer (G allele)	TAAAGCATAGGAAAGAGtCG	55	449 (G allele)	F outer primer
R inner primer (A allele)	TGAGCTGTGTCTATGTTCaCT		211 (A allele)	
F outer primer	ACCCTACCCTTGCTCACA		620 (outer primers)	
R outer primer	AGCCCATCCTGACCTTAC			
rs2288043				
F inner primer (A allele)	CATAGATGCCCTCGTTCgCA	55	292 (A allele)	F outer primer
R inner primer (G allele)	GCCAGGCCATCCCTCTAGtC		80 (G allele)	
F outer primer	CTCACTCCCAGCCATTACCTTTC		333 (outer primers)	
R outer primer	GGCACTGACCCTAACCTCACG			

The lower-case letter in the inner primer sequences represents a deliberate mismatch base, which was introduced at position -2 or -3 from the 3'-terminus, according to the principle described by Little (39). PCR, polymerase chain reaction; rs, reference single nucleotide polymorphism; F, forward; R, reverse; Ta, annealing temperature; bp, base pairs.

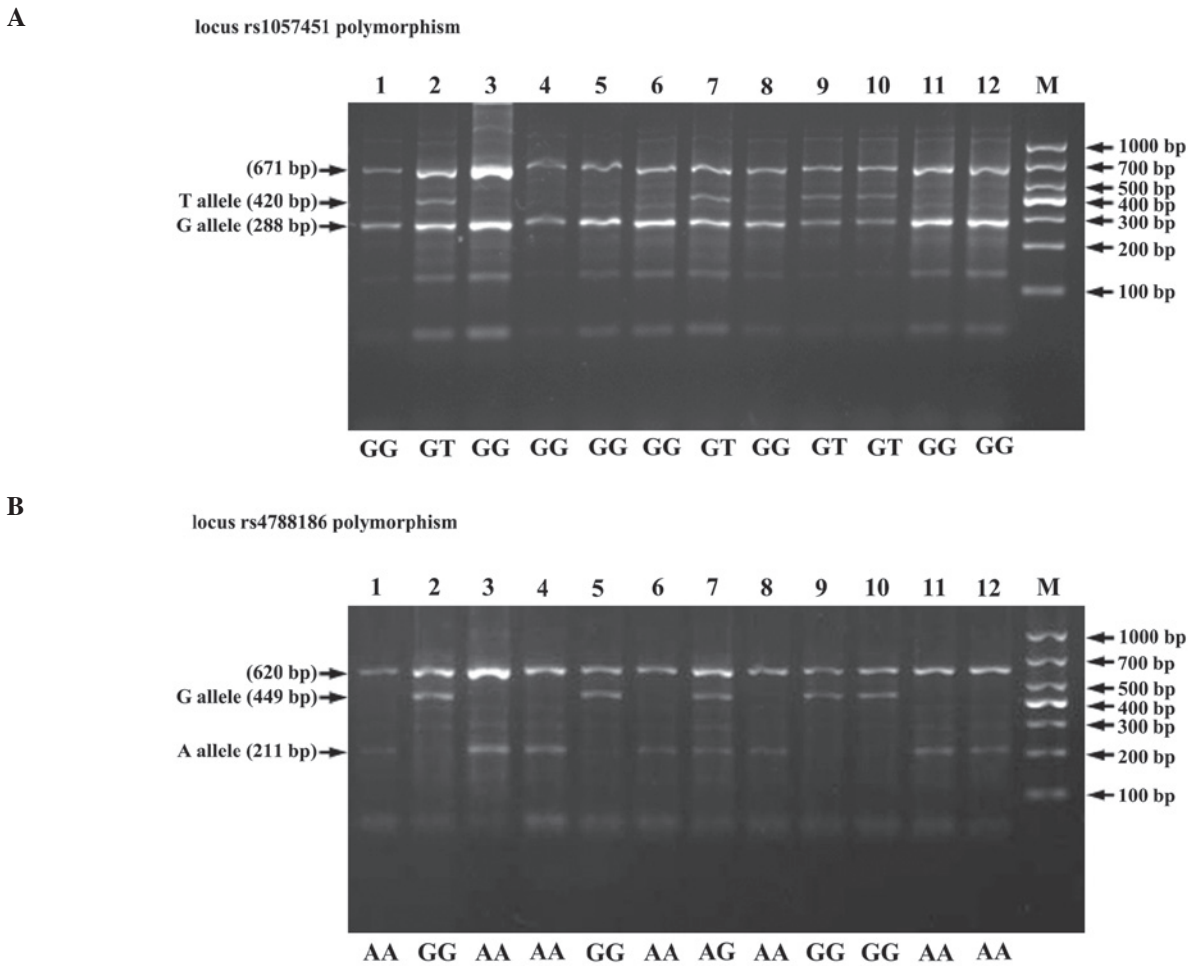

Figure 1. Results of tetra-primer amplification refractory mutation system-polymerase chain reaction for (A) locus rs1057451 polymorphism and (B) locus rs4788186 polymorphism. The extra bands not marked with an arrow represent nonspecific amplification. M, DL1,000 DNA marker; bp, base pairs; rs, reference single nucleotide polymorphism.

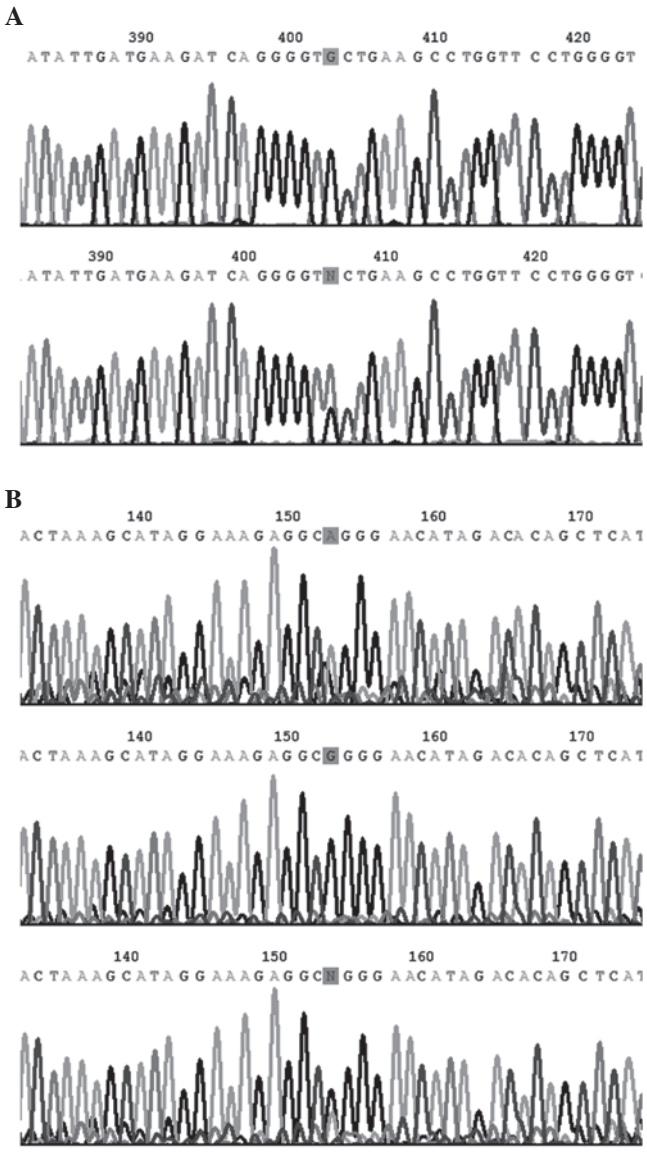

Figure 2. Results of DNA sequencing. The highlighted region in the chromatogram represents the polymorphic nucleotide. (A) Locus rs1057451 polymorphism. (B) Locus rs4788186 polymorphism. rs, reference single nucleotide polymorphism.

Table II. Clinicopathological parameters of 116 patients with epithelial ovarian cancer.

Characteristics	No. (%)
Age at diagnosis, years	
≤50	53 (45.7)
>50	63 (54.3)
FIGO stage	
I	8 (6.9)
II	17 (14.7)
III	57 (49.1)
IV	34 (29.3)
Tumor grade	
G1	3 (2.6)
G2	29 (25.0)
G3	38 (32.8)
Unknown	46 (39.7)
Histological type	
Serous	83 (71.6)
Mucinous	4 (3.4)
Endometrioid	14 (12.1)
Clear cell	10 (8.6)
Other[a]	5 (4.3)
Chemotherapy regimen	
TAX+DDP	54 (46.6)
TAX+CBP	29 (25.0)
TXT+DDP	12 (10.3)
TXT+CBP	11 (9.5)
Other platinum-based regimen	10 (8.6)
Chemotherapy response	
Resistance	31 (26.7)
Sensitivity	85 (73.3)

[a]Other histological types of epithelial ovarian cancer included undifferentiated and transitional cell carcinoma. TAX, taxol; TXT, taxetere; DDP, cisplatin; CBP, carboplatin; FIGO, International Federation of Gynecology and Obstetrics.

Direct Cycle Sequencing kit; BigDye Terminator 5X Sequencing Buffer and Hi-Di Formamide (all purchased from Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA); the equipment used was a 3730xl DNA Analyzer (Applied Biosystems)], using their respective forward outer primers as sequencing primers, with the exception of rs1057451, whose sequence differs from the others, since it contains two poly-deoxyribonucleotide structures between the two outer primers. Therefore, in order to avoid those structures, another reverse primer was designed for DNA sequencing purposes (Table I). The reverse complement sequence is presented in Fig. 2A.

Statistics. The association between each polymorphism and the clinicopathological parameters of the patients was assessed by Pearson's χ^2 test or Fisher's exact test. The allele and genotype distribution of the investigated SNPs in platinum-resistant and platinum-responsive cohorts was compared using Pearson's χ^2 test or Fisher's exact test. The combined effect of the polymorphisms on tumor response was investigated by haplotype analysis using the SHEsis software platform (date of access, November 3, 2014), which is available at http://analysis.bio-x.cn/myAnalysis.php (40). Univariate survival analysis was determined using the Kaplan-Meier method, and survival curves were compared by log-rank test. Multivariate survival analysis was performed by the Cox proportional hazards regression model to adjust for tumor stage, histological type and chemotherapy response. Statistical analyses were performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). All statistics were two-sided, and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Genotyping by tetra-primer ARMS-PCR. The tetra-primer ARMS-PCR method was successfully applied to genotype the

Table III. Genotype and allele frequencies of polymorphisms of the major vault protein gene in platinum-resistant and platinum-responsive cohorts.

Polymorphism	Non-responder, no. (%)	Responder, no. (%)	P-value
rs1057451			
GG	26 (83.9)	76 (89.4)	0.520
GT	5 (16.1)	9 (10.6)	0.520
G	57 (91.9)	161 (94.7)	0.533
T	5 (8.1)	9 (5.3)	0.533
rs4788186			
AA	15 (48.4)	48 (56.5)	0.606
AG	13 (41.9)	32 (37.6)	0.606
GG	3 (9.7)	5 (5.9)	0.606
AG+GG	16 (51.6)	37 (43.5)	0.439
AA+AG	28 (90.3)	80 (94.1)	0.439
A	43 (69.4)	128 (75.3)	0.363
G	19 (30.6)	42 (24.7)	0.363

rs, reference single nucleotide polymorphism.

Table IV. Major vault protein gene haplotypes and patients' response to platinum chemotherapy.

Haplotypes[a]	Responder (frequency)	Non-responder (frequency)	OR (95% CI)	P-value
GA	128 (0.753)	43 (0.694)	1.347 (0.708-2.561)	0.363
GG	33 (0.194)	14 (0.226)	0.826 (0.408-1.674)	0.595
TA	0 (0.000)	0 (0.000)	-	-
TG	9 (0.053)	5 (0.081)	0.637 (0.205-1.981)	0.433

[a]Haplotypes whose frequency was <0.03 were ignored in the analysis. The order of the polymorphisms was rs1057451, rs4788186, and indicates the Chromosome Reference Sequence from the SNP database of National Center for Biotechnology Information. Global χ^2=1.020, df=2, P=0.600. OR, odds ratio; CI, confidence interval; rs, reference single nucleotide polymorphism; df, degrees of freedom.

Table V. Multivariate survival analysis by Cox proportional hazards regression model.

		Progression-free survival			Overall survival		
Polymorphism	Genotype	HR	95% CI	P-value	HR	95% CI	P-value
rs1057451	GG	Reference	-	-	Reference	-	-
	GT	0.508	0.226-1.143	0.102	0.586	0.245-1.405	0.231
rs4788186	AA	Reference	-	-	Reference	-	-
	AG	0.600	0.358-1.007	0.053	0.803	0.482-1.337	0.399
	GG	0.936	0.386-2.270	0.883	1.037	0.427-2.516	0.937
	AA	Reference	-	-	Reference	-	-
	AG+GG	0.650	0.402-1.051	0.079	0.839	0.519-1.356	0.474
	GG	Reference	-	-	Reference	-	-
	AA+AG	0.888	0.370-2.129	0.790	0.882	0.371-2.093	0.775

HR, hazard ratio; CI, confidence interval; rs, reference single nucleotide polymorphism.

selected SNPs (Fig. 1). The results from DNA sequencing were consistent with those from tetra-primer ARMS-PCR (Fig. 2).

Genotype frequency distribution of the two polymorphisms and their association with clinicopathological parameters.

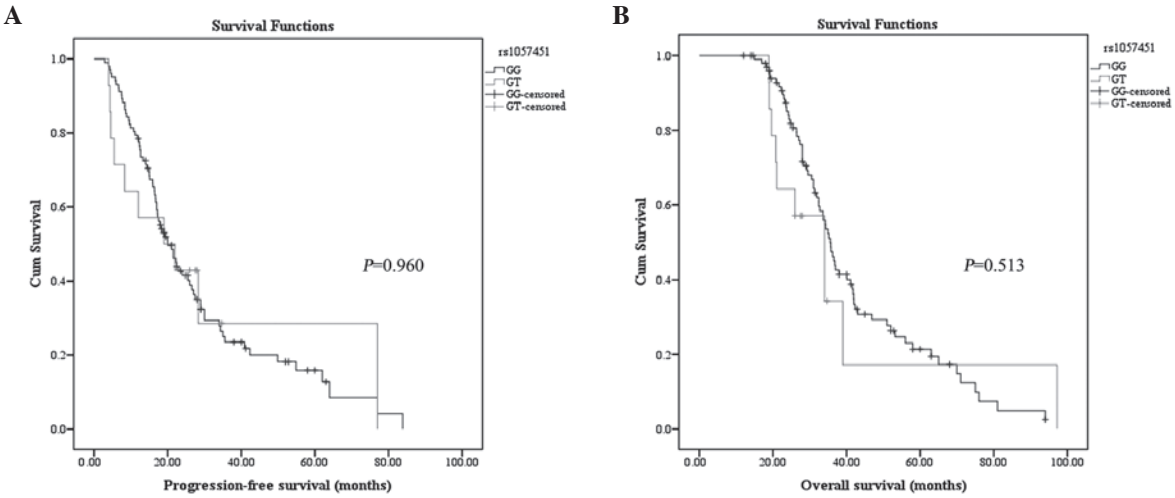

Figure 3. Kaplan-Meier analysis of (A) progression-free survival and (B) overall survival classified by the rs1057451 polymorphism in the major vault protein gene. Cum, cumulative; rs, reference single nucleotide polymorphism.

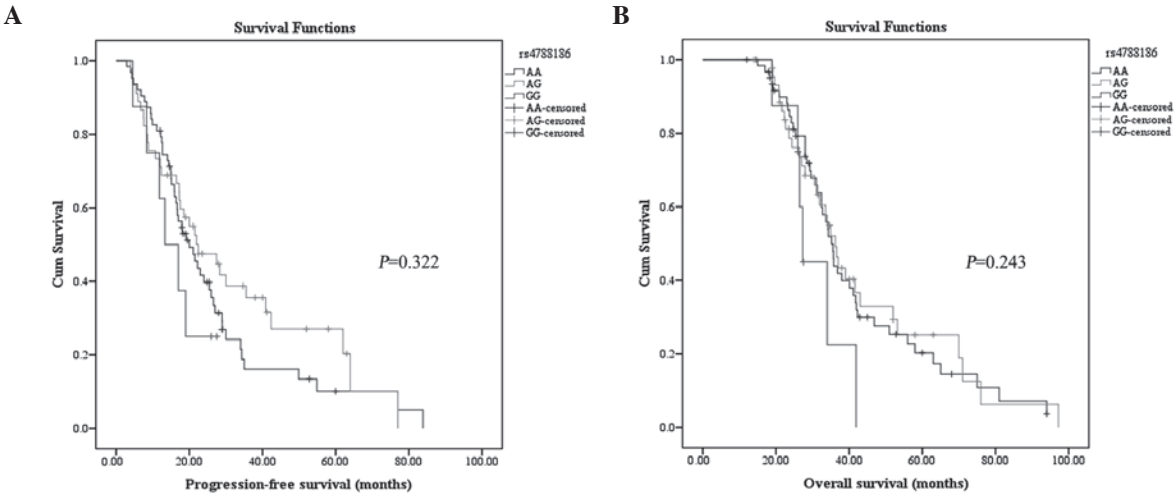

Figure 4. Kaplan-Meier analysis of (A) progression-free survival and (B) overall survival classified by the rs4788186 polymorphism in the major vault protein gene. Cum, cumulative; rs, reference single nucleotide polymorphism.

The clinicopathological parameters, including the FIGO stage (41) of the patients are summarized in Table II. Polymorphism of locus rs2288043 was not analyzed, since the mutant allele for this locus was absent in the patients selected for the present study. By contrast, the other two polymorphisms, rs1057451 and rs4788186, were evaluated. The genotype frequencies in the studied patient population were as follows: MVP locus rs1057451, 87.9% GG and 12.1% GT; and MVP locus rs4788186, 54.3% AA, 38.8% GA and 6.9% GG. Neither of these two polymorphisms in the MVP gene was associated with age or tumor stage, grade or histological type (data not shown). The overall response rate of platinum-based chemotherapy was 73.3%, with no significant difference in response rate among all platinum-based regimens (Fisher's exact test, P=0.696) (data not shown).

Association between the two polymorphisms and platinum resistance. There was no significant difference in genotype and allele distributions of the studied SNPs between platinum-resistant and platinum-responsive patients (Table III). Additionally, haplotype analysis did not reveal any association between haplotypes and platinum resistance (Table IV).

Association between rs1057451 polymorphism and survival. None of the patients was observed to be homozygous for the minor TT allele. Univariate Kaplan-Meier analysis demonstrated that PFS did not differ between patients carrying the GG genotype and those carrying the GT genotype (log-rank test, P=0.960; Fig. 3A). OS did not differ either between the two genotypes (P=0.513, Fig. 3B). When adjusting for other potential confounding variables in a multivariate Cox regression model, the locus rs1057451 polymorphism had no significant predictive value for PFS (P=0.102; Table V) or OS (P=0.231; Table V).

Association between rs4788186 polymorphism and survival. Kaplan-Meier analysis of the rs4788186 polymorphism did

not reveal a significant difference in PFS between genotypes AA, AG and GG (P=0.322; Fig. 4A). Similar trends were observed in OS for these genotypes at this locus (P=0.243; Fig. 4B). When the AG and GG subgroups were combined, the log-rank test failed to detect any significant difference in PFS (log-rank test, P=0.453) or OS (log-rank test, P=0.905). There was no statistically significant difference in PFS (log-rank test, P=0.278) or OS (log-rank test, P=0.097) between patients carrying the GG genotype and those carrying a non-GG genotype (data not shown). Multivariate Cox regression analysis revealed that rs4788186 variants were neither associated with PFS nor with OS, once adjusted by International Federation of Gynecology and Obstetrics stage, histological type and chemotherapeutic response (Table V), although a trend toward reduced risk of progression was observed for patients with the AG genotype, compared with those exhibiting the AA genotype (hazard ratio, 0.600; 95% confidence interval, 0.358-1.007; P=0.053; Table V). The multivariate survival analysis performed for genotypes AG and GG, compared with the major AA genotype, did not reveal any statistically significant difference in PFS or OS (Table V). Comparison of PFS and OS between patients with the GG genotype and those with a non-GG genotype did not reveal any significant difference (Table V).

Discussion

MVP is considered to be important in the treatment response and prognosis of various tumors (22,23,42). The majority of clinical studies published to date have evaluated the mRNA or protein levels of MVP in order to investigate the association of MVP with platinum resistance and survival in EOC (22,43). To the best of our knowledge, there are no previous studies on the association between genetic variants of the MVP gene and platinum resistance in patients with EOC. To investigate the role of MVP polymorphisms in predicting platinum response and survival, three SNPs from the genotype data derived from the phase II HapMap Project of Han Chinese population were selected in the present study. The results indicated that the mutant allele for locus rs2288043 was not present in any of the patients enrolled in the study, and neither platinum resistance nor survival were associated with the other two polymorphisms (rs1057451 and rs4788186).

These findings may be explained by the following factors: Firstly, it is important to remember that MDR is certainly involved in various mechanisms (44). Thus, the complex mechanisms involved in MDR may represent one of the obstacles in predicting the treatment response and survival by merely several genetic polymorphisms. Secondly, although MVP has been previously implicated in drug resistance, several studies have reported conflicting results (45,46). Certain studies have demonstrated that MVP has no influence on intracellular drug distribution or chemoresistance (47-50). Similarly, Siva *et al* (51) demonstrated that the upregulation of MVP is not sufficient to confer an MDR phenotype. In addition, recent studies have correlated MVP with several signaling pathways (52-56) and immune responses (57-60), which imply that the function of MVP may be more complex than expected. Thirdly, the inability to find an association between variants of the MVP gene and platinum resistance or survival in the present study may be due to the limited number of patients enrolled in the study, since the number of patients in the subgroup was below the statistical threshold. Therefore, to demonstrate the independent effect of the aforementioned polymorphisms on the chemotherapy response and prognosis of patients with EOC, a large and homogeneous cohort of patients, such as advanced stage cases following optimal cytoreductive surgery, may be required. Finally, the absence of correlations observed in the present study may be due to the SNPs selected, which may not tag the SNPs responsible for the upregulation of the protein levels of MVP.

The tetra-primer ARMS-PCR method applied in the present study is more cost- and time-effective than other commonly used genotyping methods such as PCR-restriction fragment length polymorphism and TaqMan assays (61,62). In addition, the tetra-primer ARMS-PCR method has also been demonstrated to possess a high reliability in genotyping (38,61,62), thus it may be used for detecting polymorphisms. The limitations of the present study were the absence of subgroups analysis due to the small number of patients participating in the study, and the absence of toxicity analysis.

In conclusion, no associations between the two polymorphisms in the MVP gene analyzed in the present study and platinum-resistance or survival were observed in the patients with EOC who were recruited for the study. Furthermore, the present study has demonstrated that tetra-primer ARMS-PCR is a reliable method for genotyping.

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