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Original Article

Distinct promotor methylation at tumor suppressive genes in ovarian cancer stromal progenitor cells and ovarian cancer and its clinical implication

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Abstract: Aberrant CpG-island methylation affects ovarian cancer progression. The promotor methylation changes at tumor suppressive genes in ovarian cancer stromal progenitor cells (OCSPCs) and epithelial ovarian cancer (EOC) tissues and their clinical implication remains unexplored. We systemically analyzed the promoter methylation status of 40 tumor suppressor genes (TSGs) associated with cancer in paired epithelial-like and mesenchymal-like OCSPCs and ovarian cancer cells by methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA). The effect of DNA methylation on gene expression was confirmed using qRT-PCR. The differential frequencies of TSGs' promoter methylation among matched epithelial-like or mesenchymal-like OCSPCs from tissues and ascites and ovarian cancer tissues were further validated in cancer tissues and correlated with clinicopathological features and survival outcomes of patients. According to the promoter methylation frequencies of the 40 TSGs, promoters of RASSF1A were the only significantly hypomethylated in epithelial-like OCSPCs from tissues than those from ascites and bulk tumor cells (0% vs 38% vs 45%, P=0.039 by Fisher's exact test). The most frequencies at promotor hypermethylation of TSGs in mesenchymal-like OCSPCs from ascites which processed aggressiveness were CDKN2B (73%) followed by CCND2 (45%) and RASSF1A (45%). Forty-three percent (47/110) of RASSF1A and 45% of CCND2 were validated as a frequently hypermethylated gene in an independent set of 110 EOC tissues in contrast to none (0/60) and 12% (10/60) of benign ovarian cysts (both P<0.001). Functional experiments revealed overexpression of CCND2 or CDKN2B in MSc-OCSPCs decreases EMT, invasion, and spheroid formation in EOC, and abolishes DNMT1 and COL6A3 expression. However, for the expected 5-year overall survival (OS) for patients with methylated RASSF1A, CCND2, and CDKN2B, only RASSF1A was significantly worse than those without methylated RASSF1A (56% vs 80%, p=0.022). Taken together, overexpression of CCND2 and CDKN2B decreased the aggressiveness of mesenchymal-like OCSPCs from ascites which may represent a potential therapeutic target for EOC. Promotor hypomethylation at RASSF1A in OCSPCs from EOC tissues and changes to hypermethylation of EOC and OCSPCs from ascites could predict poor survival outcomes for EOC patients compared to without those changes of CCND2 and CDKN2B.

Keywords: DNA methylation, ovarian carcinoma stromal progenitor cells, tumor suppressor genes, methylation-specific multiplex ligation-dependent probe amplification, promoter methylation, RASSF1A, CCNND2, CDKN2B

Introduction

DNA methylation is one of the most common types of epigenetic regulation that induces gene transcriptional silencing, especially in embryonic stem (ES) and tissue stem cells [1, 2]. Despite the association between epigenetic alterations and cancer being well supported by the literature, the causal relationship between epigenetic alteration and tumorigenesis is much less understood. Tumors are often caused by DNA hypermethylation of tumor suppressor genes resulting in the loss of function of these genes [3, 4]. Methylation changes of some specific tumor-related genes are also considered diagnostic markers for tumor development or prognosis [5]. Moreover, the self-renewal of cancer stem cells is also thought to be regulated by the methylation of specific genes [6].

Accumulation of aberrant gene promoter methylation and transcriptional silencing of tumor suppressor genes (TSGs) are associated with ovarian cancer progression [7, 8]. Aberrant epigenetic modifications can also be associated with genetic changes through deletions or mutations that affect TSG function which act as an inactivating "hit" leading to tumorigenesis [9]. Genetic and non-genetic alterations in both ovarian surface epithelium (OSE) and surrounding stromal tissues may determine the phenotypic characteristics and functional performance of these cells. A recent study shows that aberrant TSG hypermethylation is sufficient to transform somatic stem cells into cancer stem cells [10].

Strong evidence suggests that the recruitment of mesenchymal stem cells (MSCs) into the tumor microenvironment is an important integration of the tumor microenvironment. In an experimental model of ovarian cancer, normal human bone marrow-derived MSCs were shown to promote tumor growth through the differentiation of MSCs into tumor-associated fibroblasts that produce many growth factors to support angiogenesis and tumor cell growth [11]. Moreover, human MSCs can enhance the growth and metastasis of breast cancer cells [12, 13], and increasing the number of cancer-associated MSCs (CA-MSCs) can promote tumor growth in vitro and in vivo studies [14]. Most experiments used either MSC lines or healthy donor-derived MSCs rather than using MSCs from cancer patients or tumor-associated MSCs, however, host cells in the tumor microenvironment often have altered phenotypes that contribute to tumorigenesis [15, 16].

Cancer stem/progenitor cells (CSCs) are a subset of cells related to chemotherapy resistance in multiple cancers. An important issue has been raised for applying information from studies on CSCs for screening new biomarkers to predict or correlate patient survival. The known characteristic features of CSCs or stromal progenitor cells have self-renewal, undifferentiation, distinct epigenetic status, abnormal metabolism, and microenvironment interaction. We hypothesized that the promoter methylation of ovarian cancer-associated stromal stem/progenitor cells (OCSPCs) may involve in the progression of epithelial ovarian cancer (EOC), and the promoter methylation of OCSPCs should differ from that of bulk tumor cells. It is speculated that the promoters of polycomb repressive complex (PRC) targeted TSGs in OCSPCs remain unmethylated than those in cancer cells to increase their expressions to maintain their tumor inhibition effect and keep the stem cell-like characteristics simultaneously. We therefore systemically investigated the promoter methylation status of 40 genes, which were reported to be associated with cancer in literature, among matched epithelial-like or mesenchymal-like OCSPCs from tissues, ascites, and tumor cells of patients with EOC by methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA). The impact of promoter methylation in identified genes was correlated with clinical-pathological features and survival outcomes in a cohort study.

Materials and methods

Samples collections from EOC patients

The institutional review board of Cathay General Hospital (CGH) approved this study (CGH-P103096). Paired fresh tissue samples (n=11) and ascetic fluid (n=11) samples were collected from 11 EOC patients (age: 43-67 years) with primary stage IC (2), IIIc (8), or Stage IV (1), at CGH between March 2011 and January 2013. Two histologically proven normal ovarian tissue samples were obtained from 2 patients with early-stage EOC. Histopathology, grade, and stage of ovarian tumor were assigned according to the Federation of Gynecology and Obstetrics criteria. Of the 11 tissue samples included, 8 were from patients diagnosed with high-grade ovarian serous carcinoma (OSC), 1 was from a patient with ovarian poorly differentiated carcinoma, and 1 was from a patient with ovarian clear cell carcinoma (OCCC), and 1 was from a patient with ovarian mucinous carcinoma. Paired fresh cancer tissue and ascites samples were obtained during surgery and immediately delivered to the laboratory preparing for ovarian cancer stromal progenitor cells (OCSPCs) processing. The rest of the cancerous tissues were pathologically confirmed to contain >66% neoplastic areas which were immediately sent to a freezer at -80°C until further analysis. Cell lines (n=22) derived from mesenchymal-like OCSPCs (n=11) and epithelial-like OCSPCs (n=11) in 11 fresh ovarian cancer tissues were obtained. In addition, cell lines (n=19) derived from mesenchymal-like OCSPCs (n=11) and epithelial-like OCSPCs (n=8) derived

from 11 ascites were obtained. However, three epithelial-like OCSPCs were cultured and failed.

An independent set of 110 EOC tissues including 39 ovarian serous carcinoma and 71 non-serous carcinoma tissues and 60 ovarian cystadenomas or endometriotic cysts between 1994 and 2005 was used to verify the identified genes in OCSPCs and corresponding bulk tumor tissues. All tissues taken during surgery were divided and placed into separate Eppendorf tubes immediately frozen in a nitrogen tub and then transferred to a -80°C refrigerator until analysis, and the cancerous tissue sample was confirmed pathologically to have high neoplastic cellularity (>66%).

The clinical information, including age, preoperative CA-125 value, stage, residual tumor after debulking surgery, recurrence, and survival status were collected from medical records deposited in a centralized database. Optimal cytoreductive surgery was defined as the maximal diameter of the residual tumor of <1 cm; otherwise defined as suboptimal debulking surgery. Patients received regular follow-ups after surgery or adjuvant chemotherapy. Progression-free survival or overall survival was calculated as the period from the operation to the date of disease progression or death or the date of the last contact.

Isolation and in vitro culture conditions of cells from ascites and fresh tissues

Cells from fresh tissue and ascites samples of EOC patients were isolated using standard procedures as previously described [17]. Briefly, the cell pellets were obtained from ascitic fluid and were directly centrifuged at room temperature for 5 min at 1500 rpm. The cell pellets were re-suspended in 10 mL of ovarian culture medium with Dulbecco's modified Eagle's medium-(DMEM/F12) supplemented with EGF (10 ng/mL), FGF-b1 (10 ng/mL), and 10% FBS (Hyclone). 3×10^6 cells were contained in a T75 flask. Cultures need to be refreshed every 3 days to maintain adherent cells in a humidified chamber with 5% CO_2 at 37°C. When the adhered cells reached 85% confluence, we used 0.25% trypsin-1 mM EDTA (Sigma-Aldrich, St. Louis, MO, USA) treatment for 5 min to harvest cells.

Ovarian tissue added to Hank's Balanced Salt Solution (Invitrogen; Grand Island, NY) was minced and mixed with 1 mg/ml Collagenase 1A (Sigma, C9891) for 60 min at 37°C. The undigested tissue pieces were removed to get the dispersed cells through a 70 μm nylon mesh (BD Bioscience) filter. The dispersed cells were further centrifuged at 170 × g to obtain cell pellets. The Isolated cells seeded at a density of 5×10^4 cells per cm^2 in a T75 flask were sub-cultured when reaching approximately 80% confluence using 0.25% trypsin-1 mM EDTA (Sigma-Aldrich, St. Louis, MO, USA).

Establishment of stable SKOV3-Luc or GFP cell lines

SKOV3 cells obtained from ATCC were transfected with GFP (SKOV3-GFP) or luciferase-expressing lentivirus (SKOV3-Luc).

Invasion experiment

For invasion assays, we used transwell chambers (8 μm, 24-well format; Corning Co., USA) or Matrigel-coated transwell chambers (BD Bioscience, USA), inserted into 24-well cell culture plates. SKOV3 cells (3×10^4 cells in 0.2 mL of serum-free medium) were added to the upper chamber. The following components in 0.2 ml of medium, 5% FBS, were added to lower chambers: Conditioned medium from the following cell lines: NOSPCs (normal ovarian stromal cells), MSc-OCSPCs, MSc-OCSPCs overexpressing CCND2 (MSc-OCSPCs/CCND2), MSc-OCSPCs overexpressing CDKN2B (MSc-OCSPCs/CDKN2B), or MSc-OCSPCs/vector. Cells were cultured for 72 h, and cells that had migrated or invaded through the inserts were then fixed in methanol for 20 min, stained with crystal violet, and counted in three random microscope fields (Olympus BX3, Japan) at a magnification of 40 ×, 100 ×, or 200 ×.

Spheroid formation by EOC cells

For spheroid formation, SKOV3, cells were cocultured with MSc-OCSPCs or MSc-OCSPCs/CCND2, MSc-OCSPCs/CDKN2B, or MSc-OCSPCs/vector under spheroid inducing conditions: DMEM/F12 containing 20 ng/mL bFGF, 20 ng/mL EGF, 10 ng/mL IGF, 2% B27 (Invitrogen, Carlsbad, CA), and with or without 5-aza-dC. Spheroid numbers were counted after 7 days under an Olympus light microscope. The spheroids were harvested on day 14 for FACS analysis.

Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA)

Cells from fresh tissue and/or ascites samples of benign ovarian cysts, EOC cell lines, and EOC patients were performed using MS-MLPA. The details of the procedure of MS-MLPA were performed based on the manufacturer's guide with some modifications as previously described [17]. The methylation ratio (M-ratio) was calculated as the probe fraction value of the ligation-digestion sample divided by that of the corresponding undigested ligation sample, resulting in a "methylation ratio" (M-ratio). Promotor methylation was defined as M-ratio ≥0.25, corresponding to 25% methylated DNA. M-ratios have independently calculated the mathematical algorithm cut-off ratio for a peak height that was defined to be ≥25% when reaction-targeted genes were more than one probe as previously described [18].

Western blot analysis

Cells were lysed in PBS containing 1% Triton X-100 using an ultrasonic cell disruptor. Lysates were separated by SDS-PAGE (12.5%) and transferred to a PVDF membrane (NEN). The membranes were blocked in blocking buffer (TBS containing 0.2% Tween 20 and 1% I-block (NEN)) and incubated with the polyclonal antibodies separately for 1 hour. A purified rabbit anti-human GAPDH polyclonal Ab (Santa Cruz Biotechnology, Inc.) was also applied at the same time to normalize the signals generated from the anti-COL6A3, DNMT1, DNMT3A, DNMT3B, E-cadherin, vimentin, EZH2, PIK3Ip1, p53, CDK4/6, RB, p-RB, and cyclin D1 Abs (Cell Signaling). After washing, an alkaline phosphatase-conjugated anti-rabbit Ab (Vector Laboratories) was applied. The membranes were washed, and the bound Abs were visualized by developing with the NBT/BCIP chromogen.

In vivo animal experiments and tumor imaging

Null mice (BALB/cAnN.Cg-*Foxn1*^{nu}/CrlNarl) were purchased from the National Animal Center (Taipei, Taiwan), and all experiments were approved by the Institutional Animal Care and Use Committee of Cathay General Hospital. Null mice at 5-7 weeks of age were inoculated with cells intraperitoneally (IP). Experiments (3-5 mice/group) were carried out as previously described [19]. Bioluminescence optical images (Xenogen IVIS 2000, Caliper Life Sciences) were obtained following tumor cell injection.

RNA preparation and reverse transcription real-time quantitative (RT-qPCR) method

The details of the procedures were described previously [19]. RASSF1A gene was amplified by a QIAGEN-designed primer set: RASSF1A (Cat No: QT01016134), GAPDH (Cat No: QT01192646) was used as an internal control. Data were expressed as the mean of the copy number of the gene normalized by GAPDH of three replicates.

Statistical analysis

The SPSS statistical package (SPSS 16.0.1 for Windows 2008, Chicago, IL, USA) was used for statistical analysis. Each group of methylation frequencies of promoters of the 40 genes was compared using Fisher's exact test. The mRNA expression level was assessed using the Mann-Whitney U test. The differences in survival curves were calculated using the log-rank test, and survival curves were generated by the Kaplan-Meier method. We performed a Cox's regression analysis designed to identify meaningful prognostic subsets in the study population following the "one in ten rule", which states that logistic regression models give stable values for the explanatory variables based on a minimum of about 10 events per explanatory variable. The 4 variables were used to account for prognostic factors affecting the survival of ovarian cancer patients including stage (early stage versus advanced stage; optimal versus suboptimal debulking; age >50 years versus <50 years; RASSF1A methylated versus unmethylated). Cox's univariate and multivariate regression model was applied to evaluate prognostic factors for survival in 110 ovarian cancer patients. A *P*-value of <0.05 was used to determine statistical significance. * represents <0.05, ** represents <0.01, *** represents <0.0001.

Results

Epithelial-like and mesenchymal-like OCSPCs differentially expressed cell-specific surface marker

Cultured in selective conditional media, two morphologically different (epithelial-like and

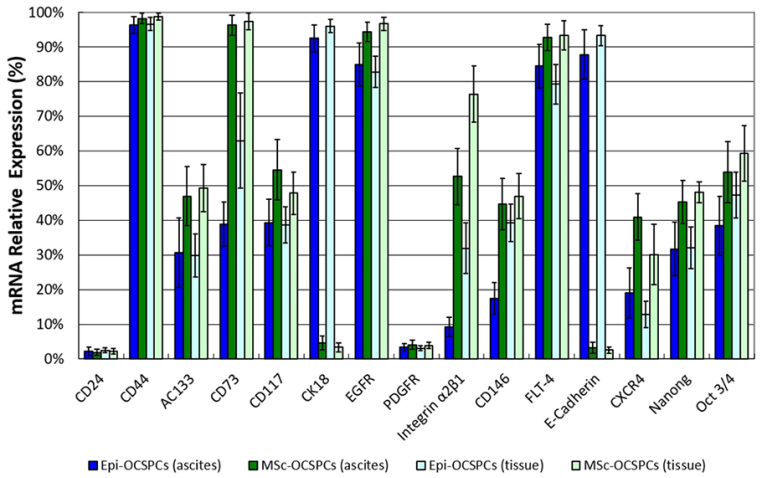

Figure 1. The differential expressions of cell-specific surface markers among epithelial-like ovarian cancer stromal progenitor cells (Epi-OCSPCs) or mesenchymal-like ovarian cancer stromal progenitor cells (MSc-OCSPCs) from ovarian cancer tissues and ascites.

mesenchymal-like) ascitic and tissues adherent cells populations displayed the typical cancer stem/progenitor cell markers $CD44^{high}$, $CD24^{low}$, and $AC133^{+}$, $CD117^{+}$ as previously described [17]. These cells had high CA125, EGFR, and Flt4 expression, and also possessed the high expression of embryonic stem cell markers Nanong and Oct3/4, and breast cancer stem cell marker SSEA4 [19]. The epithelial-like OCSPCs demonstrated higher cytokeratin 18 and E-cadherin expression than the mesenchymal-like OCSPCs. The mesenchymal-like OCSPCs, in contrast, demonstrated lower cytokeratin 18 and E-cadherin expression than the epithelial-like OCSPCs. The mesenchymal-like OCSPCs also showed higher AC133, CD73, CD117, EGFR, integrin $\alpha_2\beta_1$, CD146, CXCR4, Nanong, and Oct3/4 surface marker expression than the epithelial-like OCSPCs. The epithelial-like OCSPCs and mesenchymal-like OCSPCs from ascites showed higher integrin $\alpha_2\beta_1$, CD146, and Oct3/4 expression than those from tissues (**Figure 1**). The function of tumorigenesis is different in epithelial-like OCSPCs and mesenchymal-like OCSPCs. Epithelial-like OCSPCs could enhance tumorigenesis of EOC which could be reversed by the demethylating agent [19]. On the contrary, mesenchymal-like OCSPCs from ascites could enhance invasiveness, spheroid formation, and metastasis of EOC [20].

MS-MLPA profiles in 6 EOC cell lines

TSG promoter methylation on the EOC cell lines-SKOV3, ES2, KK, HAC-2, TOV21G, and OVCAR3 were first evaluated by MS-MLPA. The frequencies of promoters methylation of RASSFIA, FHIT, CDH13, RUNX3, SCGB3A1, ID4, SFRP4, SFRP5, CCND2, CACNA1A, and TIMP3 genes were found to be significantly higher in at least two of the six analyzed EOC cell lines than normal control (**Table 1**). The frequencies of promoters methylation of RARB, ESR1, CDKN2B, DAPK1, PTEN, GSTP1, IGSF4, and CACNA1G were significantly higher in one of the six analyzed EOC cell lines than in normal control. The different frequencies of promoter methylation of 40 tumor suppressive genes among 6 cell lines suggested those genes are not universally involved in their cancer-promoting effects (**Table 1**).

MS-MLPA profiles in OCSPCs and tumor cells of EOC

We next examine the differential frequencies of gene methylation status in OCSPCs from tissues, OCSPCs from ascites, and bulk tumor cells shown in **Table 2**. The promoter methylation frequencies of RASSFIA1 were only significantly lower in OCSPCs from tissues than OCSPCs from ascites (P=0.014) and in bulk tumor cells (P=0.026). Further analysis of the differential frequencies of gene methylation status in epithelial-like OCSPCs from tissues, epithelial-like OCSPCs from ascites, and bulk tumor cells were shown in **Table 3**. The promoter methylation frequencies of RASSFIA1 (P=0.039) were only significantly lower in epithelial-like OCSPCs from tissues than epithelial-like OCSPCs from ascites, and in bulk tumor cells (**Table 3**). The differential frequencies of gene methylation status in mesenchymal-like OCSPCs from tissues, mesenchymal-like OCSPCs from ascites, and bulk tumor cells were shown in **Table 4**. However, the promoter methylation frequencies among 40 genes were

Table 1. The frequencies of promoter methylation of 40 tumor suppressor genes between ovarian cysts and ovarian cancer cell lines

Gene name	Group 1	Group 2	*p*-value	Gene name	Group 1	Group 2	*p*-value
	number of methylation cases/all cases	Number of methylation cell lines/all cell lines			number of methylation cases/all cases	Number of methylation cell lines/all cell lines	
TP73	0/31	0/6		HIC1	0/31	0/6	
CASP8	0/31	0/6		BRCA1	0/31	0/6	
VHL	0/31	0/6		TIMP3	6/31	2/6	
RARB*	0/31	1/6	0.0212	PRDM2	0/31	0/6	
MLH1	0/31	0/6		RUNX3	0/31	3/6	<0.0001
RASSF1A	0/31	5/6	<0.0001	RARB**	0/31	0/6	
FHIT	0/31	2/6	0.0009	HLTF	0/31	0/6	
APC	0/31	0/6		SCGB3A1	4/31	6/6	<0.0001
ESR1	0/31	1/6	0.0212	ID4	0/31	4/6	<0.0001
CDKN2A	1/31	0/6		TWIST1	0/31	0/6	
CDKN2B	8/31	1/6		SFRP4	0/31	2/6	0.0009
DAPK1	0/31	1/6	0.0212	DLC1	1/31	0/6	
PTEN	0/31	1/6	0.0212	DLC2	1/31	0/6	
CD44	0/31	0/6		SFRP5	0/31	5/6	<0.0001
GSTP1	0/31	1/6	0.0212	BNIP3	1/31	0/6	
ATM	0/31	0/6		H2AFX	0/31	0/6	
IGSF4	0/31	1/6	0.0212	CCND2	2/31	6/6	<0.0001
CDKN1B	1/31	0/6		CACNA1G	0/31	1/6	0.0212
CHFR	0/31	0/6		TGIF	0/31	0/6	
BRCA2	0/31	0/6		BCL2	0/31	0/6	
CDH13	0/31	2/6	0.0009	CACNA1A	0/31	3/6	<0.0001

Note: Group 1: ovarian cysts; Group 2: ovarian cancer cell lines; *Probe 1: RARB (L10900); **Probe 2: RARB (L1698).

not significantly lower in mesenchymal-like OCSPCs from tissues than those from ascites and bulk tumor cells. Taken together, the results of promoter methylation frequencies of the 40 TSGs analyzed, the RASSF1A promoter was the only one with significant hypomethylation frequency in epithelial-like OCSPCs from tissues than those from ascites and in bulk tumor cells.

Overexpression of CCND2 or CDKN2B in MSc-OCSPCs decreases EMT, invasion, and spheroid formation in EOC, and abolishes DNMT1 and COL6A3 expression

Our previous study showed mesenchymal-like OCSPCs from ascites could enhance invasiveness, spheroid formation, and metastasis of EOC [20]. We next reasoned the potential candidates for inhibiting the aggressiveness of mesenchymal-like OCSPCs. The most common promotor hypermethylation of TSGs in ascites mesenchymal-like OCSPCs from ascites was CDKN2B (73%) followed by CCND2 (45%), RASSF1A (45%), and DLC1 (45%) (**Table 4**). Our previous study showed the cumulative methylation index (CMIs) of CDKN2B, RASSFIA, DLC1, and CCND2 in the OCSPCs from ascites were significantly higher than those in the OCSPCs from tissues (P=0.001) or bulk tumor cells (P=0.038) [19]. To clarify whether the methylation status was correlated with the expression levels of CCND2, RASSF1A, DLC1, and CDKN2B, we previously showed that mRNA levels of CCND2 and CDKN2B, but not RASSF1A and DLC1 were significantly lower in OCSPCs from ascites than those from bulk tumor tissues [19]. Moreover, CDKN2B mRNA levels were significantly lower in mesenchymal-like OCSPCs from ascites than in mesenchymal-like OCSPCs from bulk tumor tissues [19]. Therefore, we chose CDKN2B and CCND2 genes related to the CDK4/6-pRB pathway for further functional validation. We sought to determine if SKOV3

Table 2. The differential percentage of promoter methylation in tumor suppressor genes among ovarian cancer stromal progenitor cells from tissues, ovarian cancer tissues, and ovarian cancer stromal progenitor cells from ascites

Gene name	Group 1	Group 2	Group 3	*p*-value	
	OCSPC (T) (%)	OC (T) (%)	OCSPC (A) (%)	Group 1 vs 2	Group 1 vs 2 vs 3
DLC1	5/22 (23%)	3/11 (27%)	9/19 (47%)	0.097	0.223
RASSF1A*	2/22 (9%)	5/11 (45%)	8/19 (42%)	0.014	0.026
CDH13	0/22 (0%)	3/11 (27%)	1/19 (5%)	0.276	0.019
BRCA1	0/22 (0%)	2/11 (18%)	3/19 (16%)	0.053	0.129
TIMP3	3/22 (14%)	0/11 (0%)	4/19 (21%)	0.529	0.266
SCGB3A1	5/22 (23%)	2/11 (18%)	5/19 (26%)	0.790	0.878
ESR1	2/22 (9%)	0/11 (0%)	2/19 (11%)	0.877	0.551
CDKN2A	1/22 (5%)	3/11 (27%)	2/19 (11%)	0.463	0.154
CCND2**	12/22 (55%)	6/11 (55%)	8/19 (42%)	0.427	0.689
CDKN2B	9/22 (41%)	6/11 (55%)	11/19 (58%)	0.278	0.524
RUNX3	4/22 (18%)	1/11 (9%)	1/19 (5%)	0.207	0.417
RARB	3/22 (0%)	0/11 (0%)	1/19 (5%)	0.368	0.338
HLTF	2/22 (9%)	0/11 (0%)	0/19 (0%)	0.178	0.242
SFRP4	0/22 (0%)	1/11 (9%)	0/19 (0%)	NA	0.150
CACNA1A	0/22 (0%)	1/11 (9%)	1/19 (5%)	0.639	0.883
H2AFX	0/22 (0%)	1/11 (9%)	0/19 (0%)	NA	0.150
FHIT	0/22 (%)	0/11(0%)	0/19 (0%)	NA	NA
ID4	0/22 (%)	0/11(0%)	0/19 (0%)	NA	NA
SFRP5	0/22 (0%)	1/11 (9%)	0/19 (0%)	NA	0.150

Note: *represents probe RASSF1A382; **represents probe CCND2142.

invasiveness was inhibited by overexpression of CCND2 or CDKN2B in MSc-OCSPCs. To assess this, we cocultured SKOV3 cells with MSc-OCSPCs/CCND2 or MSc-OCSPCs/CDKN-2B cells in a transwell experiment (**Figure 2A**). The number of spheroids was significantly reduced when SKOV3 cells were cocultured with MSc-OCSPCs/CCND2 or MSc-OCSPCs/CDKN2B cells compared to cocultures of SKOV3 cells with MSc-OCSPCs/mock ($P<0.005$ for MSc-OCSPCs/CCND2, $P<0.001$ for MSc-OCSPCs/CDKN2B) or compared to cocultures of SKOV3 cells with MSc-NOSPCs (**Figure 2B** and **2C** left). In the animal experiment in which SKOV3-Luc cells and OCSPCs/CDKN2B cells were coinjected intraperitoneally, tumor growth was decreased compared to SKOV3-Luc cells coinjected with OCSPCs/mock cells ($P<0.05$. **Figure 2C** middle and right). However, we did not get consistent results from those with MSc-OCSPCs/CCND2 (data not shown). DNMT (all isoforms) mRNA expression levels were reduced in spheroids formed by either MSc-NOSPCs or by SKOV3 cells cocultured with MSc-OCSPCs/CCND2 or MSc-OCSPCs/CDKN-2B cells than in spheroids formed by SKOV3 cells cocultured with OCSPCs/mock cells (all $P<0.01$) (**Figure 3A**). Moreover, coculture of SKOV3 cells with MSc-OCSPCs/CCND2 or MSc-OCSPCs/CDKN2B decreased vimentin expression and increased E-cadherin expression resulting in the inhibition of EMT (**Figure 3B**). In addition, coculture of SKOV3 cells with MSc-OCSPCs/CCND2 or MSc-OCSPCs/CDKN2B almost completely abolished phosphorylation of RB (p-RB) and decreased DNMT and COL6A3 expression, but did not alter the expression of P53, CDK4/6, cyclin D1, or CDKN2A (**Figure 3B**). 5AZA-dC treatment of MSc-OCSPCs reduced their ability to induce the invasiveness of SKOV3 cells (**Figure 3C** and **3D**). Taken together, hypermethylation of CDKN2B or CCND2 with loss of tumor suppressor expression in MSc-OCSPCs induced EOC spheroid formation, EMT, invasion, and tumor growth through decreasing expression of DNMTs, p-RB, and COL6A3 in vivo and/or in vitro. Demethylating agent treatment of MSc-OCSPC could reduce the invasiveness of SKOV3 cells.

Table 3. The differential percentage of promoter methylation in tumor suppressor genes among epithelial-like ovarian cancer stromal progenitor cells from tissues, ovarian cancer tissues, and epithelial-like ovarian cancer stromal progenitor cells from ascites

Gene name	Epi-OCSPC (T) (%)	OC (T) (%)	Epi-OCSPC (A) (%)	*p*-value
DLC1	2/11 (18%)	3/11 (27%)	4/8 (50%)	0.318
RASSF1A*	0/11 (0%)	5/11 (45%)	3/8 (38%)	0.039
CDH13	0/11 (0%)	3/11 (27%)	0/8 (0%)	0.056
BRCA1	0/11 (0%)	2/11 (18%)	1/8 (13%)	0.351
TIMP3	0/11 (0%)	0/11 (0%)	0/8 (0%)	NA
SCGB3A1	4/11 (36%)	2/11 (18%)	3/8 (38%)	0.560
ESR1	0/11 (0%)	0/11 (0%)	0/8 (11%)	NA
CDKN2A	1/11 (9%)	3/11 (27%)	1/8 (13%)	0.485
CCND2**	9/11 (82%)	6/11 (55%)	3/8 (38%)	0.135
CDKN2B	3/11 (27%)	6/11 (55%)	3/8 (38%)	0.420
RUNX3	3/11 (18%)	1/11 (9%)	0/8 (0%)	0.197
RARB	0/11 (0%)	0/11 (0%)	1/8 (13%)	0.241
HLTF	1/11 (9%)	0/11(0%)	0/8 (0%)	0.409
SFRP4	0/11 (0%)	1/11(9%)	0/8 (0%)	0.409
CACNA1A	0/11 (0%)	1/11(9%)	0/8 (0%)	0.409
H2AFX	0/11 (0%)	1/11(9%)	0/8 (0%)	0.409
FHIT	0/11 (%)	0/11(0%)	0/8 (0%)	NA
ID4	0/11 (%)	0/11(0%)	0/8 (0%)	NA
SFRP5	0/11 (0%)	1/11 (9%)	0/8 (0%)	0.409

Note: Epi-OCSPC (T): Epithelial-like Ovarian Cancer Stromal Progenitor Cells from Tissues; OC (T): Ovarian Cancer Tissues; Epi-OCSPC (A): Epithelial-like Ovarian Cancer Stromal Progenitor Cells from Ascites; *represents probe RASSF1A382; **represents probe CCND2142.

The clinical characteristics of 110 EOC patients and MS-MLPA profiles in 110 EOC tissues and 60 benign ovarian cysts

The mean age of the 110 EOC patients was 54 (range, 29-90) years. The distribution of stages was 13 at stage IA, 1 at IB, 24 at IC, 3 at IIA, 1 at IIB, 12 at IIC, 3 at IIIA, 1 at IIIB, 46 at IIIC, and 6 at IV. The percentages of optimal and suboptimal debulking surgeries were 66.3% (73/110) and 33.7% (37/110). Forty-one percent (41/100) of 100 patients had preoperative CA125 serum levels available higher than 500 IU/ml. We further evaluated the TSG promoter methylation in an independent set of 110 EOC tissues using MS-MLPA. Eight of the 40 TSGs promoters showed significantly higher frequencies of hypermethylation in 110 EOC tissues compared to those in 60 benign ovarian cysts as normal control (**Table 5**). The genes with the most frequently hypermethylated promoter in the 110 EOC tissues analyzed were in the order of CCND2 (49/110, 45%), RASSFIA (47/110, 43%), CDH13 (27/110, 26%), CDKN2B (16/110, 25%), CACNA1A (27/110, 25%), HIN-1 (24/110, 22%), SFRP5 (15/110, 14%), CDKN2A (7/46, 11%), ID4 (12/110, 11%), BRCA1 (11/110, 10%), APC (11/110, 10%), and so on. CCND2 and RASSF1A were validated as two genes with the most frequently hypermethylated promoter in the independent set of 110 EOC tissues. When stratified by different histology subtypes, the promoter methylation frequencies of RASSFIA, ID4, CACNA1A, and HIN-1 were found significantly higher in non-serous type EOC specimens than those of serous type EOC, but the frequencies of promoter methylation of CDKN2A and BRCA1 were significantly higher in serous type EOC specimens than those of non-serous type EOC (**Table 6**).

The mRNA level of 5 genes in 6 ovarian cancer cell lines and normal ovarian tissue

The mRNA expression levels of CCND2, RASSF1A, RUNX3, CDKN2B, and DLC1 were found higher in normal ovarian tissue than those in EOC cell lines by RT-qPCR, which supported those genes were TSGs (**Figure 4**).

Table 4. The differential percentage of promoter methylation in tumor suppressor genes among mesenchymal-like ovarian cancer stromal progenitor cells from tissues, ovarian cancer tissues, and mesenchymal-like ovarian cancer stromal progenitor cells from ascites

Gene name	MSc-OCSPC (T) (%)	OC (T) (%)	MSc-OCSPC (A) (%)	*p*-value
DLC1	3/11 (27%)	3/11 (27%)	5/11 (45%)	0.580
RASSF1A*	2/11 (18%)	5/11 (45%)	5/11 (45%)	0.308
CDH13	0/11 (0%)	3/11 (27%)	1/11 (9%)	0.137
BRCA1	0/11 (0%)	2/11 (18%)	2/11 (18%)	0.320
TIMP3	3/11 (27%)	0/11 (0%)	4/11 (36%)	0.095
SCGB3A1	1/11 (9%)	2/11 (18%)	2/11 (18%)	0.790
ESR1	2/11 (18%)	0/11 (0%)	2/11 (18%)	0.320
CDKN2A	0/11 (0%)	3/11 (27%)	1/11 (9%)	0.137
CCND2**	3/11 (27%)	6/11 (55%)	5/11 (45%)	0.420
CDKN2B	6/11 (55%)	6/11 (55%)	8/11 (73%)	0.602
RUNX3	1/11 (18%)	1/11 (9%)	1/11 (9%)	1
RARB	3/11 (27%)	0/11 (0%)	0/11 (0%)	0.037
HLTF	1/11 (9%)	0/11 (0%)	0/11 (0%)	0.357
SFRP4	0/11 (0%)	1/11 (9%)	0/11 (0%)	0.357
CACNA1A	0/11 (0%)	1/11 (9%)	1/11 (9%)	0.587
H2AFX	0/11 (0%)	1/11 (9%)	0/11 (0%)	0.409
FHIT	0/11 (%)	0/11(0%)	0/11 (0%)	NA
ID4	0/11 (%)	0/11(0%)	0/11 (0%)	NA
SFRP5	0/11 (0%)	1/11 (9%)	0/11 (0%)	0.409

Note: MSc-OCSPC (T): Mesenchymal-like Ovarian Cancer Stromal Progenitor Cells from Tissues; OC (T): Ovarian Cancer Tissues; MSc-OCSPC (A): Mesenchymal-like Ovarian Cancer Stromal Progenitor Cells from Ascites; *represents probe RASSF1A382; **represents probe CCND2142.

The mRNA levels of RASSF1A, CCND2, CDKN2B, DLC1, and RUNX3 in the benign ovarian cysts, early-stage, and advanced-stage EOC tissues

The mRNA levels of RASSF1A were analyzed by RT-qPCR and found significantly lower in advanced stage EOC (n=24) than in benign ovarian cyst tissues (n=24) (0.034±0.017 vs 0.376±0.110, P<0.000, Mann-Whitney U test), and also for those in early-stage EOC tissues (n=12) (0.034±0.017 vs 0.207±0.419 vs P= 0.004, Mann-Whitney U test) (**Figure 5**). However, the mRNA levels of CCND2 were analyzed by RT-qPCR and found significantly higher in advanced stage EOC (n=24) than in benign ovarian cyst tissues (n=24) (1.361±0.155 vs 0.139±0.239, P<0.000, Mann-Whitney U test), but not significant difference for those in early-stage EOC tissues (n=12) (1.361±0.155 vs 1.220±0.140, P>0.05, Mann-Whitney U test). The mRNA levels of CDKN2B were analyzed by RT-qPCR and found significantly lower in advanced stage EOC (n=24) than in benign ovarian cyst tissues (n=24) (1.145±0.305 vs 41.1±65.918, P<0.000, Mann-Whitney U test), but not a significant difference for those in early-stage EOC tissues (n=12) (1.145±0.305 vs 0.948±0.313, P>0.05, Mann-Whitney U test). The mRNA levels in DLC1 were significantly higher in benign ovarian cysts than those in an advanced stage of EOC (1.227±0.017 vs 0.224±0.627, P=0.002, Mann-Whitney U test). However, the mRNA levels in RUNX3 were not significantly higher in benign ovarian cysts than those in an advanced stage of EOC (0.121±1.394 vs 0.204±1.010, P=0.866, Mann-Whitney U test).

Methylation status associated with patient outcomes

The most common promotor hypermethylation of TSGs in ascites mesenchymal-like OCSPCs from ascites were CDKN2B followed by CCND2, RASSF1A, and DLC1, which represent potential correlation with poor survival outcomes. We excluded DLC1 for survival analysis because the percentage of promoter methylation of DLC1 between ovarian cyst tissues and ovarian cancer tissues was not significantly different

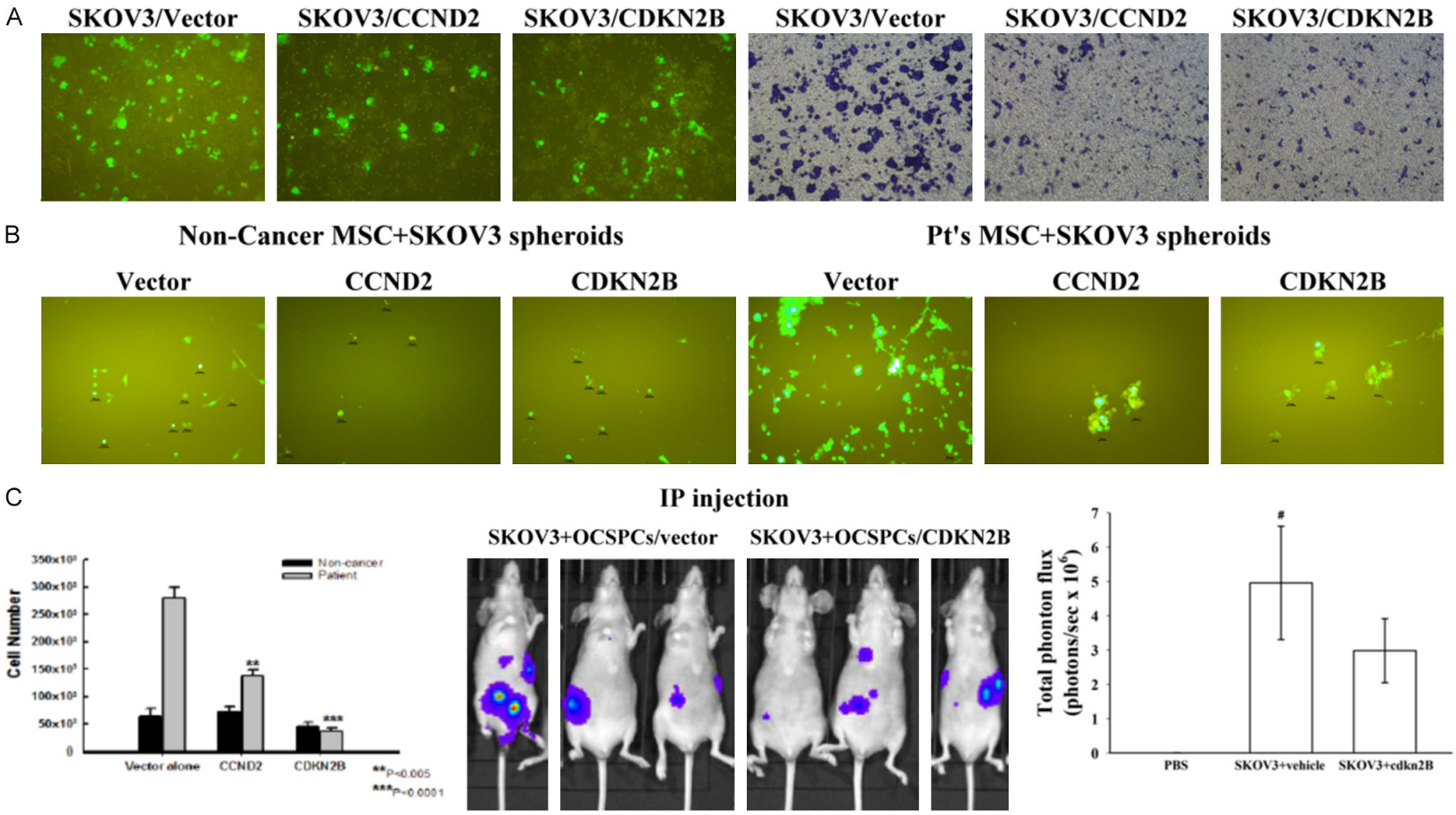

Figure 2. (A) The effect of vector-MSc-OCSPCs, MSc-OCSPCs/CCND2, or MSc-OCSPCs/CDKN2B on the invasive ability of SKOV3 cells. (B, C left) Numbers of spheroids developed from SKOV3 cells cocultured with non-cancer MSC (NOSPCs) or cocultured with mesenchymal-like OCSPCs (MSc-OCSPCs) transfected with either CCND2 or CDKN2B compared to vector controls. (C middle and right) Tumor growth in the peritoneal cavity visualized by luciferase activity after intraperitoneal (IP) coinjection of mice with OCSPCs/CDKN2B cells and SKOV3-Luc cells, versus coinjection of OCSPCs/mock cells and SKOV3-Luc cells.

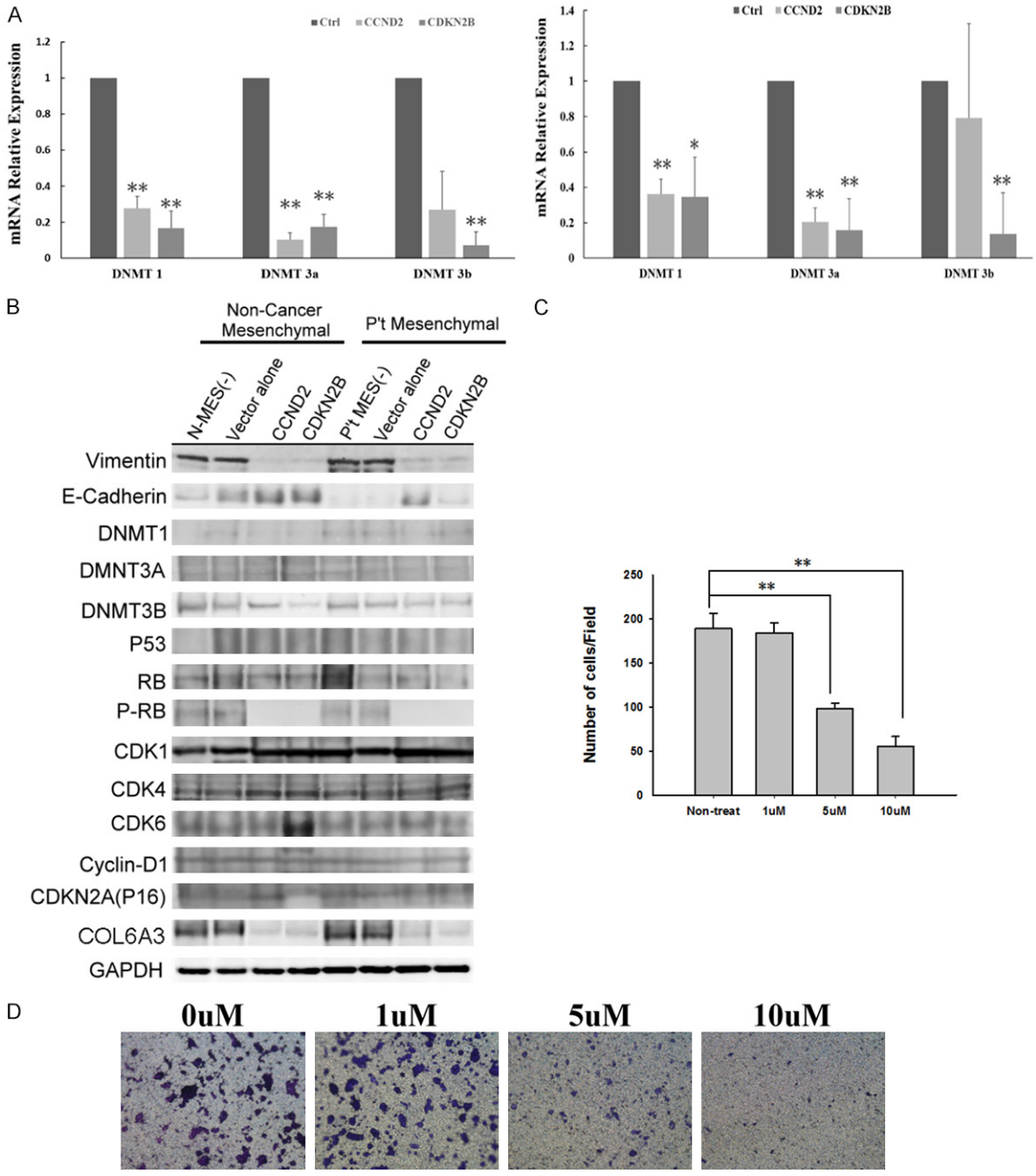

Figure 3. A. Differences in DNMT mRNA expression levels in spheroids formed in cocultures of SKOV3 cells with mesenchymal-like NOSPCs (lower left) or OCSPCs (lower right) transfected with either vector alone or with CCND2 or CDKN2B. B. Expression levels of vimentin, E-cadherin, Rb, phosphorylated RB, COL6A3, DNMTs, P53, CDK4/6, cyclin D1, and CDKN2A in non-cancer mesenchymal-like NOSPCs and MSc-OCSPCs transfected with CCND2 or CDKN2B. C, D. Invasiveness of SKOV3 cells cocultured with MSc-OCSPCs treated with 0, 1, 5, or 10 μM 5AZA-dC for 3 days.

(**Table 5**). To examine the associations between promoter methylation status and EOC patients' outcomes, Kaplan-Meier curves were generated to assess the impact of gene methylation on patient survival. The expected 5-year OS for 110 ovarian cancer patients with methylated RASSF1A promoter was significantly worse than those for patients without methylated RASSF1A (56% vs 80%, p=0.022) (**Figure 6**). In contrast, the expected 5-year overall survival for 110 ovarian cancer patients with unmethylated promoters of CCND2 and CDKN2B was

Table 5. The significantly differential percentage of promoter methylation in tumor suppressor genes between ovarian cyst tissues and ovarian cancer tissues

Gene name	Number of methylation/all (%)		*p*-value
	Group 1	Group 2	
RASSF1A*	0/60 (0%)	44/110 (40%)	<0.001
RASSF1A**	0/60 (0%)	47/110 (43%)	<0.001
APC	0/60 (0%)	11/110 (10%)	0.011
CDH13	0/60 (0%)	27/110 (26%)	<0.001
BRCA1	0/60 (0%)	11/110 (10%)	0.011
TIMP3	10/60 (17%)	3/110 (3%)	0.001
SCGB3A1	0/60 (0%)	10/110 (9%)	0.016
SFRP5	0/60 (0%)	15/110 (14%)	0.003
CCND2#	2/60 (3%)	27/110 (25%)	<0.001
CCND2##	10/60 (12%)	49/110 (45%)	<0.001
CACNA1A	0/60 (0%)	27/110 (25%)	<0.001

Note: group 1: ovarian cysts tissues; group 2: ovarian cancer tissues. *: probe 1: RASSF1A(328); **: probe 2: RASSF1A: RASSF1A(382). #: probe1: CCND2(220); ##: probe 2: CCND2(142).

Table 6. The significantly differential percentage of promoter methylation in tumor suppressor genes between serous type ovarian cancer tissues and non-serous type ovarian cancer tissues

Gene name	Number of methylation/all (%)		*p*-value
	Group 1	Group 2	
RASSF1A*	10/39 (26%)	34/71 (48%)	0.023
RASSF1A**	11/39 (28%)	36/71 (51%)	0.022
CDKN2A	4/39 (10%)	1/71 (1%)	0.033
BRCA1	11/39 (28%)	0/71 (0%)	<0.001
SCGB3A1	3/39 (8%)	21/71 (30%)	0.008
ID4	1/39 (3%)	11/71 (15%)	0.038
CACNA1A	3/39 (8%)	24/71 (34%)	0.002

Note: *group 1: serous type epithelial ovarian cancer tissues; group 2: non-serous type epithelial ovarian cancer tissues; *: probe 1: RASSF1A(328); **: probe 2: RASSF1A: RASSF1A(382).

significantly worse than those for patients with methylated CCND2 and CDKN2B (P=0.038 for CCND2; P=0.009 for CDKN2B) (**Figures 7** and **8**). Univariate and multivariate Cox proportional hazard modeling showed that methylated RASSF1A promoter was an independent poor prognostic factor after adjusting by FIGO stage (early vs advanced), age (<60 years old vs >60 years old), and surgical debulking (optimal vs suboptimal) relative risk (95% CI): 2.81 (1.27-6.21) in 110 ovarian cancer patients (**Table 7**).

Taken together, these data support the diagnostic role of RASSF1A promoter methylation as an independent predictor of poor survival outcomes in ovarian cancer patients (**Table 7**).

Discussion

This study points out that methylation frequencies in RASSF1A were significantly different among normal ovarian tissues or ovarian cysts, OCSPC, and cancer cells (0% (0/60) vs 9% (2/22) vs 43% (47/110), P<0.0001 by Chi-Square test). This result indicated that the RASSF1A promoter switches from unmethylation of normal tissues and ovarian cysts, hypomethylation of RASSF1A in ovarian cancer stromal progenitor cells (OCSPCs) to promoter hypermethylation status in EOC tissues. Although we did not experimentally validate the functional role of RASSF1A in OCSPCs, our data support the hypothesis that hypomethylated specific TSGs in the stromal progenitor cells function to maintain their tumor inhibition function and keep stem cell-like characteristics simultaneously, ultimately losing their tumor-suppressive functions and causing cancer progression. The most frequent promoter methylation in MSc-OCSPCs derived from ascites was CDKN2B (73%) and CCND2 (45%). We did the experimental functional study to verify overexpression of CDKN2B and CCND2 tumor suppressive function in MSc-OCSPCs cocultured with SKOV3 cells decreased invasiveness and intraperitoneally metastatic tumor burden, which can be used as a therapeutic target. However, those in vitro and in vivo data cannot translate promoter methylation of CDKN2B and CCND2 into survival benefits and become prognostic markers.

Previous studies suggest that de novo DNA methylation of gene CpG islands within the promoter region may serve as biomarkers in gynecologic malignancies [21, 22]. Yasuda et al compared the DNA methylation and chromatin structure of 10 TSGs in sorted $CD24^{-}CD44^{+}$ cancer stem cells (CSC) and MCF7 and Huh7 cancer cell lines and found that significantly lower DNA methylation was detected in CSC through bivalent chromatin structure were more common in cancer cells [23]. Our previous study also indicated that the cumulative methylation index of DNA methylation in 40 TSGs was significantly lower in OCSPCs derived from tissues than those from ascites (P<0.000)

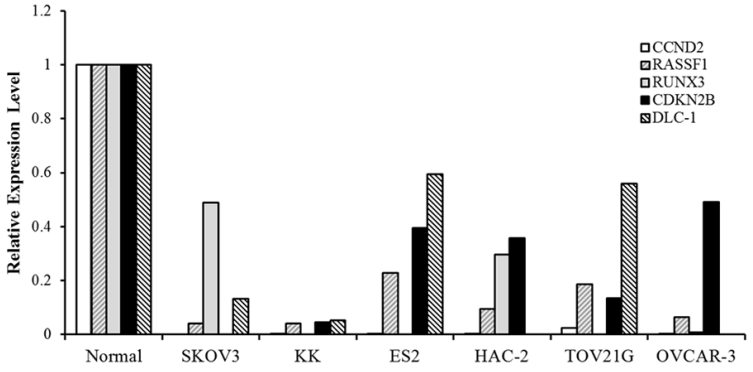

Figure 4. The relative mRNA expression folds in CCND2, RASSF1A, RUNX3, CDKN2B, and DLC1 were shown in normal ovarian tissue and epithelial ovarian cancer cell lines.

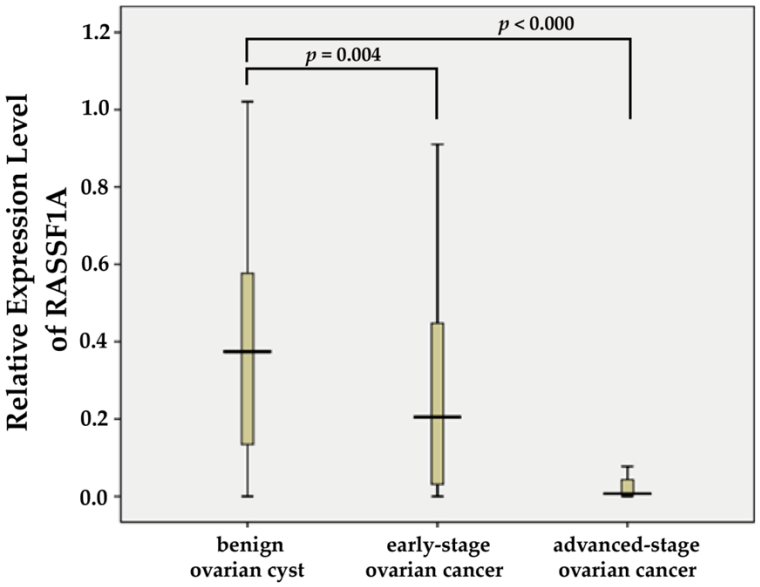

Figure 5. The mRNA expression levels in RASSF1A were significantly lower in advanced-stage EOC (n=24) than in benign ovarian cyst tissues (n=24) (P<0.000) and those in early-stage EOC tissues (n=12) (P<0.004).

and in bulk tumor tissues (P<0.05) [19]. Among the 40 TSGs tested, RASSF1A was the only one with a significant hypomethylation frequency in OCSPCs from tissues than those from ascites and in bulk tumor cells. RASSF1A was further validated as one of two genes with the most frequently hypermethylated promoter in an independent set of 110 EOC tissues. The expected 5-year OS for patients with methylated promoters of RASSF1A has significantly worse survival than those without methylated RASSF1A (56% vs 80%, P=0.022). Univariate and multivariate Cox proportional hazard modeling showed that only RASSF1A with methylated promoters was an independent poor prognostic factor after adjusting by FIGO stage, age, and surgical debulking relative risk (95% CI): 2.81 (1.27-6.21). This study provides cogent evidence that promoter methylation at the RASSF1A tumor suppressor gene was involved in early EOC tumorigenesis. RASSF1A can be detected across different stages from no methylation in epithelial-like OCSPCs from tissues which may undergo EMT change to 18% methylation in mesenchymal-like OCSPCs from tissues, then progressed to 45% of EOC tissues and MSc-OCSPCs from ascites. We directly compared promoter methylations of 40 well-known specific TSGs among matched epithelial-like or mesenchymal-like OCSPCs from tissue and corresponding ascites, and EOC tissues, respectively. Only the percentage of RASSF1A promoter methylation showed a statistically significant difference among 0 percent of epithelial-like OCSPCs from EOC tissues, 38% of epithelial-like OCSPCs from ascites, and 45% of EOC tissues (P=0.039) (**Tables 3** and **4**). The result supports our hypothesis that hypomethylated specific TSGs in the epithelial-like stromal progenitor cells in the EOC tissues switched to hypermethylated status in corresponding EOC tissues and epithelial-like stromal progenitor cells from ascites were associated with tumorigenesis, progression and poor survival outcome of EOC patients. On the other hand, CCND2 or CDKN2B promoter methylation did not show a significant difference among epithelial-like stromal progenitor cells in the EOC tissues, in corresponding EOC tissues, and epithelial-like stromal progenitor cells from ascites (82% vs 55% vs 38% for CCND2; 27% vs 55% vs 38% for CDKN2B). That implied CCND2 and CDKN2B were not driver genes involved in tumorigenesis and disease progres-

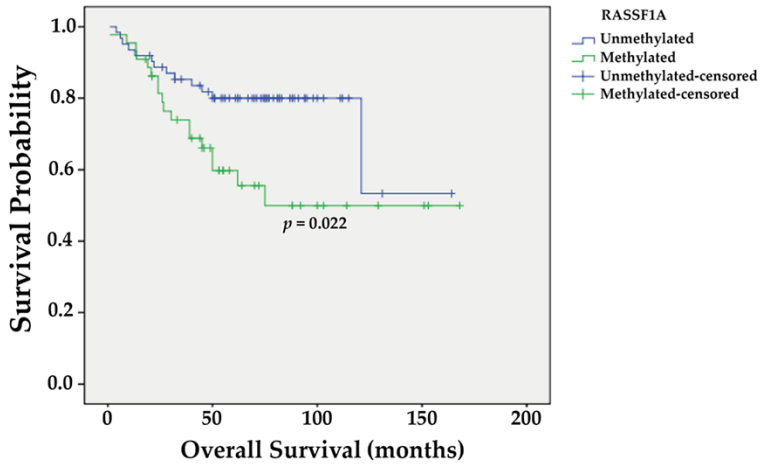

Figure 6. The expected 5-year overall survival for 110 ovarian cancer patients with methylated promoters of RASSF1A1 was significantly worse than those for patients without methylated RASSF1A (56% vs 80%, P=0.022).

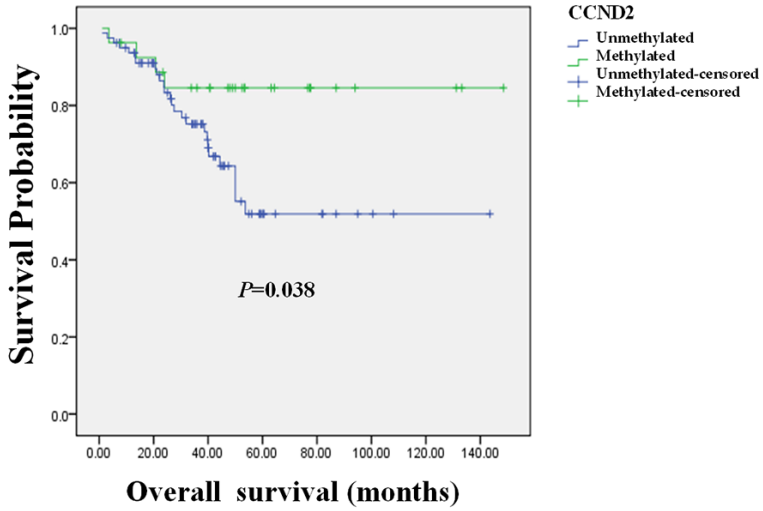

Figure 7. In contrast, the expected 5-year overall survival for 110 ovarian cancer patients with unmethylated promoters of CCND2 was significantly worse than those for patients with methylated CCND2 (*P*=0.038 for CCND2).

sion in terms of the overall survival of EOC patients. This model could be used to comprehensively search for new biomarkers to early detect and predict survival outcomes in EOC patients in the future. Furthermore, we used univariate and multivariate Cox proportional hazard modeling to verify promoter methylation of RASSF1A as an independent poor survival outcome in EOC which has never been done before.

The frequently aberrant promoter methylation of the RASSF1A and APC gene has been reported as an accumulated epigenetic event and suggested to associate with the malignant transformation of benign cysts and low malignant potential tumors to carcinomas [24, 25]. Previous studies have shown that platinum resistance can be overcome by demethylating agents in ovarian cancer [26, 27]. Patients with higher baseline methylation levels of RASSF1A, HOXA10, and HOXA11 genes in tumors that received the demethylating agent treatment were correlated with PFS for more than 6 months [26]. The hypomethylation signature of tumor-initiating cells was reported to be able to predict the poor prognosis of ovarian cancer patients [28]. However, the clinical significance of DNA methylation in ovarian cancer-associated stromal progenitor cells (OCSPCs) from tumor tissues remains unexplored. A study stated that methylated RASSF1A was seen in surrounding high-grade serous carcinoma tumors [29]. Our previous study revealed that OCSPCs from ascites usually presented with promoter methylation and decreased TSG expressions in the ovarian tumor microenvironment enhanced tumor growth and dissemination [19, 20]. This study provides further evidence to support the hypothesis to test the feasibility of examing DNA methylation alterations in ovarian tumor stromal progenitor cells and tumor tissue cells as a potential prognostic method for EOC.

Most of the 40 TSGs analyzed in MS-MLPA are polycomb group targeted genes (PcGTs), and some TSGs are thought to be targeted and epigenetically regulated by polycomb repressive complex (PRC). Cancer stem or progenitor cells may transiently silence these important growth regulatory genes with specific chroma-

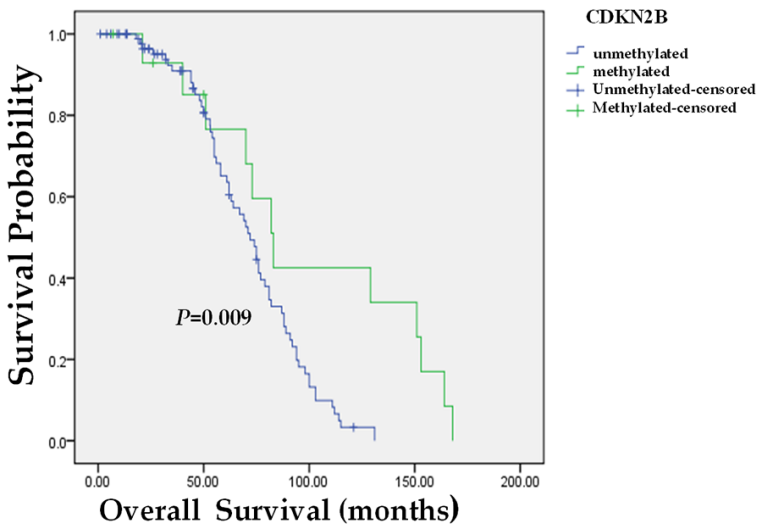

Figure 8. In contrast, the expected 5-year overall survival for 110 ovarian cancer patients with unmethylated promoters of CDKN2B was significantly worse than those for patients with methylated CDKN2B (*P*=0.009 for CDKN2B).

tin patterns in tumors. Undifferentiated cells like cancer stem or progenitor cells are marked by bivalent chromatin structure and vulnerable to aberrant DNA hypermethylation with gene silencing in differentiated adult cancer cells [30]. The polycomb group proteins are believed to function as gene silencers epigenetically by keeping genes in lower DNA methylation state in ES and undifferentiated embryonic cancer cells but heavily methylated rather than in cancer cells [31, 32]. The methylation instability index at PcGTs, specifically, epigenetic silencing of RASSF1A, BRCA1, DAPK, OPCML, and development-associated transcription factors, HOXA10 and HOXA11 [8, 33, 34] are associated with clinical outcome in both cancer initiation and chemotherapy resistance of ovarian cancer [8, 35]. Easwaran et al reported that preferentially DNA hypermethylation at a certain subset of PcG genes whose functions are developmental regulators may dedicate to the cancer stem-like cells [36]. Ovarian carcinoma stromal stem/progenitor cells are thought to be crucial in ovarian cancer development, chemotherapy-resistant, and disease progression. To eradicate ovarian cancer cells, ovarian carcinoma stromal stem/progenitor cells targeted chemotherapy is highly anticipated. In this report, we provide evidence showing epigenetic alteration in OCSPCs from ascites and tumor tissues and bulk tumors on the survival outcome of patients with OEC.

Taken together, the OCSPCs in the ovarian tumor microenvironment have a unique methylation profile with decreased expression of TSGs *in vitro*. The profiles of methylated genes in OCSPCs and bulk tumor cells are different. The most frequently methylated genes in OCSPCs from ascites in advanced serous ovarian carcinomas were CDKN2B, RASSFIA, DLC1, and CCND2; while the aberrantly methylated genes such as RASSF1A, APC, CDH13, BRCA1, HIN-1, SFRP5, CDND2, and CACNA1A were rarely detected in benign ovarian cysts. Overexpression of CCND2 and CDKN2B decreased the aggressiveness of mesenchymal-like OCSPCs from ascites which may represent a potential therapeutic target for EOC. In this study, we found promoter of RASSF1A was hypermethylated in cancer cells and ascites but remained in hypomethylated status in OCSPCs, especially for epithelial-like OCSPCs from bulk tumor tissues, and was an independent poor prognostic factor, and potentially could be used as a prognostic marker in the future. However, it should be validated in a large series of cohort studies in the future.

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Disclosure of conflict of interest

None.

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Table 7. Univariate and multivariate Cox proportional hazard modeling of survival outcomes in 110 ovarian cancer patients

	univariate analysis		multivariate analysis	
	relative risk (95% CI)	*p*-value	relative risk (95% CI)	*p*-value
Surgery (suboptimal v.s. optimal)	3.34 (1.20-9.29)	0.021	2.03 (0.75-5.53)	0.17
FIGO stage (III/IV v.s. I/II)	3.05 (1.03-9.02)	0.044	0.97 (0.97-1.18)	0.72
Age (>50 y/o v.s. <50 y/o)	2.26 (0.71-7.23)	0.17	1.02 (0.09-1.05)	0.12
RASSF1A (methylated v.s. unmethylated)	2.25 (1.10-4.62)	0.027	2.81 (1.27-6.21)	0.011

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