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Original Article

Gene methylation profiles as prognostic markers in ovarian clear cell and endometrioid adenocarcinomas

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Abstract: Ovarian cancer is a cancer of high mortality. Aberrant gene methylation of tumor suppressor genes has been shown to be related to the development of malignancy. This study aimed to investigate the methylation of various genes in ovarian clear cell adenocarcinoma (OCCA) and ovarian endometrioid adenocarcinoma (OEA) and evaluate methylation biomarkers in terms of patient chemo-response and outcome. Eight candidate genes from 66 OCCA and 51 OEA patients were evaluated by methylation-specific polymerase chain reaction and capillary electrophoresis. Clinico-pathological parameters and patient outcomes were analyzed. The frequencies of gene methylation in RASSF1A (79% vs. 59%, p=0.025), E-cadherin (30% vs. 10%, p=0.011), and DLEC1 (71% vs. 43%, p=0.003) were higher in the patients with OCCA than in those with OEA. The chemo-resistant group had a significantly higher percentage of E-cadherin methylation (36.7% vs. 16.1%, p=0.036) than the chemo-sensitive group. In multivariate analysis (log-rank test), advanced stage (4.79 [2.10-10.94], p<0.001) was the only risk factor for mortality. Those with methylation of more than two out of three genes (E-cadherin, DLEC1, and SFRP5) had a shorter disease-free survival (1.89 [1.07-3.32], p=0.028) and overall survival (3.29 [1.57-6.87], p=0.002) than those with methylation of one or no gene. In advanced-stage malignancies, those with more than two out of the three gene methylations also had a shorter overall survival (3.86 [1.63-9.09], p=0.002) than those with methylation of only one or no gene. Patients with OCCA have different patterns of gene methylation than those with OEA. Methylation of the E-cadherin, DLEC1 and SFRP5 genes can be a prognostic biomarker for OCCA and OEA.

Keywords: E-cadherin, DLEC1, RASSF1A, SFRP5, ovarian endometrioid adenocarcinoma, ovarian clear cell adenocarcinoma, methylation

Introduction

Increasing attention has been paid to epithelial ovarian carcinoma in recent years due to a five-year overall survival rate of less than 50% [1, 2]. Current treatments of cyto-reduction surgery and adjuvant platinum-based chemotherapy have been reported to have a response rate of 80% [3]. However, relapse is common after the initial treatment leading to recurrence-related mortality, especially in those with an advanced stage [4]. Although serous adenocarcinoma is the most prevalent type of epithelial ovarian cancer, the incidence rates of ovarian clear cell carcinoma adenocarcinoma (OCCA) and ovarian endometrioid adenocarcinoma (OEA) in Taiwan are estimated to be 10-15% and 15-20%, respectively, which are higher than the 2-5% and 7-12% reported in Western countries [5-8]. Although both are regarded to develop from endometriosis [9, 10], OCCA patients tend to have a poorer prognosis than OEA patients due to resistance to chemotherapy [11, 12]. Differences in the tumor biology between OCCA and OEA remain unclear. Thus, investigating the carcinogenesis of OCCA and OEA will help in the differential diagnosis and the understanding of the tumor biology, and guide the development of new strategies for individualized therapy.

Epigenetics is the study of heritable changes in gene expression that are not coded in the DNA sequence itself. The methylation of gene pro-

Table 1. Characteristics and gene methylation of the 117 OCCA and OEA women

	OCCA	OEA	*p*
Patient numbers	66	51	
Age (years old)*	49.7±9.1	52.2±11.6	0.19
CA 125 (U/ml)#	888 (16.6-21041)	1821 (12.9-39370)	0.28
Grade			
I	NA	22 (44%)	NA
II	NA	13 (25%)	NA
III	NA	16 (31%)	NA
FIGO stage$			
Early (I & II)	33 (50%)	27 (52%)	0.85
Advanced (III & IV)	33 (50%)	24 (48%)	
Debulking surgery$			
Optimal	54 (82%)	45 (88%)	0.29
Suboptimal	12 (18%)	6 (12%)	
Platinum-based chemotherapy$			
With paclitaxel	45 (68%)	32 (63%)	0.56
Without paclitaxel	21 (32%)	19 (37%)	
Prognosis@			
Median disease-free survival (months)	15 (0-212)	14 (0-147)	0.31
Median overall survival (months)	26 (1-216)	26 (1-147)	0.94
Mean numbers of methylated genes	4.50	4.12	0.16
Frequency of gene methylation$			
RASSF1A	52 (79%)	30 (59%)	0.025
E-cadherin	20 (30%)	5 (10%)	0.011
DLEC1	47 (71%)	22 (43%)	0.003
RUNX3	33 (50%)	25 (49%)	1.00
SFRP1	36 (55%)	34 (67%)	0.25
SFRP5	27 (41%)	27 (53%)	0.26
PAX	40 (61%)	39 (77%)	0.07
LMX1A	42 (64%)	28 (55%)	0.35

Abbreviations: OCCA, ovarian clear cell carcinoma; OEA, ovarian endometrioid adenocarcinoma; NA: not available; n: patient number. Note: Age is presented as mean ± SD. CA 125, disease-free survival, and overall survival are presented as median (minimum-maximum). Other values are presented as number (percentage). *P* values indicate comparisons between patients with ovarian clear cell carcinoma (OCCA) and those with ovarian endometrioid adenocarcinoma (OEA). *Student t-test; #ANOVA; $Chi-square test; @log-rank test.

moters has been reported to be involved in mediating epigenetic transcriptional silencing [13], and aberrant gene methylation of tumor suppressor genes has been shown to promote the development of malignancy [14]. In addition, the profiles of gene methylation have been shown to be different in various malignant tumors and to be associated with clinical outcomes [15]. Distinct gene methylation profiles may exist between different pathways of ovarian tumorigenesis [16].

The methylation of FANCF has been reported to contribute to chemo-selectivity in ovarian cancer [17], and DNA methylation has been shown to be a diagnostic prognostic biomarker in ovarian cancer [18]. However, no studies have focused on the methylation profiles in different histological sub-types of ovarian cancer except for the serous type. This study was therefore conducted to investigate gene methylation in patients with OCCA and OEA, and correlate gene methylation with clinical outcomes.

Methods

Patients and specimens

Patients with OCCA or OEA who underwent debulking surgery and adjuvant chemotherapy at

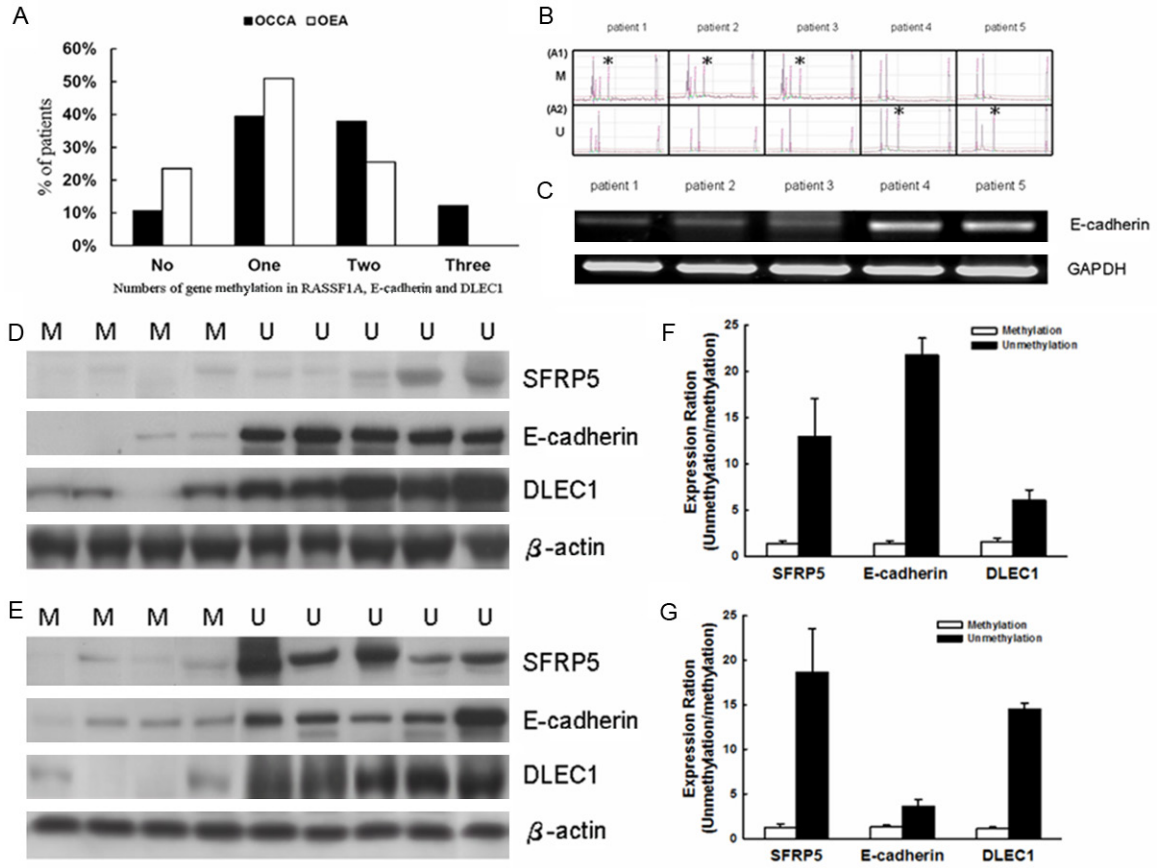

Figure 1. (A) Bar figure of the number of RASSF1A, E-cadherin, and DLEC1 gene methylation in 66 OCCA and 51 OEA patients. The OCCA patients had a higher amount of RASSF1A, E-cadherin, and LEC1 methylation than the OEA patients (p=0.01, Mann-Whitney test). (B) Representative figures of capillary electrophoresis of MS-PCR for E-cadherin. There was E-cadherin methylation in Patents 1, 2, and 3, whereas Patient 4 had unmethylated E-cadherin promoter. M, primers for the methylated gene promoter for E-cadherin; U, primers for the unmethylated gene promoter; *Positive findings of MS-PCR products. (C) Representative figures of reverse-transcription polymerase chain reaction for E-cadherin in ovarian cancerous tissues. Representative figures of immunoblotting for DLEC1, SFRP5 and E-cadherin in (D) OCCA and (E) OEA cancerous tissues (M: methylation, U: unmethylation). Bar figure of the mean protein expression levels of three genes in the unmethylated and methylated samples in (F) OCCA and (G) OEA patients. *Note:* The related protein expression levels in the methylated samples were significantly lower than those in the unmethylated samples in the OCCA group (SFRP5 p=0.031, E-cadherin p=0.007, DLEC1 p=0.015 by one-way ANOVA) and in the OEA group (SFRP5 p=0.024, E-cadherin p=0.021, DLEC1 p=0.011 by one-way ANOVA).

the study institute were enrolled. Clinical information was retrieved from medical records in the hospital's centralized database. The Institutional Review Board of the National Taiwan University Hospital approved the study protocol. And all of the patients recruited in this study had singed and provided informed consents before collecting the samples.

Debulking surgery was defined as being optimal when the maximal diameter of the residual tumor was <1 cm. Otherwise, it was defined as sub-optimal debulking surgery. Histological grading was based on the International Union against Cancer criteria, and staging was based on the criteria of the International Federation of Gynecology and Obstetrics. Abnormal imaging study results (including computerized tomography and magnetic resonance imaging), elevated tumor markers (more than 2-fold higher than the upper limit of normal) of two consecutive tests at 2-week intervals, or tissue proven from biopsies were defined as recurrence.

Patients with disease progression or recurrence ≤6 months after completing adjuvant chemotherapy were defined as the chemo-resistant group, and those without recurrence or recurrence >6 months were defined as the chemo-sensitive group. Progression-free sur-

Table 2. Correlations between gene methylation and stage, debulking surgery, and chemo-response

Frequency of gene methylation			
Stage	Early (n=60)	Advanced (n=57)	*p*
RASSF1A	46 (76.7%)	36 (63.2%)	0.16
E-cadherin	10 (16.7%)	15 (26.3%)	0.26
DLEC1	36 (60.0%)	33 (57.9%)	0.85
RUNX3	31 (51.7%)	27 (47.4%)	0.71
SFRP1	38 (64.4%)	32 (56.1%)	0.45
SFRP5	29 (48.3%)	25 (43.9%)	0.71
PAX	43 (71.7%)	36 (63.2%)	0.43
LMX1A	36 (60.0%)	34 (59.7%)	1.00
Optimal debulking surgery (Advanced stage)	Yes (n=40)	No (n=17)	
RASSF1A	27 (67.5%)	9 (52.9%)	0.30
E-cadherin	8 (20.0%)	7 (41.2%)	0.097
DLEC1	20 (50.0%)	13 (76.4%)	0.064
RUNX3	19 (47.5%)	8 (47.1%)	0.98
SFRP1	23 (57.5%)	9 (52.9%)	0.75
SFRP5	20 (50.0%)	5 (29.4%)	0.15
PAX	26 (65.0%)	10 (58.8%)	0.66
LMX1A	24 (60.0%)	10 (58.8%)	0.93
Platinum-based chemotherapy (Advanced stage)	Sensitive (n=32)	Resistant (n=25)	
RASSF1A	20 (62.5%)	16 (64.0%)	0.91
E-cadherin	4 (12.5%)	11 (44.0%)	0.007
DLEC1	15 (46.9%)	18 (72.0%)	0.057
RUNX3	15 (46.9%)	12 (48.0%)	0.93
SFRP1	19 (59.4%)	13 (52.0%)	0.58
SFRP5	15 (46.9%)	10 (40.0%)	0.60
PAX	20 (62.5%)	16 (64.0%)	0.91
LMX1A	20 (62.5%)	14 (56.0%)	0.62

Abbreviations: n, patient number.

vival was defined as the period from the date of completing chemotherapy to the date of confirmed recurrence, disease progression, or last follow-up. Overall survival was defined as the period from surgery to the date of death associated with the disease or the date of the last follow-up.

Selection of candidate genes for the detection of gene methylation

We searched the literature to identify the potential tumor suppressor genes that were known to be silenced by gene methylation in several cancer types, including ovarian carcinoma. The methylation status of RASSF1A [19, 20], E-cadherin [21], DLEC1 [22], RUNX3 [23], SFRP1 [24], SFRP5 [25], PAX [24], and LMX1A [24] were evaluated. The primer sequences are summarized in Supplementary Table 1.

Extraction of DNA in ovarian cancerous tissues

Cancer tissue samples were collected during surgery, frozen, and stored at -70°C until analysis. All of the samples were from primary ovarian cancerous tissues, and not tissues from peritoneal or metastatic sites. Histological examinations were performed to calculate the percentage of malignancy of the frozen cancerous tissues. Tissues containing more than 80% malignant cells were used for further analysis. Genomic DNA was isolated using a Qiagen EZ1 DNA Tissue Kit (Qiagen, Valencia, CA) according the manufacturer's instructions.

Methylation specific-polymerase chain reaction (MS-PCR)

MS-PCR were conducted following a previously published protocol with some modifications

Table 3. Univariate and multivariate analysis for DFS and OS of all women with OCCA and OEA (n=117)

		DFS				OS			
		Univariate		Multivariate		Univariate		Multivariate	
	n	HR (95% C.I.)	*p*	HR (95% C.I.)	*p*	HR (95% C.I.)	*p*	HR (95% C.I.)	*p*
Age									
<50 years (reference)	50	1				1			
≥50 years	67	1.20 (0.67-2.13)	0.53			1.92 (0.85-4.31)	0.11		
CA125									
<35 U/ml (reference)	19	1				1			
≥35 U/ml	98	5.24 (0.72-38.15)	0.12			22.98 (0.45-11795.8)	0.32		
Histology									
OEA (reference)	51	1				1			
OCCA	66	1.51 (0.69-3.30)	0.31			1.13 (0.41–3.14)	0.81		
Grade									
I (reference)	22	1				1			
II	13	0.59 (0.12-2.96)	0.52			1.29 (0.29-5.77)	0.73		
III	16	2.83 (0.89-8.09)	0.15			1.73 (0.48-6.14)	0.39		
FIGO stage									
Early (reference)	60	1		1		1		1	
Advanced	57	4.22 (1.94-9.15)	<0.001	4.26 (2.09-8.74)	<0.001	6.57 (2.29-18.86)	<0.001	8.03 (2.94-21.92)	<0.001
Debulking surgery									
Optimal (reference)	99	1		1		1			
Suboptimal	18	2.22 (0.92-5.36)	0.07	2.82 (1.39-5.68)	0.004	1.04 (0.38-2.90)	0.93		
Platinum-based chemotherapy									
Without Paclitaxel (reference)	40	1				1			
With Paclitaxel	77	0.95 (0.44-2.05)	0.89			1.99 (0.79-4.99)	0.14		
E-cadherin, DLEC1, SFRP5 gene methylation									
<2 methylated gene (reference)	75	1		1		1		1	
≥2 methylated gene	42	1.52 (0.97-2.65)	0.10	1.89 (1.07-3.32)	0.028	2.40 (1.17-4.92)	0.016	3.29 (1.57-6.87)	0.002

Abbreviations: n, patient number; DFS, disease-free survival; OS, overall survival; OEA, ovarian endometrioid adenocarcinoma; OCCA, ovarian clear cell adenocarcinoma.

[25]. Briefly, genomic DNA was treated with sodium bisulfite, de-sulforated with NaOH, precipitated with ethanol, and re-suspended in water. After treatment, DNA methylation was determined by primers specific to methylated and unmethylated alleles of each gene. MS-PCR of the isolated genomic DNA of the cancer tissues was performed using an EZ DNA Methylation Kit™ (Zymo Research, Orange, CA) following the manufacturer's instructions. Bisulfite-modified, Sss I (New England Biolabs, MA)-treated normal lymphocyte DNA served as the methylated control, and bisulfite-treated normal lymphocyte DNA served as the unmethylated control.

Capillary electrophoresis

Capillary electrophoresis (CE) was performed to analyze the MS-PCR products using an HDA system with a GCK-5000 cartridge kit (eGene, Irvine, CA). The gel matrix in the gel cartridge was composed of proprietary linear polymer with ethidium bromide dye. The PCR products were diluted 20-fold with deionized water and placed in the instrument's sample chamber. The DNA samples were then injected into the capillary channels and subjected to electrophoresis based on the manufacturer's protocol. The BioCalculator Graphing software was used to automatically label peak sizes.

The bisulfite treatment converted unmethylated cytosine (C) into uracil (U) that was detected as thymidine (T) after the MS-PCR. In contrast, cytosine (C) in the methylated gene promoter was not converted after the C-T conversion agent, and it was detected by the primers used for methylated gene promoters. Gene methylation was defined as a positive result with the primers used for methylated gene promoters. Direct sequencing in 25% of the samples was performed as an independent method to validate the MS-PCR and CE results.

Extraction of RNA and reverse-transcription polymerase chain reaction in ovarian cancerous tissues

Cancer tissue specimens were collected, frozen, and stored as described earlier. Total RNA of the tissues was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions.

The RNA was first reverse-transcribed to cDNA using a Moloney murine leukemia virus reverse transcriptase kit (Invitrogen Life Technologies, San Diego, CA). The primers of these genes were used for 30 cycles. Glyceraldehyde-3-phosphate dehydrogenase was used as the housekeeping gene for comparison using the primer sets 5'-ACCCAGAAGACTGTGGATGG-3' and 5'-TGCTGTAGCCAAATTCGTTG-3' for 30 cycles. The PCR products were then analyzed in 1% agarose gel with ethidium bromide staining in the TBE solution. The PCR products of around 400 and 500 bp were regarded as exact products. Differences in transcription levels were compared using electrophoresis.

Immunoblotting

Frozen ovarian cancerous tissues were homogenized with lysis buffer as described earlier [26]. The protein extracts were quantified using a bicinchoninic acid protein assay kit (Pierce). Fifty μg of each lysate was then resolved by SDS/PAGE (12% gel), transferred to a PVDF/nylon membrane (Millipore), and probed with antibodies specific to DLEC-1 (1:100, Abcam, Cambridge, MA), E-cadherin (1:500, Abcam), SFRP5 (1:200, Abcam), or β-actin (1:20000, Abcam). The membrane was then probed with either horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit antibodies. The specific bands were visualized by an ECL (enhanced chemiluminescence) Western blotting system (GE Healthcare). The relative protein expression levels of DLEC-1, E-cadherin, and SFRP5 compared to β-actin protein in the respective samples were further calibrated and quantitated by densitometry.

Statistical analysis and clinical correlation

All statistical analyses were done using the Statistical Package for Social Sciences software package (SPSS for Windows, version 15.0.0, SPSS Inc., Chicago, IL). The Student's *t* test or one-way ANOVA was used for continuous variables and chi-square test for categorical variables. Survival curves were generated using the Kaplan-Meier method, and differences were calculated using the log rank test. A multivariate Cox's regression model was used to evaluate the prognostic factors for disease-free survival (DFS) and overall survival (OS). The variables which had p values less than 0.10 in univariate analysis were further analyzed in the multivariate analysis. Statistical significance was set at a p value of less than 0.05.

Table 4. Univariate and multivariate analysis for DFS of the women with early- (n=60) or advanced-stage (n=57) OCCA and OEA

		DFS				OS			
		Univariate		Multivariate		Univariate		Multivariate	
	n	HR (95% C.I.)	*p*	HR (95% C.I.)	*p*	HR (95% C.I.)	*p*	HR 95% C.I.)	*p*
Early stage	60								
Histology									
OEA (reference)	27	1				1			
OCCA	33	2.15 (0.69-6.71)	0.18			1.08 (0.25–4.65)	0.91		
Platinum-based chemotherapy									
Without Paclitaxel (reference)	29	1				1			
With Paclitaxel	31	103 (0.38-2.77)	0.94			0.74 (0.17-3.14)	0.68		
E-cadherin, DLEC1, SFRP5 gene methylation									
<2 methylated genes (reference)	38	1				1			
≥2 methylated genes	22	1.35 (0.50-3.66)	0.55			1.91 (0.44-8.12)	0.38		
Advanced stage	57								
Histology									
OEA (reference)	24	1				1			
OCCA	33	1.44 (0.72-2.88)	0.29			1.09 (0.47–2.50)	0.83		
Debulking surgery									
Optimal (reference)	40	1		1		1			
Suboptimal	17	2.35 (1.16-4.75)	0.017	2.34 (1.14-4.77)	0.020	1.23 (0.51-3.00)	0.65		
Platinum-based chemotherapy									
Without Paclitaxel (reference)	11	1				1		1	
With Paclitaxel	46	0.68 (0.29-1.57)	0.37			0.37 (0.14-0.96)	0.042	0.31 (0.11-0.84)	0.022
E-cadherin, DLEC1, SFRP5 gene methylation									
<2 methylated genes (reference)	37	1		1		1		1	
≥2 methylated genes	16	1.96 (1.00-3.86)	0.049	1.95 (0.99-3.86)	0.055	3.55 (1.54-8.17)	0.003	3.85 (1.66-9.05)	0.002

Abbreviations: n, patient number; DFS, disease-free survival; OS, overall survival; OEA, ovarian endometrioid adenocarcinoma; OCCA, ovarian clear cell adenocarcinoma.

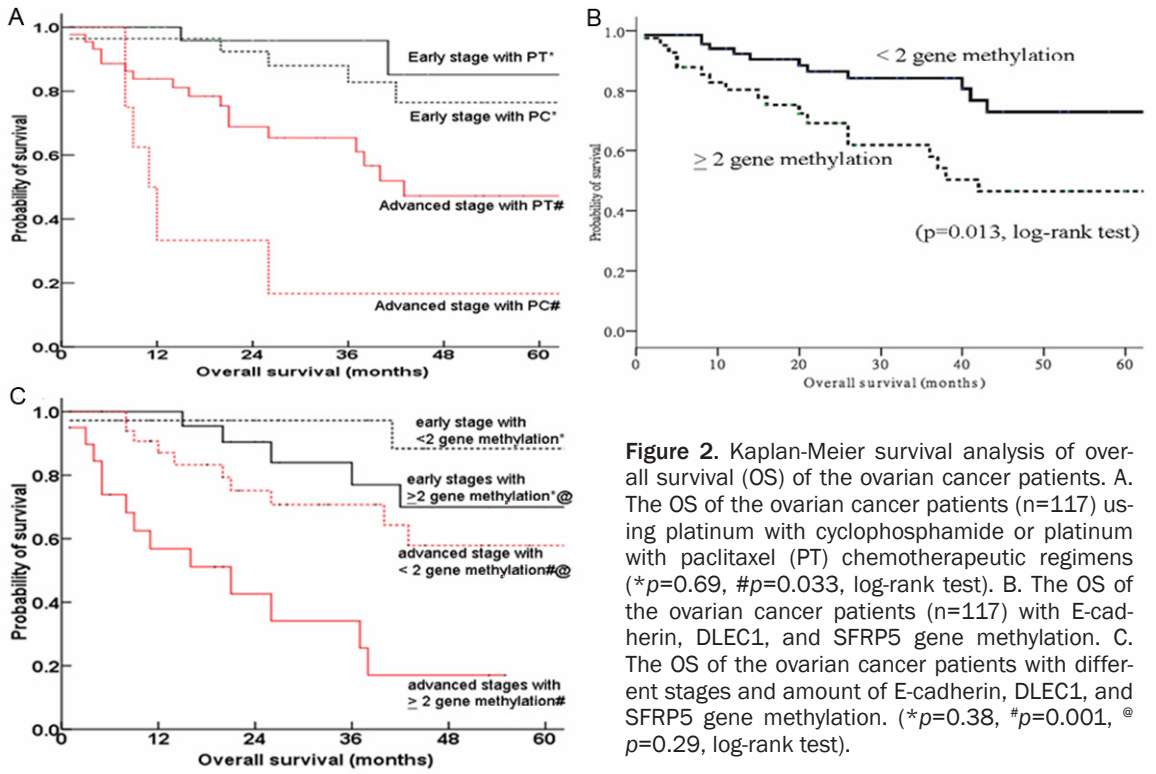

Figure 2. Kaplan-Meier survival analysis of overall survival (OS) of the ovarian cancer patients. A. The OS of the ovarian cancer patients (n=117) using platinum with cyclophosphamide or platinum with paclitaxel (PT) chemotherapeutic regimens (*p=0.69, #p=0.033, log-rank test). B. The OS of the ovarian cancer patients (n=117) with E-cadherin, DLEC1, and SFRP5 gene methylation. C. The OS of the ovarian cancer patients with different stages and amount of E-cadherin, DLEC1, and SFRP5 gene methylation. (*p=0.38, #p=0.001, @ p=0.29, log-rank test).

Results

Clinical characteristics of the 117 OCCA and OEA patients

In total 117 patients were enrolled, including 66 with OCCA and 51 with OEA, with a mean age of 50.8±10.3 years and median pre-treatment CA125 value of 254 U/ml. Of the 117 patients, 51% had early stage disease, 85% (99/119) received optimal debulking surgery, and 66% (77/119) received platinum with paclitaxel chemotherapeutic regimens. The median DFS and OS of all of the patients were 15 (range: 0-212) and 26 (range: 0-216) months, respectively. The overall percentages of mdethylation in RASSF1A, E-cadherin, DLEC1, RUNX3, SFRP1, SFRP5, PAX, and LMX1A were 70%, 21%, 59%, 50%, 60%, 46%, 68%, and 60%, respectively.

Clinical characteristics and patterns of gene methylation between OCCA and OEA patients

The characteristics of the 66 OCCA and 51 OEA patients are summarized in **Table 1**. There were no significant differences in mean age at diagnosis (p=0.19, Student's t-test), median pre-treatment CA 125 value (p=0.28, one-way ANOVA), percentages of early or advanced stages (p=0.85, chi-square test), percentages of optimal debulking surgery (p=0.29, chi-square test), or percentages of different chemotherapeutic regimens between the OCCA and OEA groups. There were also no significant differences in DFS or OS between the two groups (p=0.31 and p=0.94, respectively, log-rank test).

The status and percentage of methylation in each gene of all 117 patients are summarized in **Table 1**. The mean number of methylated genes of the eight genes were 4.12 and 4.50 in the OEA and OCCA groups, respectively (p=0.16, one-way ANOVA). There was no significant difference in the mean number of methylated genes between the two groups. The OCCA group had significantly higher percentages of RASSF1A (79% vs. 59%, p=0.025), E-cadherin (30% vs. 10%, p=0.011), and DLEC1 (71% vs. 43%, p=0.003) gene methylation than the OEA group (all by chi-square test). The OCCA patients also had higher percentages of RASSF1A, E-cadherin and DLEC1 gene methylation than the OEA patients (p=0.01, Mann-Whitney test) (**Figure 1A**). However, the percentage of methylation of any gene in the OEA group was not higher than that in the OCCA group.

Decreased transcription and protein expressions in methylated and unmethylated genes in cancerous tissues

We further explored the influence of gene methylation on RNA transcription and protein expression levels. Comparisons of the results of MS-PCR by CE analysis (**Figure 1B**) and reverse-transcription polymerase chain reaction for E-cadherin transcriptional levels in various ovarian cancerous tissues (**Figure 1C**) revealed that the cancer tissues with methylated E-cadherin had lower E-cadherin RNA transcription levels than those without methylated E-cadherin. The expression levels of DLEC1, SFRP5, and E-cadherin in the unmethylated and methylated OCCA and OEA cancerous tissues are shown in **Figure 1D** and **1E**. The related protein expression levels in the methylated samples were significantly lower than those in the unmethylated samples in the patients with OCCA (**Figure 1F**) (SFRP5 p=0.031, E-cadherin p=0.007, DLEC1 p=0.015 by one-way ANOVA) and in the patients with OEA (**Figure 1G**) (SFRP5 p=0.024, E-cadherin p=0.021, DLEC1 p=0.011 by one-way ANOVA).

This indicated that methylation inhibited both gene and protein expression levels of the DLEC1, SFRP5, E-cadherin genes in the patients with OCCA and OEA.

Correlations between gene methylation and disease severity, debulking surgery or chemo-response

There were no significant differences in the percentages of methylation of each gene between those with early- and advanced-stage disease (**Table 2**). The optimal debulking group had a trend of a lower percentage of DLEC1 methylation than the sub-optimal group (50.0% vs. 76.4%, p=0.064, chi-square test) (**Table 2**). The chemo-resistant group had a significantly higher percentage of E-cadherin methylation than the chemo-sensitive group (44.0% vs. 12.5%, p=0.007, chi-square test) (**Table 2**).

E-cadherin, DLEC1, and SFRP5 gene methylation as molecular markers for recurrence or death

We further evaluated whether the methylation of single or multiple genes was correlated with the OS of these 117 ovarian cancer patients. There were no statistically significant differences between the methylation of single genes and the OS of the patients in univariate analysis (Supplementary Table 2). The genes with hazard ratios (HR) of methylation versus unmethylation that were higher than 1 were entered into combination analysis. Only the combination of E-cadherin, DLEC1 and SFPR5 showed a significantly poorer OS in univariate analysis (Supplementary Table 2).

We further evaluated whether a combination of the methylation of various genes and the clinical variables were independent prognostic factors for the outcomes of the 117 patients (**Table 3**). Advanced stage (HR: 4.26 [2.09-8.74], p<0.001), sub-optimal debulking surgery (HR: 2.82 [1.39-5.68], p=0.004), and two or more methylated genes (E-cadherin, DLEC1, and SFRP5) (HR: 1.89 [1.07-3.32], p=0.028) were found to independent prognostic factors of a shorter DFS in multivariate analysis. Advanced stage (HR: 8.03 [2.94-21.92], p<0.001) and two or more methylated genes (E-cadherin, DLEC1, and SFRP5) (HR: 3.29 [1.57-6.87], p=0.002) were found to be independent prognostic factors of a poorer OS in multivariate analysis.

Association of chemotherapeutic regimens with the outcomes of OCCA and OEA

The OS of the 117 patients with ovarian cancer under platinum and cyclophosphamide or platinum and paclitaxel chemotherapeutic regimens were assessed. The patients with early-stage ovarian carcinoma had a significantly longer OS than those with advanced stage, regardless of the chemotherapeutic regimen (**Figure 2A**) (p<0.001, log-rank test). The OS of the patients with early-stage ovarian carcinoma treated with platinum and paclitaxel was not different to that of the patients under a platinum and cyclophosphamide regimen (p=0.69, log-rank test). However, the patients with advanced-stage ovarian carcinoma who were treated with platinum and paclitaxel had a longer OS than those treated with platinum and cyclophosphamide (p=0.033, log-rank test).

Combination analysis of E-cadherin, DLEC1, and SFRP5 gene methylation with the outcomes of the OCCA and OEA patients

The patients with two or more methylated genes (E-cadherin, DLEC1, and SFRP5) had a significantly shorter OS than those with only

one or no gene methylation when stratified by early and advanced stage (p=0.013, log-rank test) (**Figure 2B**). The OS of the ovarian cancer patients with different stages and amount of E-cadherin, DLEC1, and SFRP5 gene methylation were shown in **Figure 2C**. The OS of the 60 patients with early-stage ovarian carcinoma was not different with regards to the number of methylated genes (p=0.38, log-rank test) (**Figure 2C**). Among the patients with advanced-stage ovarian carcinoma, those with one or no methylated genes had a longer OS than those with two or more methylated genes (p=0.001, log-rank test) (**Figure 2C**). However, the OS of those with early-stage carcinoma and two or more methylated genes was not statistically different compared to the patients with advanced-stage disease with one or no methylated genes (p=0.29, log-rank test) (**Figure 2C**).

We further evaluated whether the combination of methylated genes was a prognostic factor for outcomes in the 60 patients with early-stage disease and 57 patients with advanced-stage disease (**Table 4**). No significant risk factor was identified in the patients with early-stage disease in either DFS or OS. In the patients with advanced-stage disease, sub-optimal debulking surgery (HR: 2.34 [1.14-4.77], p=0.020) was the only independent prognostic factor of a poorer DFS in multivariate analysis. The patients with two or more methylated genes (E-cadherin, DLEC1, and SFRP5) had a marginally shorter DFS (HR: 1.95 [0.99-3.86], p= 0.055) than those with only one or no methylated genes. A paclitaxel-based regimen was a protective factor for the OS of patients with advance-stage disease (HR: 0.31 [0.11-0.84], p=0.022) in multivariate analysis. However, the patients with two or more methylated genes (E-cadherin, DLEC1, and SFRP5) still had a poorer OS (HR: 3.85 [1.66-9.05], p=0.002) compared to those with only one or no methylated genes in multivariate analysis.

Discussion

The mechanisms of carcinogenesis of epithelial ovarian carcinoma differ between histological subtypes, the four major subtypes being serous, mucinous, endometrioid, and clear cell. A proposed mechanism of pathogenesis includes type I and II tumor pathways containing different histological subtypes [27]. Type I tumors are composed of low-grade serous, low-grade endometrioid, clear cell, mucinous and Brenner carcinomas characterized by an indolent behavior and lesions usually confined to the ovary on initial presentation. It has been reported that ovarian clear cell and endometrioid carcinomas develop from endometriosis [9, 10]. However, differences in tumor biology between OCCA and OEA are still not well understood. The present study evaluated the tumor biology between OCCA and OEA using epigenetics.

OCCA and OEA have different genetic profiles. While epidemiologic and molecular evidence supports the hypothesis that OCCA and OEA develop from endometriosis, these two histological types of epithelial ovarian carcinoma have similar as well as distinct characteristics, implying that there may be different underlying mechanisms with regards their respective carcinogenesis [9, 10, 23]. Epigenetic control is like a switch that turns on or off the expression of DNA coding genetic information [15]. Aberrant epigenetic alterations are now viewed as being crucial processes in carcinogenesis in addition to genetic alterations [28].

Different histological types of ovarian carcinomas have different methylation profiles. The current study found that the majority of normal ovarian tissues did not show gene methylation compared with cancerous tissues (data not shown). In addition, the patients with OCCA had higher frequencies of RASSF1A, E-cadherin, and DLEC1 gene methylation compared to the patients with OEA. The RAS association domain family protein 1A (RASSF1A), a putative tumor suppressor gene located on 3p21, is regarded to play important roles in the regulation of different types of human tumors [29, 30]. It has also been suggested that RASSF1A inactivation may be a tumorigenic mechanism that is distinct from the oncogenic activation of Ras signaling in tumors [31].

E-cadherin is a trans-membrane glycoprotein and a member of the family of calcium-dependent adhesion molecules. Mature E-cadherin protein is encoded by the tumor suppressor gene CDH1 located on chromosome 16q22.1, and the hyper-methylation of E-cadherin has recently been shown to be an important molecular mechanism in the transcriptional inactivation of key tumor suppressor genes in many cancers [32, 33]. Lastly, DLEC1, located in

3p22.3, is reportedly down-regulated by epigenetic alterations in ovarian, colon, and gastric cancers [22, 34]. This implies that the Wnt/β-catenin and Ras signaling pathways are involved in the carcinogenesis of ovarian carcinomas, and specifically those of the clear cell histological type.

Methylation of E-cadherin can be a predictive biomarker for the personalized management of OCCA and OEA patients. Chemo-resistance is an important obstacle in ovarian cancer treatment, and several potential agents or combined regimens are currently under investigation [4]. The results of the current study show that chemo-resistant patients have a higher prevalence of E-cadherin gene methylation than chemo-sensitive patients, indicating that transcriptional silencing of E-cadherin by gene methylation may play an important role in the resistant response to paclitaxel. Ho et al. reported that paclitaxel-based chemotherapy improved survival among patients with ovarian clear cell carcinoma and positive E-cadherin immuno-reactivity [35]. Paclitaxel acts through the stabilization of microtubules, cell-cycle arrest in the G2/M-phase, and the activation of pro-apoptotic signaling [36]. The dysregulation of E-cadherin has been reported to have an effect on drug resistance in *in vitro* studies on cancer cell lines [37].

DLEC1 is a functional tumor suppressor gene involved in multiple tumorigenesis, however, the mechanism underlying its role remains largely unknown. Ying et al. observed that the introduction of DLEC1 to silenced tumor cell lines strongly suppressed their growth in colony formation assays [34]. Similar properties have been observed in esophageal, renal and lung cancer cell lines [22, 38]. The predicted protein sequence of DLEC1 has no significant homology to any known proteins or domains. An earlier report showed that 27 potential CK2 (formerly known as casein kinase II) phosphorylation sites are present in the predicted sequence of DLEC1 [38]. Litchfield et al. reported that CK2is required for multiple transitions in the cell cycle, including G0/G1, G1/S and G2/M [39]. This indicates that DLEC1 may be involved in cell cycle arrest.

Methylation profiles can act as predictive markers for outcomes in patients with ovarian carcinoma. The results of the current study indicate that patients with a greater number of methylated E-cadherin, DLEC1, and SFRP5 genes have a poorer prognosis, especially among those with advanced-stage disease. Furthermore, patients with early-stage disease and two or more of these methylated genes have an OS similar to that of patients with advanced-stage disease and one or no methylated genes. Gene methylation may be a more sensitive predictor than conventional clinicopathologic factors such as surgical stage.

Secreted Frizzled-related protein 5 (SFRP5) is a member of the SFRP protein family, and contains a cysteine-rich domain homologous to the putative Wnt-binding site of Frizzled proteins. By antagonizing Wnt signaling, it acts as a tumor suppressor. Previous studies have identified associations between SFRP5 promoter hyper-methylation with ovarian [40], pancreatic [41], and breast cancers [42]. The E-cadherin and SFRP5 genes are antagonists against the Wnt/β-catenin pathway. However, the molecular mechanism underlying the tumor suppressive function of DLEC1 remains unknown. Nonetheless, the level of tumor suppressor genes may play a role in the prognosis of ovarian carcinoma patients.

Our results revealed that the combination of E-cadherin, DLEC1 and SFRP5 gene methylation was a prognostic marker of OCCA and OEA. Oncogenic activation of the Wnt/b-catenin signaling pathway is common in cancers, and over activation of Wnt/b-catenin signaling has been reported to be a major factor in oncogenesis of the ovaries, especially in that of a clear cell histology [43, 44]. SFRP5 can antagonize the Wnt signaling pathway [45], and binding of the Wnt ligand to SFRP5 leads to stabilization of b-catenin and its translocation into the nucleus. This transcription factor complex transactivates a host of target genes governing cancer-relevant processes. In addition, E-cadherin can anchor β-catenin, preventing its availability for nuclear entry and oncogenic transcriptional activity [46]. We hypothesize that DLEC1 may also act through the pathway of Wnt/b-catenin signaling to interact with oncogenesis of the ovaries. The more methylated genes of E-cadherin, DLEC1 and SFRP5 resulted in a reduced inhibiting effect on the Wnt/b-catenin signaling pathway of ovarian carcinogenesis and the outcomes of the patients.

The limitations of the present study include the limited number of screened genes, small patient number, and the use of qualitative assays for gene methylation. DNA methylation chips can provide large-scale screening, and other quantitative assays are also under development [25]. The MS-PCR and CE used in this study could only identify whether or not a gene was methylated, but not the degree of methylation. Periodic direct sequencing and the use of methylated and unmethylated controls in this study validated our results and avoided false positive and negative results. Based on the results of this study, more patients with ovarian clear cell carcinoma and endometrioid carcinoma should be recruited in prospective studies to comprehensively investigate the difference in methylation profiles between these subtypes of ovarian carcinoma, and methylation chips and methylation-sensitive high-resolution melting-curve analysis should be used.

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Disclosure of conflict of interest

No potential conflicts of interest were disclosed.

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Supplementary Table 1. Primer sequences and PCR conditions for methylation-specific PCR

Genes		Sense	Anti-sense	Tm (°C)	Size (b.p.)	genomic location	a CpG island site
RASSF1A	M	5'-GGGTTCGTTTTGTGGTTTCGTTC-3'	5'-TAACCCGATTAAACCCGTACTTCG-3'	62	76	75 b.p. in front of ATG site	Yes
	U	5'-GGGGTTTGTTTTGTGGTTTTGTTT-3'	5'-AACATAACCCAATTAAACCCATACTTCA-3'		81		
E-cadherin	M	5'-TGTAGTTACGTATTTATTTTTAGTGGCGTC-3'	5'-CGAATACGATCGAATCGAACCG-3'	63	112	From 145 b.p.in front of ATG site	Yes
	U	5'-TGGTTGTAGTTATGTATTTATTTTTAGTGGTGTT-3'	5'-ACACCAAATACAATCAAATCAAACCAAA-3'		120		
DLEC1	M	5'-GATTATAGCGATGACGGGATTC-3'	5'-ACCCGACTAATAACGAAATTAACG-3'	60	197	From 112 b.p. in front of ATG site	Yes
	U	5'-TGATTATAGTGATGATGGGATTTGA-3'	5'-CCCAACCTAATAACAAAATTAACACC-3'		197		
RUNX3	M	5'-ATAATAGCGGTCGTTAGGGCGTCG-3'	5'-GCTTCTACTTTCCCGCTTCTCGCG-3'	60	115	From 74 b.p.in front of	Yes
	U	5'-ATAATAGTGGTTGTTAGGGTGTTG-3'	5'-ACTTCTACTTTCCCACTTCTCACA-3'		115	transcription start site	
SFRP1	M	5'-GTGTCGCGCGTTCGTCGTTTCGC-3'	5'-AACGTTACCCGACTCCGCGACCG-3'	55	172	From 283 b.p.in front of ATG site	Yes
	U	5'-GAGTTAGTGTTGTGTGTTTGTTGTTTTGT-3'	5'-CCCAACATTACCCAACTCCACAACCA-3'		181		
SFRP5	M	5'-AAGATTTGGCGTTGGGCGGGACGTTC-3'	5'-ACTCCAACCCGAACCTCGCCGTACG-3'	58.8	136	From 146 b.p. in front of ATG site	
	U	5'-GTAAGATTTGGTGTTGGGTGGGATGTTT-3'	5'-AAAACTCCAACCCAAACCTCACCATACA-3'		141		
PAX	M	5'-TATTTTGGGTTTGGGGTCGC-3'	5'-CCCGAAAACCGAAAACCG-3'	64	153	From 5 b.p.after ATG site	Yes
	U	5'-GTTTATTTTGGGTTTGGGGTTGTG-3'	5'-CACCCAAAAACCAAAAACCAC-3'	53	158		
LMX1A	M	5'-TTTAGAAGCGGGCGGGAC-3'	5'-CCGAATCCAAACACGCG-3'	62	130	From 353 b.p. in front of	Yes
	U	5'-GAGTTTAGAAGTGGGTGGGATG-3'	5'-CAACCAAATCCAAACACACAAAAC-3'	65	153	transcription start site	

Abbreviations: b.p., base pair; U, unmethylated primers; M, methylated primers.

Supplementary Table 2. Univariate analysis of gene methylation and OS of all women with OCCA and OEA women (n=117)

Gen methylation	HR (95% C.I.)	*P* value
RASSF1A		
No	1	0.13
Yes	0.55 (0.56-0.26)	
Ecadherin		
No	1	
Yes	1.93 (0.86-4.35)	0.11
DLEC1		
No	1	0.58
Yes	1.26 (0.56-2.83)	
RUNX3		
No	1	0.96
Yes	0.98 (0.45-2.14)	
SFRP1		
No	1	0.98
Yes	0.99 (0.45-2.16)	
PAX		
No	1	0.48
Yes	0.76 (0.35-1.64)	
LMX1A		
No	1	0.34
Yes	0.68 (0.32-1.48)	
SFRP5		
No	1	0.22
Yes	1.63 (0.75-3.55)	
E-cadherin, DLEC1 and SFPR5		
<2 methylated gene	1	0.016
≥2 methylated gene	2.40 (1.17-4.92)	
Ecadherin+RUNX3+SFRP5		
<2 methylated gene	1	0.20
≥2 methylated gene	1.68 (0.76-3.73)	
Ecadherin+SFRP1+SFRP5		
<2 methylated gene	1	0.098
≥2 methylated gene	1.93 (0.89-4.22)	
Ecadherin+SFRP1+DLEC1		
<2 methylated gene	1	0.19
≥2 methylated gene	1.68 (0.77-3.67)	
Ecadherin+RUNX3+DLEC1		
<2 methylated gene	1	0.53
≥2 methylated gene	1.29 (0.59-2.78)	
SFRP1+DLEC1+SFRP5		
<2 methylated gene	1	0.15
≥2 methylated gene	1.81 (0.80-4.10)	
RUNX3+DLEC1+SFRP5		
<2 methylated gene	1	0.36
≥2 methylated gene	1.43 (0.66-3.10)	

HR: hazard ratio, CI: confidence interval.