Cumulative methylation alternations of gene promoters and protein markers for diagnosis of epithelial ovarian cancer

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ABSTRACT. DNA methylation plays an important role in carcinogenesis and cancer development. In this study, we examined gene methylation in epithelial ovarian cancer (EOC) using cationic conjugated polymer (CCP)-based fluorescence resonance energy transfer (FRET) to evaluate the application of cumulative methylation alternations of genes associated with cancer antigen 125 for early cancer diagnosis. The methylation status of 3 genes (Ras association domain family 1 isoform A, RASSF1A; opioid-binding protein/cell adhesion molecule, OPCML; homeobox A9, HOXA9) were examined and compared in 35 EOC samples and 11 normal ovarian tissue samples using CCP-based FRET. Gene methylation levels were clustered into 3 sections and assigned a value; values for the 3 genes were summed. Although methylation of the OPCML gene was significantly associated with stage, histological types, grade, and ascites and that of RASSF1A and HOXA9 was not, the sum for the 3 genes was significantly associated with stage and ascites. The sum showed higher sensitivity (85.7%) and specificity (100%) for discriminating EOC from normal ovarian tissues than did the methylation status of RASSF1A, OPCML, and HOXA9.
(48.6, 77.1, 77.1, and 100, 88.1, 100%, respectively). Combining cancer antigen 125 levels with the sum increased the sensitivity to 94.3%. The detection and analysis of a panel of genes’ methylation status with the CCP-based FRET technique may be useful for diagnosis and screening of EOC; the associated cancer antigen 125 can be used to increase diagnostic sensitivity.

**Key words:** Cancer antigen 125; Methylation level detection; Cationic conjugated polymer-based fluorescence resonance energy transfer; Epithelial ovarian cancer; DNA methylation

**INTRODUCTION**

Epithelial ovarian cancer (EOC) is the most lethal gynecologic malignancy. Because of the lack of early diagnosis methods and no obvious symptoms, EOC, which accounts for 90% of ovarian cancer, is diagnosed at an advanced stage (FIGO III and IV) in 75% of all cases, when the disease has spread throughout the abdomen. Patients with advanced-stage disease have a 5-year survival rate of only 30% in contrast to early-stage disease confined to the ovaries, for which 5-year survival rate exceeds 80% (Jemal et al., 2010). The overall survival of women with ovarian cancer has not changed in over 50 years (Kurman, 2013). Over the past 2 decades, numerous studies, including large clinical trials, have been conducted in an effort to reduce mortality by developing screening tests such as serum cancer antigen 125 (CA125) and transvaginal ultrasound to detect early-stage ovarian cancer that is confined to the ovaries. Unfortunately, these methods show high false-negative rates, as well as low sensitivity and specificity in the clinic, and have not demonstrated the capacity to reduce population morbidity and/or mortality (Menon and Jacobs, 2000; Jacobs and Menon, 2004; Munkarah et al., 2007).

DNA methylation is an important epigenetic modification that affects gene expression without changing the DNA sequence (Egger et al., 2004). During cancer development, cells undergo profound alterations in DNA methylation patterns, with functional consequences on the activity of key genes intimately involved in the carcinogenic process (Bast Jr. et al., 2005). Recent studies have shown that aberrant DNA methylation, which typically occurs before patients develop clinical manifestations and radiographic evidence, provides a new molecular approach for the early diagnosis of cancer (Issa, 2007; Zhu and Yao, 2007). The DNA methylation profile of a large number of genes have been associated with the molecular, clinical, and pathological features of ovarian cancer (Widschwendter et al., 2009; Cul’bová et al., 2011; Ho et al., 2012).

It is likely that determining the methylation status of a panel of genes, rather than an individual gene, will result in more sensitive and specific diagnosis (Montavon et al., 2012; Bhagat et al., 2012). We developed a cationic conjugated polymer (CCP)-based fluorescence resonance energy transfer (FRET) method for detecting the methylation levels of Ras association domain family 1 isoform A (RASSF1A), opioid-binding protein/cell adhesion molecule (OPCML), and homeobox A9 (HOXA9) of EOCs and associated analysis of DNA methylation (Zhang et al., 2014). We examined the correlation between the data generated by our technique and the clinical and pathological features of EOC. The study provides a rational basis for the development of a protocol for the diagnosis and screening of EOC.
MATERIAL AND METHODS

Sample collection and gene selection

Based on a retrospective study, 35 EOC samples and 11 normal ovarian samples were obtained from the Changzhou Maternal and Child Health Care Hospital from 2004-2011. All patients provided consent and the study was approved by the hospital Ethics Committee. Histologic classification of EOC was conducted according to the World Health Organization criteria, and tumor stages were established according to the International Federation of Gynecology and Obstetrics. Because high-grade serous ovarian cancer is the subtype with highest prevalence, estimated at approximately 70% of all cases (Landen Jr. et al., 2008), the grade of differentiation was defined as high-grade serous cancers and other grades. Eleven cases of normal ovarian tissues were collected from patients without cancer who underwent salpingo-oophorectomy at the time of surgery for benign gynecological diseases. Clinical characteristics, including age and preoperative CA125 levels, and pathological characteristics, including histopathologic diagnosis, grade, and stage, were determined for each patient. The promoter of the selected gene was expected to be unmethylated in normal cases and methylated in EOC cases.

DNA preparation

Genomic DNA samples of formalin-fixed and paraffin-embedded tissue sections were isolated and extracted following the TIANamp Genomic DNA kit (QIAGEN, Hilden, Germany) instructions. DNA concentration was quantified by measuring absorbance at 260 nm using a Nanodrop spectrophotometer (ND2000; Thermo Scientific, Waltham, MA, USA). Genomic DNA was divided into 2 identical sections: section A for HpaII treatment and section B for non-HpaII treatment. Both reaction systems were incubated at 37°C for 12 h, followed by heat inactivation of HpaII at 85°C for 15 min. The digested DNA was used for polymerase chain reaction (PCR) amplification or stored at -80°C.

Detection of methylation level

PCR primers were designed using DNAMAN (Lynnon Biosoft, San Ramon, CA, USA). The digested DNA was amplified using 2-round PCR. The methylation levels of selected genes were detected using the CCP-based FRET. Ninety microliters CCP working solution and 10 μL amplified sample were mixed on a 96-well plate. Absorbance on the 96-well plate was then read using the Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA) at an excitation wavelength of 380 nm and emission wavelengths of 424 and 530 nm. Emission filter 1 (440/30 nm) was used to detect emission at 424 nm, and emission filter 2 (528/20 nm) was used to detect emission at 530 nm. We calculated FRET ratios (I_{530 nm}/I_{424 nm}) to determine the methylation level “E” as shown:

\[
E = \frac{\text{FRET ratio}_{\text{HpaII}} - \text{FRET ratio}_{\text{blank}}}{\text{FRET ratio}_{\text{non-HpaII}} - \text{FRET ratio}_{\text{blank}}}
\]
The methylation level was clustered into 3 sections, and each section was assigned a value of (-), (+), or (2+). Value (-) represented a low methylation level below 0.3, value (+) represented a moderate methylation level from 0.3-0.7, and value (2+) represented a high methylation level above 0.7. For each sample, the 3 values were summed.

Statistical analysis

The SPSS version 19.0 software was used for statistical analysis (SPSS, Inc., Chicago, IL, USA). The association between the methylation level, CA125, and clinical and pathological characteristics was evaluated using a chi-square test. The level of statistical significance was set at P < 0.05.

RESULTS

Based on published literature, 3 tumor suppressor genes, RASSF1A, OPCML, and HOXA9, were selected. Promoter methylation of these genes was observed in high frequency, was stable in all histological types of EOC, and played an important role in ovarian carcinogenesis (Table 1).

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Chromosome location</th>
<th>Methylation in EOC</th>
<th>Histological type</th>
<th>Assay</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RASSF1A</td>
<td>3p21.3</td>
<td>49% (23/47)</td>
<td>S, M, C, E</td>
<td>MSP</td>
<td>(Wu et al., 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>58% (50/86)</td>
<td>S, M, C, E</td>
<td>MSP</td>
<td>(Bhagat et al., 2012)</td>
</tr>
<tr>
<td>OPCML</td>
<td>7p15-p14</td>
<td>78.4% (80/102)</td>
<td>S, M, C, E</td>
<td>Quantitative MSP</td>
<td>(Zhou et al., 2014)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>83.3% (20/24)</td>
<td>S, M, C, E</td>
<td>Nested MSP</td>
<td>(Zhou et al., 2011)</td>
</tr>
<tr>
<td>HOXA9</td>
<td>11q25</td>
<td>51% (26/51)</td>
<td>S, M, C, E</td>
<td>MSP</td>
<td>(Wu et al., 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95% (75/79)</td>
<td>S*</td>
<td>Headloop MSP</td>
<td>(Montavon et al., 2012)</td>
</tr>
</tbody>
</table>

S = serous carcinoma; M = mucinous carcinoma; C = clear cell carcinoma; E = endometrioid carcinoma; MSP = methylation-specific PCR. *We only detected high-grade serous ovarian cancer.

CCP-based FRET was used to determine the methylation patterns in DNA isolated from 35 EOC patients with complete clinical pathological information and 11 normal samples. Normal samples showed no methylation in RASSF1A and HOXA9 and 2 moderate methylations in OPCML. Thus, the sum of the 2 normal sample was (+). EOC samples showed increased methylated loci compared to normal samples, with 17 tumor samples showing methylation in RASSF1A, 27 in OPCML, and 27 in HOXA9. There were 32 [91.4%, sum ≥ (+)] tumor samples showing methylation of at least 1 gene. Compared to the sum of the normal samples, the individual gene threshold was set as (+) [≥ (+)], while the sum threshold of EOC samples was set as (2+) [sum ≥ (2+)]. Consequently, the sensitivity and specificity of the protocol for diagnosing EOC was 85.7% (30/35) and 100% (11/11), respectively. CA125 levels in 11 patients corresponding to 11 normal ovarian tissues were all less than 200 U/mL, and those in 25 of 35 EOC patients were more than 200 U/mL, including 2 EOC patients with a sum of (+) and 1 EOC patient with a sum of (-) (Figure 1).

Across all genes analyzed, all groups showed methylation of the 3 genes. Hypermethylation of the OPCML gene was significantly associated with stage, histological type, grade, and ascites, while that of the RASSF1A and HOXA9 genes showed no correlation with clinic-
pathological features. The sum was significantly associated with stage and ascites. The same trend was observed for grade. No association was observed between methylation frequency and CA125 level (Table 2).

![Figure 1](image-url) Values of RASSF1A, OPCML, and HOXA9 genes, the sums of 3 genes, and levels of CA125 (U/mL). Thirty-five cancer samples are labeled from 1-35, and 11 normal samples are labeled from N1-N11.

The sensitivity and specificity of the sum for diagnosing EOC were higher than the values for the individual genes. CA125 showed a sensitivity of 71.4% and a specificity of 100%. However, combining CA125 levels with methylation status for the sum increased the sensitivity to 94.3% (Table 3).
DISCUSSION

In this study, we analyzed the correlation between the methylation status of candidate genes and clinicopathological features in order to confirm the feasibility of CCP-based FRET. Based on published studies, the 3 genes RASSFIA, OPCML, and HOXA9 were selected because they show a high frequency of methylation in EOC and no methylation in normal tissues (Rathi et al., 2002; Teodoridis et al., 2005; Wu et al., 2007; Liggett et al., 2011). In EOC, inactivation of the RASSFIA, OPCML, and HOXA9 gene promoters was reported to be related to carcinogenesis or early cancer (Sellier et al., 2003; Montavon et al., 2012; Bhagat et al., 2012; Zhang et al., 2013).

OPCML is an opioid binding protein cell adhesion molecule. Over-expression of OPCML decreases the proliferation of EOC cells in vitro and tumor growth in vivo while increasing cellular aggregation (Sellier et al., 2003). Zhou et al. (2011) found that the methylation frequency of the OPCML gene in ovarian cancer samples differed in different assays, with values of 58.3% (14/24) by methylation-specific PCR and 83.3% (20/24) by nested
methylation-specific PCR, respectively, and no methylation was observed in normal ovarian tissues using either method. In our study, the percentages of promoter hypermethylation of \textit{OPCML} were 77.1\% in EOC and 18.2\% in normal ovarian tissues. This indicates that our more sensitive method improved the methylation frequency compared with the methods used in other studies (Teodoridis et al., 2005; Chen et al., 2007). Another study found that \textit{OPCML} promoter methylation was significantly associated with an older patient age, advanced stage of ovarian cancer, and poor overall survival of ovarian cancer patients (Zhou et al., 2014). Similarly, we found that the methylation of \textit{OPCML} was significantly associated with advanced stage, high-grade, serous cancer and mucous cancer, and positive ascites. Loss of \textit{OPCML} simultaneously reduces intercellular adhesion and accelerates cell growth, not only promoting the early steps of ovarian carcinogenesis but also causing cancer development.

\textit{RASSF1A} acts as a tumor suppressor gene in the RAS pathway and can regulate proliferation, induce apoptosis, and bind to and stabilize microtubules (Liu et al., 2005). Previous studies showed that the frequency of promoter hypermethylation of \textit{RASSF1A} in ovarian carcinomas was 40-58\% (Yoon et al., 2001; Bhagat et al., 2012). In our study, epithelial ovarian cancers exhibited \textit{RASSF1A} gene promoter methylation frequencies of 48.6\% (17/35). Ibanez de Caceres et al. (2004) reported a significant correlation between methylation status, tumor grade, CA125 levels, and stage in ovarian cancer. However, we found that the methylation status of \textit{RASSF1A} was not related to age, grade, stage, histological type, ascites, and CA125, which was similar to the results of Bhagat et al. (2012). Homeodomain-containing genes encode transcriptional factors that function during embryonic development to control patterning, differentiation, and proliferation (Gorski and Walsh, 2003). A study by Montavon et al. (2012) found a 95\% frequency of methylation of \textit{HOX9} in high-grade serous ovary cancer, as \textit{HOXA9} regulates serous differentiation of the Müllerian ducts to Fallopian tubes (Du and Taylor, 2004; Chen et al., 2005) and \textit{HOX9} methylation in ovarian cancer may reflect a loss of transcriptional plasticity during disease development and a shift towards epithelial cell de-differentiation or high-grade classification (Coolon et al., 2010). Our study used CCP-based FRET to detect the 4 types of EOC. Methylation frequency of the \textit{HOX9} gene was 77.14\%, which was higher than the value of 51\% (26/51) by methylation-specific PCR found in a previous report (Wu et al., 2007). There was no significant association between gene methylation and the high grade of EOC, but methylation frequency in the high-grade serous ovarian cancers (81\%) was higher than that of other EOCs (71\%). The small number of samples in our study may account for this trend. No normal ovarian samples were included in the methylation of \textit{RASSF1A} and \textit{HOXA9}.

In our study, the technique for detecting methylation was more sensitive and the methylation status of 3 genes agreed with previously reported values, although different techniques such as methylation-specific PCR were used in these studies. The thresholds, (+) for individual genes and (2+) for the sum, were reasonable. Our protocol is advantageous because it takes the degree and contribution of promoter methylation in different candidate gene into account. The methylation of \textit{OPCML} was significantly associated with stage, histology type, grade and ascites, while \textit{RASSF1A} and \textit{HOXA9} were not. The sum could be used for comprehensive analysis, exhibiting a significant association with stage, ascites, and grade, but no relevance with histological type. The sum also balanced the different effects of methylation of individual gene in specificity and improved the sensitivity and specificity of the method.

The sum of all normal samples was less than (+), and 30 of 35 cancer samples exceeded (+). Thus, a sum of \(\geq (2+)\) was set as a threshold. If the sum of a sample exceeded (+),
cancer diagnosis was confirmed. The sensitivity of the protocol was calculated to be 85.7% (30/35), with a specificity of 100% (11/11). However, 4 samples showed a sum of (+), with 2 samples in the cancer group and 2 in the normal group. The sum of (+) was borderline, making it difficult to determine whether the disease was cancerous or benign. To assess whether gene methylation could be used to improve existing molecular biomarkers, we investigated the discrimination properties of pre-operative serum CA125 levels in our study. CA125 level was not related to the sum of methylation level. Combining CA125 with the methylation level sum revealed 2 EOC cases whose sums were (+) and 1 EOC case whose sum was (-). The sensitivity increased to 94.3% (33/35), while specificity remained at 100%.

Recent studies have shown that aberrant DNA methylation, which typically occurs before patients develop clinical manifestations and radiographic evidence, can be detected in cell-free serum (Vlassov et al., 2010; Liggett et al., 2011; Zhang et al., 2013). The amount of available cell-free serum DNA is often very low. The CCP-based FRET technique is sensitive, thus small DNA samples obtained from serum, plasma, or stool can be examined. This method can also detect low methylation levels of the gene promoter, meeting the requirement for early cancer diagnosis. In the future, we will continue to examine this protocol for detecting early EOC using cell-free serum DNA and investigate the potential application in monitoring EOC treatment.

Conflicts of interest

The authors declare no conflict of interest.

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