Detection of OPCML methylation, a possible epigenetic marker, from free serum circulating DNA to improve the diagnosis of early-stage ovarian epithelial cancer

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Abstract. The aim of the present study was to identify the appropriate DNA sequence and design high-quality primers for methylation-specific polymerase chain reaction (MSP). These primers may be used to examine and identify patients with early-stage epithelial ovarian carcinoma (EOC). Opioid binding protein/cell adhesion molecule like (OPCML), Runt-related transcription factor 3 and tissue factor pathway inhibitor 2 were selected as possible molecular markers. MSP primer sets were designed to monitor the methylation of the three markers. Free circulating DNA (fcDNA) from 194 patients with epithelial ovarian carcinoma and healthy donors were templates in the nested MSP. OPCML MSP was effective with respect to screening methylated fcDNA. One-way ANOVA P-values indicated that the difference in cancer antigen 125 (CA125), a biomarker for EOC diagnosis, level between early EOC and healthy donors was not significant. The methylation of OPCML was significantly altered in early-stage EOC compared with healthy donors ($P<0.0001$), and this supported the hypothesis that specific fcDNA methylation was able to distinguish patients with early-stage EOC from healthy donors. With respect to detecting early EOC, compared with the results of the CA125 test, MSP increased the κ coefficient from 0.140 to 0.757. Therefore, OPCML combined with fcDNA may be used to establish an improved clinical assay compared with the current CA125 test.

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Introduction

Ovarian cancer is the most lethal type of female reproductive system cancer. In the United States of America, 14,180 patients were estimated to succumb to ovarian cancer and 21,290 new patients were estimated to be diagnosed in 2015 (1). A histological study indicated that 90-95% of ovarian cancer cases were of epithelial ovarian carcinoma (EOC) (2). The International Federation of Gynecology and Obstetrics (FIGO) staging system categorizes ovarian cancer into four stages (I-IV) from the least to the most advanced (3). The more advanced the ovarian cancer, the lower the five-year survival rate of patients. The five-year survival rate at FIGO stage I is 90-95%, stage II is 70-80%, stage III is 20-50% and Stage IV is only 1-5% (4). However, patients with early-stage EOC often do not exhibit noticeable symptoms and it is therefore difficult to diagnose. Thus, the majority of patients with EOC are diagnosed at an advanced stage.

Cancer antigen 125 (CA125), also termed MUC16, is a plasma membrane glycoprotein on ovarian epithelial cells and the most common type of biomarker currently used for EOC diagnosis (5). A CA125 level ≥35 U/ml is a marker of ovarian cancer (6). Ozols (7) reported that the sensitivity of the CA125 blood test is 50% with respect to the early stages and 80% with respect to the advanced stages of EOC. Therefore, the current diagnosis assay presents an issue for patients, as it cannot efficiently inform optimally-timed cancer treatment. An improved assay that is sensitive to early-stage EOC has yet to be established. The present study aimed to explore certain alternative markers, with potentially improved sensitivity to early and advanced malignancy.

Serum free circulating DNA (fcDNA) is a type of sample that may be accessed without surgical biopsy. Numerous studies have identified a correlation between fcDNA and cancer, including ovarian, uterine, colorectal, breast, lung and prostate cancer, cervical and malignant gastrointestinal

tumors, glioma, hepatocellular carcinoma, metastatic melanoma, leukemia and lymphoma (8-13). The characteristics of fcDNA may also make this type of DNA a promising tool for the diagnosis of early-stage EOC.

In addition, in human somatic cells, methylation primarily occurs at the cytosine of the 5'-C-phosphate-G-3' (CpG) site (14,15). The regulatory regions of genes are rich in unmethylated CpGs, which form CpG islands (16). A total of three tumor suppressor genes, Runt-related transcription factor 3 (RUNX3), tissue factor pathway inhibitor 2 (TFPI2) and opioid binding protein/cell adhesion molecule-like (OPCML), were considered as candidate biomarkers in the present study. RUNX3 is a transcription factor, which has methylated CpG islands in various types of cancer with percentages ranging from 73 to 2.5% (17). A previous study reported that 53.1% patients with primary ovarian cancer exhibited methylated RUNX3 (18). The expression of TFPI2, a serine proteinase inhibitor, is inversely correlated with the degree of tumor malignancy (19). OPCML is a plasma membrane protein functioning as an opioid receptor. Previous studies revealed that the expression of these genes was suppressed by DNA methylation of their regulatory regions in ovarian cancer (19-21).

Herman *et al* (22) developed a type of polymerase chain reaction (PCR), methylation-specific PCR (MSP), to identify the methylation status of CpG islands. This method requires two pairs of primers, the M pair and the U pair. The M pair corresponds to the modified and methylated sequence, and the U pair corresponds to the modified and unmethylated sequence. Cytosine of CpG in the U pair converts to thymine. A primer design program for MSP, MethPrimer, is available at present (23). The present study investigated potential new biomarkers on fcDNA to improve the sensitivity, specificity and accuracy of early stage EOC screening.

Materials and methods

Patients and sample collection. Patients that were admitted to the Affiliated Hospital of Guiyang Medical College (GMCAH; Guiyang, China) between June 2011 and December 2014, comprising of 80 healthy donors, 43 donors with benign ovarian tumors and 71 donors with ovarian epithelial carcinoma, provided informed consent to take part in the present study. The healthy donor samples were collected from the Blood Transfusion Station of GMCAH. Their median age was 46 years (range 18-61 years). The median age of the patients with benign tumors was 34 years (range 18-52 years) and of the patients with malignant cancer was 54 years (range 28-67 years). The blood and tissue samples of the patients with ovarian cancer were collected and processed in the Department of Medical Laboratory of GMCAH, and stored at -80˚C prior to use. Based on the FIGO staging system, out of the 71 patients with ovarian cancer, 39 had stage I or II, and 32 patients had stage III or IV disease. The present study regarded stages I and II as early-phase cancer, and stages III and IV as advanced-phase cancer. A total of 3 ml blood was collected from each patient and the serum was isolated by 3,500 x *g* centrifugation at 4˚C for 5 min. In addition, 100 mg tissue sample from each patient was collected during surgery and then soaked in 4˚C RNAlater Stabilization Solution (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) overnight prior to storage at -80˚C until required.

Cell culture. The epithelial ovarian cancer cell line HO8910 was acquired from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). This cell line was cultured in RPMI-1640 media (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, China), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Cell cultures were maintained in an atmosphere with 5% CO_2 at 37˚C.

DNA isolation and quantification. fcDNA from sera collected from the aforementioned 194 healthy donors and patients with ovarian epithelia carcinoma or benign ovarian tumors was extracted using the QIAamp DNA blood mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. Briefly, 200 μl patient serum was mixed with 200 μl Buffer AL plus 20 μl QIAGEN Protease from the kit described above and incubated at 56˚C for 10 min. The mixture was applied to the QIAamp Mini spin column subsequent to the addition of 200 μl 100% ethanol. Centrifugation at 6,000 x *g* at room temperature for 1 min was performed to remove the majority of impurities and bind the DNA to the column. The column was washed withAW1 and AW2 buffers. Finally, the DNA was collected in buffer AE. Genomic DNA from 3 randomly-chosen patient tissues from each category and HO8910 cell cultures was purified with a QIAamp DNA mini kit (Qiagen GmbH) according to the manufacturer's protocol. To calculate the DNA concentration, absorbance at a wavelength of 260 nm was measured with a SmartSpec Plus spectrophotometer (Bio-Rad Laboratories, Inc.) and compared with a standard curve. Only DNA with an A260/A280 ratio of 1.7-2.0 was used in the present study.

Methylation status analysis of tumor suppressor genes. The cytosine residues in target DNA were converted into uracil and the product was purified with an EpiTect Bisulfite kit (Qiagen GmbH). In total, 1 μg fcDNA was mixed with 85 μl Bisulfite mix in a 200-μl PCR tube. The mixture was incubated at 60˚C for 25 min, 1 h 25 min and 2 h 55 min with 5 min denaturation at 95˚C between each incubation in a C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Inc.). Once the reaction was completed, the DNA was recovered by EpiTect spin column.

Methylation of DNA was detected using nested PCR. The PCR primers used in this study were listed in Table I. The PCR reactions included 0.2 μM forward and reverse primers, 0.1 μg template, 12.5 μl Taq 2X Master mix (New England BioLabs, Inc., Ipswich, MA, USA) and nuclease-free water. The total volume was 25 μl. PCR mix without template DNA was used as a negative control. The external round PCR reaction conditions were: i) Initialization at 95˚C for 5 min; ii) denaturation at 95˚C for 30 s, annealing at 50˚C for 1 min, elongation at 72˚C for 45 s, total 25 cycles; iii) final elongation at 72˚C for 10 min. The internal round PCR reaction conditions were similar to the external round PCR, except that the total cycle number was 35.

DNA gel quantification analysis. The nested MSP products were separated by gel electrophoresis. Images of the DNA bands observed were captured using the Gel DocXR+ Imaging System (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Table I. Primer sets used in the present study.

Protein	Sequence
OPCML	
M system	5'-ACC **G**CT AAA C**G**T AAC **G**TC C**G**-3'
U system	5'-ACC **A**CT AAA C**A**T AAC **A**TC C**A**-3'
Nested F	5'-AAA AGT TTT AAG GYG GGG AT-3'
Nested R	5'-TCA AAA CTA CTC CCA AAA AA-3'
RUNX3	
M system	5'-T**C**G GGA **C**GT ATA ATA TTT T**C**G-3'
U system	5'-T**T**G GGA **T**GT ATA ATA TTT T**T**G AG-3'
Nested F	5'-GGT TAG GGG TTT TTT AAT TTT AAT TYG-3'
Nested R	5'-CAC CRC RAA TAA AAT ACR AAC-3'
TFPI2	
M system	5'-TGG **C**GA AGT TGT TAT TAG T**C**-3'
U system	5'-T TGG **T**GA AGT TGT TAT TAG T**T**-3'
Nested F	5'-ATT TTT TGT AGA AAG TGA GAT G-3'
Nested R	5'-AAT ACA CAC AAA ACT ACC AC-3'

Letters that are bold and underlined indicate sites where it was initially believed methylation would occur. OPCML, opioid binding protein/cell adhesion molecule like; TFPI2, tissue factor pathway inhibitor 2; RUNX3, Runt-related transcription factor 3; M, methylated; U, unmethylated; F, forward; R, reverse.

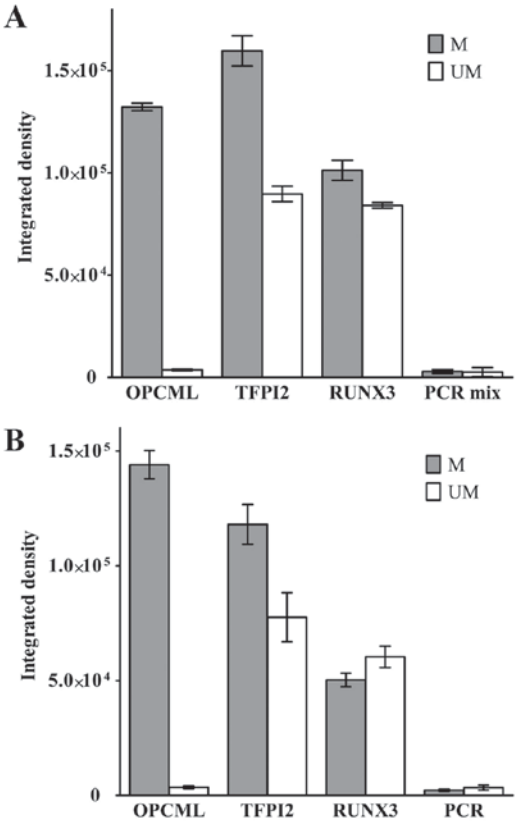

Figure 1. MSP primer set detects OPCML methylation status in epithelial ovarian cancer HO8910 cell line. (A) Three sets of nested MSP primers, corresponding to OPCML, TFPI2 and RUNX3, and separate reactions were set up to test each single set of primers. (B) Three sets of primers were mixed in one MSP reaction. Integrated density represented relative PCR product amount. PCR mix was the negative control, without DNA template. Error bars represent standard deviation of the mean. OPCML, opioid binding protein/cell adhesion molecule like; TFPI2, tissue factor pathway inhibitor 2; RUNX3, runt-related transcription factor 3; PCR, polymerase chain reaction; MSP, methylation-specific PCR; M, methylated; UM, unmethylated.

JPEG-formatted images were used for quantification analysis by ImageJ software [National Institutes of Health (NIH), Bethesda, MD, USA]. Based on expected PCR product size, DNA bands in images were selected and the Analyze>Set Measurements command in ImageJ was used to record area, mean gray value and integrated density. An integrated density <9,000 was regarded as a negative result.

CA125 assay. A CA125 II assay kit was purchased from Ortho Clinical Diagnostics, Inc., Raritan, NJ, USA. All assays were performed according to the protocol of the manufacturer. The principle of this assay was to evaluate CA125 antigen expressionin samples using streptavidin-coated wells and biotinylated M11 mouse anti-CA125 antibody, which was then incubated with horseradish peroxidase (HRP)-labeled mouse anti-CA125 antibody to form a sandwich. HRP catalyzed the signal reagent to generate luminescence, which was monitored using a VITROS 5600 Integrated System (Ortho Clinical Diagnostics, Inc.).

Statistical analysis. Microsoft office Excel 2013 (Microsoft Corporation, Redmond, WA, USA) and GraphPad Prism version 6 (GraphPad Software, Inc., La Jolla, CA, USA) were used for data calculation, and table and chart construction. Data obtained from small sample sizes were presented as the mean ± standard deviation. Data obtained from large sample sizes were presented as a box and whisker plot, which was analyzed by one-way analysis of the variance (ANOVA) to statistically determine the significance of differences between groups. $P<0.05$ was considered to indicate a statistically significant difference. Cohen's κ coefficient was used to compare the degree of agreement between observed and expected results. The following κ values were considered to indicate the following: <0.20, poor agreement; 0.20-0.40, fair agreement; 0.40-0.60, moderate agreement; 0.60-0.80, good agreement; and 0.80-1.00, very good agreement.

Results

Evaluating effectiveness of RUNX3, TFPI2 and OPCML MSP primer sets to HO8910 cell line genomic DNA. To verify if the chosen primers were able to evaluate known methylation of tumor suppressor genes in patients with EOC, genomic DNA was extracted from HO8910 cell culture. HO8910 is an established human epithelial ovarian carcinoma cell line. Bisulfite-modified genomic DNA from the HO8910 cells and three sets of primers were used in the MSP. The three sets of primers corresponded to non-coding regions of the tumor suppressor genes OPCML, TFPI2 and RUNX3. Fig. 1A demonstrates the quantitative results of separate MSP experiments using each set of primers. Each reaction was repeated three times. These results were analyzed by the NIH Java-based image processing software, ImageJ. A 190-bp PCR

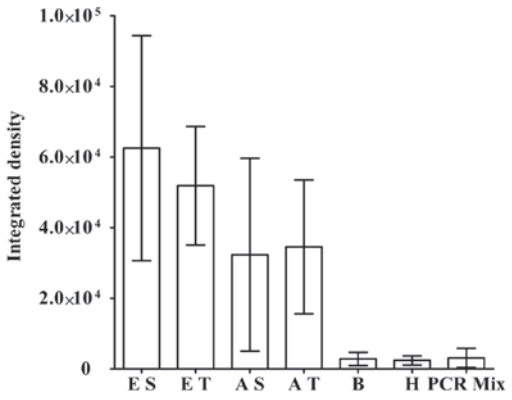

Figure 2. OPCML MSP primer set may also be valid for samples from patients with early- and advanced-stage EOC. Nested MSP reactions examined OPCML methylation status in various types of patients with EOC. Relative PCR product amount was compared with respect to integrated density. Values indicate the mean ± standard deviation. OPCML, opioid binding protein/cell adhesion molecule like; EOC, epithelial ovarian carcinoma; PCR, polymerase chain reaction; MSP, methylation-specific PCR; E, patients with early-stage EOC; A, patients with advanced-stage EOC; fcDNA, free circulating DNA; S, fcDNA as template; T, tissue genomic DNA as template; B, fcDNA of patients with benign ovarian tumors as template; H, fcDNA of healthy donors as template; PCR mix, PCR reaction without templates.

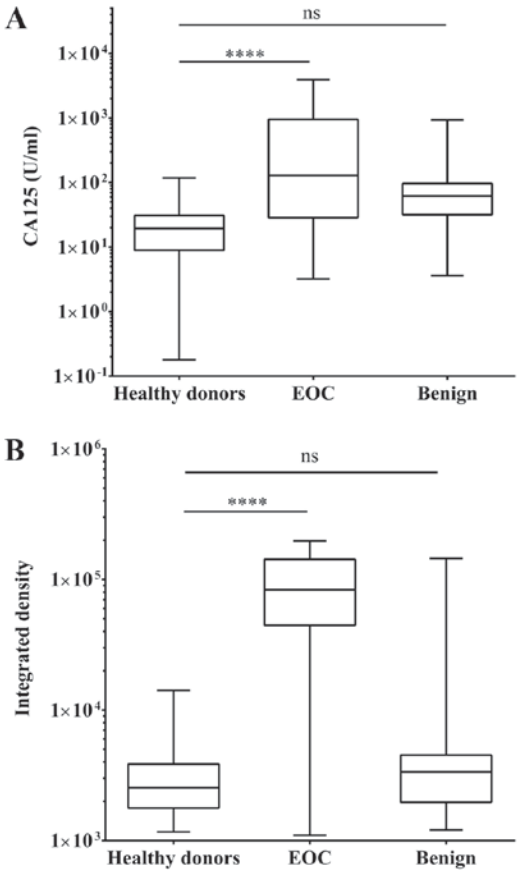

Figure 3. Comparison of EOC verification by OPCML methylation-specific polymerase chain reaction and by traditional CA125 test. (A) CA125 level of patients with EOC and controls. (B) OPCML methylation status in healthy donors, patients with EOC and patients with benign ovarian cancer. The upper error bars are the 4th quartiles and maximum of data. The lower error bars are the 1st quartiles and minimum of data. ****P<0.0001, one-way analysis of variance. EOC, epithelial ovarian carcinoma; OPCML, opioid binding protein/cell adhesion molecule like; CA125, cancer antigen 125; ns, not significant.

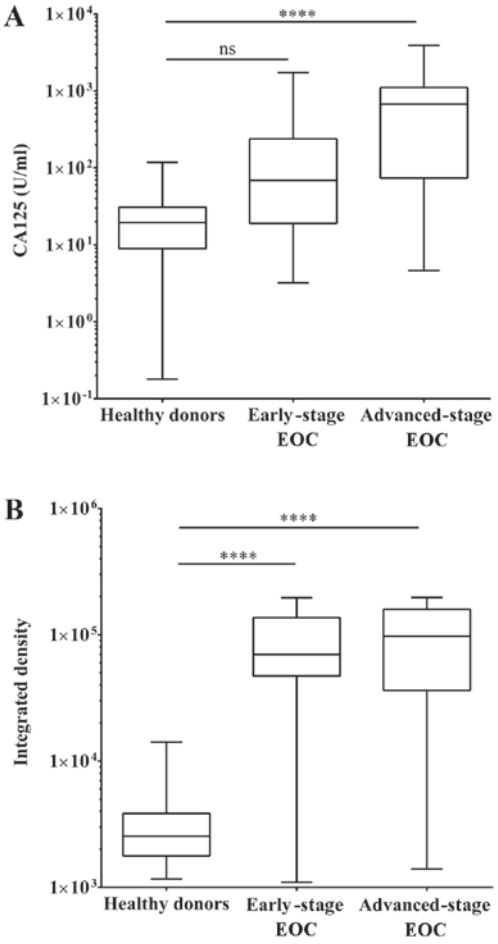

Figure 4. MSP detection of opioid binding protein/cell adhesion molecule like methylation improves diagnosis of early-stage EOC cases. (A) Serum CA125 levels in healthy donors, patients with early-stage EOC and patients with advanced-stage EOC. (B) Integrated density of the 190 bp MSP products from various types of patients with EOC. The top error bars show the 4th quartiles and maximum values of the groups, and the lower error bars indicate the 1st quartiles and minimum values of the groups. ****P<0.0001, one-way analysis of variance. ns, not significant; MSP, methylation-specific polymerase chain reaction; EOC, epithelial ovarian carcinoma.

product band was identified when OPCML M pair primers were applied. The average integrated density was ~130,000. No band was identified using the U pair primers. Using the TFPI2 and RUNX3 M primer pairs, 140- and 80-bp bands were amplified. However, same size PCR products were also present when U primer pairs were used.

For manipulation convenience, the present study also tested a PCR system with all three sets of primers in one reaction. The results are presented in Fig. 1B, and were similar to those for separate reactions. These data suggested that the primer set for OPCML was appropriate for additional investigation, and not the primer sets for TFPI2 and RUNX3.

Validating OPCML MSP primer set in patient samples. To examine if the MSP primer set for OPCML was effective in real patient samples, plasma and tissue from patients were tested. Sampling involved 3 randomly chosen patients in each category (Fig. 2). In this experiment, patients with EOC were classified as early or advanced EOC based on their clinical signs and symptoms. fcDNA from plasma or genomic DNA

Table II. Performance of fcDNA MSP for diagnosing patients with early-stage EOC.

	EOC		Early-stage EOC		Advanced-stage EOC	
Variables	CA125 test	fcDNA MSP	CA125 test	fcDNA MSP	CA125 test	fcDNA MSP
Sensitivity, %	67.61 (48/71)	90.14 (64/71)	53.85 (21/39)	87.18 (34/39)	81.25 (26/32)	93.75 (30/32)
Specificity, %	63.41 (78/123)	91.87 (113/123)	63.41 (18/39)	91.87 (5/39)	63.41 (78/123)	91.87 (113/123)
Accuracy, %	64.95 (126/194)	91.24 (177/194)	61.11 (99/162)	90.74 (147/162)	67.10 (104/155)	92.26 (143/155)
PPV, %	51.61 (48/93)	86.49 (64/74)	31.82 (21/66)	77.27 (34/44)	36.62 (26/71)	75.00 (30/40)
NPV, %	63.40 (123/194)	63.40 (123/194)	75.93 (123/162)	75.93 (123/162)	79.35 (123/155)	79.35 (123/155)
κ	0.291	0.813	0.14	0.757	0.308	0.784
Strength of agreement	Fair	Very good	Poor	Good	Fair	Good

EOC, epithelial ovarian carcinoma; CA125, cancer antigen 125; fcDNA, free circulating DNA; MSP, methylation-specific polymerase chain reaction; PPV, positive predictive value; NPV, negative predictive value.

from cancer tissue were templates in the MSP. The integrated density of PCR products corresponding to fcDNA from early and advanced patients with EOC was ~63,000 and 52,000, respectively. The integrated density of MSP products amplified through genomic DNA from patients with early and advanced EOC was 32,000 and 35,000, respectively. Although there was a relatively wide range of standard deviation, the scores were still significantly higher compared with those from reactions utilizing fcDNA of healthy donors, patients with benign tumors or the background integrated density scores (~3,000).

Methylation status of OPCML in patients with EOC screened by MSP. To determine the efficacy of MSP compared with the CA125 test with respect to the diagnosis of epithelial ovarian carcinoma, plasma samples and fcDNA from 80 healthy donors, 43 patients with benign ovarian tumors and 71 ovarian epithelial carcinoma donors were assessed by the two methods. The box and whisker plot in Fig. 3A illustrates that the median value of CA125 in healthy donors was 19.45 U/ml. Comparing the aforementioned value with 128.93 U/ml CA125 in patients with EOC, the P-value subsequent to one-way ANOVA was <0.0001. Additionally, the median CA125 level in patients with benign ovarian tumors was 62.13 U/ml, which was not statistically significant compared with the healthy donors. One-way ANOVA for the MSP results revealed that there was a statistically significant difference between healthy donors and patients with EOC (P<0.0001), but not between healthy donors and patients with benign ovarian tumors (Fig. 3B).

Significance of OPCML methylation status in early-stage EOC reflected by MSP. The conventional CA125 test is not efficient to detect early-stage EOC. To evaluate the detection sensitivity of early-stage EOC using MSP and CA125 tests, patients with EOC in the cohort of the present study were sorted into 39 early- and 32 advanced-stage patients based on the FIGO stages. Patients exhibiting stages I and II were early-stage, while stages III and IV were considered advanced-stage EOC. The median serum CA125 level in patients with advanced-stage EOC reached up to 672.02, however the median level of patients with early-stage EOC was 147 (Fig. 4). One-way ANOVA indicated that the P-value of CA125 level with respect to the difference between healthy donors and patients with advanced-stage EOC was <0.0001, but no statistical significance was observed between healthy donors and patients with early-stage EOC. The median integrated densities of PCR products from healthy donors and patients with early- and advanced-stage EOC were 2540.54, 69795.43 and 96921.52, respectively. The P-values between healthy donors and patients with early- or advanced-stage EOC were all <0.0001.

Diagnostic performance of MSP method. To assess the diagnostic performance of the MSP test in terms of early, advanced and overall EOC cases, the sensitivity, specificity and accuracy of the CA125 test and the OPCML MSP test were analyzed (Table II). A CA125 level <35 U/ml or integrated density <9,000 was defined as negative results in the present study. The sensitivity of the CA125 test with respect to patients with early, advanced and overall EOC was 53.85, 81.25 and 67.61%, respectively. The sensitivity of the fcDNA MSP test, which was 87.18, 93.75 and 90.14%, respectively, was higher than that of CA125 test for each group of patients. The results of Cohen's κ coefficient for the CA125 test, whereby κ represents the degree of agreement for categorizing subjects into groups, were 0.14, 0.308 and 0.291 in the aforementioned order, therefore the degrees of agreement are poor, fair and fair. However, κ of the fcDNA MSP were 0.757, 0.784 and 0.813 and the degrees of agreement were good, good, and very good, respectively.

Discussion

Epithelial ovarian carcinoma is the most common type of ovarian cancers, accounting for 90% of cases. Due to few or

no symptoms in the majority of early cases, 85% patients were revealed to be diagnosed at an advanced stage (24). However, an issue with respect to diagnosis and medical intervention is that the five-year survival rate of advanced-stage patients with EOC may be <50%, whereas the five-year survival rate of patients with early-stage EOC may be >95% (4). At present, serum CA125 level is a common marker used to assist clinical diagnosis. The inability of this measurement to screen for early-stage EOC makes it unsatisfactory to aid resolving this challenge. The aim of the present study was to reveal another biomarker to improve the diagnosis of early-stage EOC.

Herman *et al* (22) reported MSP as a novel assay to detect hypermethylation in tumor suppressor genes, which are often silenced in patients with cancer. In the present study three promising markers, methylated OPCML, TFPI2 and RUNX3, were selected in an attempt to improve the efficacy of early-stage EOC diagnosis assays. The initial study with HO8910 genomic DNA as a template suggested that the OPCML MSP primer set may be an appropriate marker, but TFPI2 and RUNX3 MSP primer sets may introduce too many false positive results.

Furthermore, test results from patient tissue and fcDNA samples indicated that nested MSP with the OPCML primer set clearly differentiated patients with advanced-stage EOC from patients with benign ovarian tumors and healthy donors, and patients with early-stage EOC from healthy and non-malignant samples. To validate the methylated OPCML maker and the MSP method in screening early-stage EOC, the sample size was expanded to 80 healthy donors, 43 patients with benign tumors, 39 patients with early-stage EOC and 32 patients with advanced-stage EOC. CA125 level results were consistent with previously known clinical data (7). A total of 35 U/ml is an appropriate threshold value to distinguish between healthy donors and patients with EOC. Notably, even though the differences between patients with benign ovarian tumors and healthy donors were not statistically significant, >75% of patients with benign tumors exhibited CA125 levels >30 U/ml, leading to false positive results. Similar to the CA125 assay, the MSP test for methylated OPCML also distinguished healthy donors and patients with benign tumors from patients with EOC. In addition, the novel approach made this difference more marked.

In diagnostic performance analysis, compared with the CA125 test, the MSP assay increased Cohen's κ coefficient from 0.291 to 0.813. A κ value is considered good or excellent when it is >0.81. Further investigation confirmed that the CA125 test failed to provide unambiguous evidence of early-stage EOC; however, the test was revealed to highlight patients with advanced-stage EOC. Notably, the κ value of the MSP assay in early-stage EOC, 0.757, suggested a good agreement with the expected test results, contrasted with 0.14 in the CA125 test, which is extremely low. Thus, the present study revealed that the hypermethylation of OPCML is a promising biomarker to identify early-stage EOC. Additionally, the biomarker exhibits an excellent degree of agreement when testing patients with EOC at all stages.

In addition, the present study also demonstrated that multiple biomarkers may be tested for in a single MSP reaction, as long as the sizes of the amplified DNA fragments are different. This system may reduce the time required and increase the sensitivity and accuracy of clinical diagnosis. Therefore, this MSP assay for EOC may be a novel supplement or replacement for the traditional CA125 test.

To develop an effective diagnostic kit, the present authors aimed to improve the screening technique to provide a higher sensitivity and a κ value >0.81 for early-stage EOC. A potential method is the investigation of additional biomarkers. Sung *et al* (25) reported the absence of p150 expression in ovarian cancer cells. This occurs due to the methylation of the P2 promoter in a putative tumor suppressor gene, spalt-like transcription factor 2. Other potential candidates include p16 (26), cyclin dependent kinase inhibitor 2B, cadherin 13 or ras association domain family member 1 (27). Multiple biomarkers may compensate for individual differences among patients, which may account for some of the false negative cases in single biomarker assays.

Investigating and designing MSP primers may be another key aspect to improve the assay developed in the present research. TFPI2 and RUNX3 MSP primers were not adopted for screening patient cohorts since they introduced unacceptable false positive results. One of the criteria for the design of MSP primers is the inclusion of at least one CpG site at the 3'-end (22,23). Unfortunately, the RUNX3 U pair of MSP primers does not exhibit a CpG site at the 3'-end. In addition, adequate CpG sites are necessary for good quality MSP primers (23). The U pair of the TFPI2 MSP primers exhibits two CpG sites, which may be not be competent enough to differentiate methylated DNA templates from unmethylated ones. As a result, the two primers may be imperfect MSP primers. Therefore, designing and testing high-quality MSP primers may be part of prospective investigation to optimize the assay of the present study. The present study demonstrates a preliminary effort to identify a promising novel biomarker and develop an improved screening method for EOC. Building upon the results of the present study, a clinical screening test kit for EOC, particularly early stage EOC, will be investigated.

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