Comprehensive methylome analysis of ovarian tumors reveals hedgehog signaling pathway regulators as prognostic DNA methylation biomarkers

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Women with advanced stage ovarian cancer (OC) have a five-year survival rate of less than 25%. OC progression is associated with accumulation of epigenetic alterations and aberrant DNA methylation in gene promoters acts as an inactivating “hit” during OC initiation and progression. Abnormal DNA methylation in OC has been used to predict disease outcome and therapy response. To globally examine DNA methylation in OC, we used next-generation sequencing technology, MethylCap-sequencing, to screen 75 malignant and 26 normal or benign ovarian tissues. Differential DNA methylation regions (DMRs) were identified, and the Kaplan–Meier method and Cox proportional hazard model were used to correlate methylation with clinical endpoints. Functional role of specific genes identified by MethylCap-sequencing was examined in in vitro assays. We identified 577 DMRs that distinguished (p < 0.001) malignant from non-malignant ovarian tissues; of these, 63 DMRs correlated (p < 0.001) with poor progression free survival (PFS). Concordant hypermethylation and corresponding gene silencing of sonic hedgehog pathway members ZIC1 and ZIC4 in OC tumors was confirmed in a panel of OC cell lines, and ZIC1 and ZIC4 repression correlated with increased proliferation, migration and invasion. ZIC1 promoter hypermethylation correlated (p < 0.01) with poor PFS. In summary, we identified functional DNA methylation biomarkers significantly associated with clinical outcome in OC and suggest our comprehensive methylome analysis has significant translational potential for guiding the design of future clinical investigations targeting the OC epigenome. Methylation of ZIC1, a putative tumor suppressor, may be a novel determinant of OC outcome.

Introduction

Ovarian cancer (OC) causes more deaths than any other gynecologic malignancy.1 Five-year survival rates have only marginally improved over the past three decades, and women with advanced stage OC have a five-year survival rate of less than 25%.1 Although most patients respond to platinum-based chemotherapy, relapses are common, leading to platinum-resistant OC, which is uniformly fatal.2,3

Similar to other malignancies, OC progression is associated with a redistribution of DNA methylation, characterized by genomic hypomethylation with localized CpG island (CGI) hypermethylation.4–6 Such changes are associated with both OC initiation and progression to chemotherapy resistance.7 Hypermethylation of specific CGI-containing genes in OC has been shown to repress the expression of tumor suppressors, such as RASSFIA, BRCA1, DAPK and OPCML, and development-associated transcription factors, such as HOXA10 and HOXA11.6,8,9

Individual methylated genes with possible prognostic value in OC include HOXA11, linked with post-surgical residual tumor and overall poor prognosis,9 MCJ, strongly associated with chemoresistance,10 and 14-3-3-σ, significantly correlated with elevated serum CA-125 levels and high grade malignancy.11 In addition, panels of hypermethylated loci, identified using various
In the present study, we used a next generation sequencing approach, methyl-CpG binding domain of the MBD2 protein to capture double-stranded DNA followed by high-throughput next-generation sequencing (MethylCap-seq) to assess the methylation status of malignant and benign ovarian tumors and normal ovarian tissues. We identified differential DNA methylation profiles as well as candidate hypermethylated loci. We validated the functional consequence of hypermethylation-mediated silencing of the Hedgehog (Hh) signal transduction pathway which has been shown to be involved in human ovarian carcinogenesis and chemotherapy drug sensitivity. In addition, we have generated perhaps the most comprehensive methylome analysis to date in OC, which we believe has strong translational potential for guiding the design of future clinical investigations targeting the epigenome of this deadly female malignancy.

**Results**

MethylCap-seq analysis of the ovarian tumor methylome. To investigate methylation signatures associated with poor outcome in OC, genome-scale DNA methylation profiles were obtained for 75 tumors, 20 benign ovarian samples (unmatched) and 6 normal (unmatched) ovarian tissues (Fig. 1A; Table S1). A sequencing depth of ten million uniquely mapped reads provided sufficient coverage for methylation mapping (Fig. 1B, histogram). In addition, repeated sequencing was performed on two samples (Fig. S1A), and the data were highly (R² > 0.9) correlated.

To identify differentially methylated regions (DMRs) in malignant vs. non-malignant (20 benign and 6 normal ovarian tissues) ovarian tissues, an area spanning 4 kb upstream or downstream of the nearest transcription start site (TSS) was used. This approach yielded 577 DMRs in the tumors, and these DMRs represented the vast majority hypermethylated CGIs (92%) and 86% of hypomethylated tumor CGIs (Fig. 1B, pie charts). Methylation profiles of the 577 DMRs were more similar in benign and normal tissues compared with tumor (Fig. 1C; Fig. S1B and C). Furthermore, as shown in Figure 1D, malignant from non-malignant samples could be distinguished with 87% accuracy by using the average of 577 normalized methylation levels (AUC = 0.87, 95% CI = 0.79 to 0.93, p < 0.001), and with 89% accuracy when the top 10% of significantly methylated DMRs were used (AUC = 0.89; 95% CI = 0.81 to 0.94, p < 0.001).

To examine the association between DMRs and progression-free survival (PFS), methylation regions closer to the TSS (2 Kb upstream or downstream in the 577 DMRs) were used. This analysis yielded 63 DMRs significantly associated with PFS (Fig. 2A), as determined by the Cox regression model (Table S2, adjusted for stage, grading, age and histological type). The relationship between survival probability and methylation frequency was then examined using Kaplan-Meier estimates (Fig. 2B; Fig. S2A). DMRs were first dichotomized into high- or low-methylation using the 60th percentile cut-off value (described above), and the number of high methylation DMRs (HM-DMRs) was calculated for each patient. Next, as the area under the ROC curve (AUC) indicated that 17 HM-DMRs were highly accurate (p < 0.001) predictors of recurrence (Fig. S2B), HM-DMRs were dichotomized as high or low frequency. At 60 mo, high frequency HM-DMRs correlated (p < 0.001) with reduced PFS (17.5% survival probability), and low frequency HM-DMRs correlated (p < 0.001) with long PFS (87.3% survival probability) (Fig. 2B, top panel). Only two HM-DMRs correlated (p < 0.05) with long PFS (Fig. S3). In addition, when only advanced stage tumors were assessed, high frequency HM-DMRs correlated (p < 0.001) with reduced PFS (11% PFS probability at 40 mo), compared with an 80% PFS probability at 40 mo for low frequency HM-DMRs (Fig. 2B, bottom panel). A similar trend was observed in early stage tumors, as high frequency HM-DMRs tended to be associated with shorter PFS and low frequency HM-DMRs with longer PFS (Fig. S4); however, statistical significance was not achieved (p = 0.092) due to low rate of recurrence in Stage 1, 2 OC, and a smaller sample size.

Identification and validation of functionally relevant DMR genes in ovarian cancer. Based on the MethylCap-seq results, we selected 14 genes not previously shown to be hypermethylated in ovarian tumors and two genes (HNF1B and HOXB5) reported to be methylated in OC. Epigenetic (5-aza-dc +/- HDAC1) drug treatment restored expression of all 16 genes in at least one of the ovarian cell lines examined (Fig. 2C), and SPON2 and ZIC1 reexpression was observed in five cells. In addition, functional roles of genes associated with the 53 DMRs (10 DMRs were eliminated because they corresponded to hypothetical proteins or noncoding RNA) and OC pathogenesis were investigated using the Protein Interaction Network Analysis (PINA) to search for DMR-pathway associations. As shown in Figure 2D, a significant number of genes (23/63 genes) were connected to functional networks involving cancer-relevant pathways such as mTOR, MAPK, VEGF, ErbB, Adherens Junction, NOTCH, Wnt, TGF-β, Cell Cycle and Hedgehog Signaling.

ZIC1 and ZIC4 methylation and clinical correlations. PINA network analysis identified a differentially methylated gene, the putative tumor suppressor ZIC1 (Fig. 2D, lower right corner), as closely linked with hedgehog (Hh) signaling. Detailed analysis of the MethylCap-seq data showed that both ZIC1 and ZIC4 (another ZIC family member) were arranged in a “head-to-head” configuration (Fig. 3A) with overlapping CGIs (referred to as CpG islands 1–9). These regions were hypermethylated in OC, in contrast to modest methylation of ZIC family members ZIC2 and ZIC5 (located at 13q32 and overlapping CGIs 10–17) in benign and normal tissues (Fig. 3A). A strong correlation (Pearson’s correlation r > 0.80) was observed between methylation of CGI 4 and CGIs 1, 2, 5, 6, and 8 (Fig. 3B). Interestingly, CGI 4 overlapped the ZIC1 promoter and first exon, as well as the putative ZIC4 promoter, and CGIs 1, 2, 5, and 6 overlapped the ZIC1 and ZIC4 coding regions, with only CGI 8 located in an intergenic region (Fig. 3A).

Because the sonic hedgehog pathway plays an important role in OC cell proliferation and the above results indicated that methylation-mediated silencing of ZIC1, a tumor suppressor, might result in loss of its inhibitory effects and
ZIC1 expression negatively correlates with GLI methylation patterns and transcription. To broaden the scope of our findings, we analyzed gene expression and methylation array data from The Cancer Genome Atlas (TCGA; 406 ovarian tumors). Sample groups (n = 5) based on increasing ZIC1 methylation levels also showed reduced (p < 0.001) ZIC1 expression (Fig. 4A; Fig. S5). In addition, by treating ovarian cells with the demethylating agent 5-aza-dc and observing ZIC1 and ZIC4 re-expression, we further confirmed that ZIC1 and ZIC4 were silenced by DNA methylation (Fig. S6A).

Figure 1. Strategy for discovering novel methylated genes in ovarian cancer using MethylCap-seq. (A) Flowchart of the experimental design. (B) Histograms show the number of mapped reads in 101 ovarian samples. At least 10 million uniquely mapped reads per sample were used in the analysis. Pie charts illustrating the percentage of differential methylation regions (DMRs) of promoter CpG islands (CGI) and non-promoter CGI. (C) Methylation profiles of 577 DMRs locate at ± 4 kb spanning from transcription start site (TSS) in malignant, benign and normal ovarian samples. Each row shows a DMR region overlapping a TSS. Average of the methylation levels was calculated for 75 malignant (left), 20 benign (middle) and 6 normal (right) ovarian tissues. Each 200 base pairs is a bin to show the methylation profiles in an 8K region. (D) Area under the receiver operating curves (AUC) shows the accuracy for distinguishing malignant samples and non-malignant samples (containing benign and normal). The average methylation level of 577 DMRs and top 10% DMRs (n = 57) shows higher 87% accuracy for distinguishing malignant and non-malignant ovarian tissue.
or ZIC4 expression suppressed GLI1 and GLI2 levels in Cp70 (Fig. 4C). Taken together, the data indicate that ZIC1 and ZIC4 negatively regulate Hh signaling by suppressing the expression of GLI2 and possibly GLI1 in OC.

Functional consequences of ZIC1 and ZIC4 overexpression. We next investigated the effects of transient ZIC1 and ZIC4 overexpression on cell proliferation, migration and invasion. Overexpression of ZIC1 inhibited (p < 0.05) proliferation of both OC cell lines tested (SKOV3 and Cp70) compared with vector control, while ZIC4 only reduced Cp70 cell proliferation (Fig. S7). However, overexpressing either ZIC1 or ZIC4 in SKOV3 cells reduced (p < 0.001) both migration and invasion (Fig. 5A and B). Taken together, the above data indicated that silencing

As ZIC and GLI proteins have been shown to transcriptionally regulate one another, it was of interest to determine whether ZIC1 and ZIC4 regulate the expression of the Hh effectors GLI1, GLI2 and GLI3 in OC. Treatment of ovarian cell lines with the Hh inhibitor cyclopamine resulted in repressed GLIs expression but no corresponding change in expression of ZIC1 and ZIC4 (Fig. 4B). However, the combined treatment of Hh inhibitor plus 5-aza-dc resulted in reexpression of ZIC1 and ZIC4 in all three cell lines examined (Cp70, SKOV3, IOSE), reduced GLI2 expression in SKOV3 and Cp70, and reduced GLI3 expression in SKOV3 (Fig. 4B). Furthermore, transfection of ZIC1 or ZIC4 resulted in reduced expression of GLI2 in both OC cell lines as well as IOSE, albeit to a lesser extent (Fig. 4C). Enforced ZIC1 or ZIC4 expression suppressed GLI1 and GLI2 levels in Cp70 (Fig. 4C). Taken together, the data indicate that ZIC1 and ZIC4 negatively regulate Hh signaling by suppressing the expression of GLI2 and possibly GLI1 in OC.

**Figure 2.** A panel of DMRs associated with prognostic outcome and pathologically functional pathways. (A) Heatmap illustrating the methylation levels of 63 DMRs at promoter regions (± 2Kb spanning the transcription start site (TSS). The DMRs are listed in Table S2. High methylation-DMR (HM-DMR) was defined by 60th percentile of each gene in 75 malignant samples for prognostic analysis. Malignant tissues derived from patients with recurrent disease were associated with HM-DMRs (black gradient). (B) High frequency HM-DMRs (high freq HM-DMRs) were associated with poor progression-free survival (PFS). freq, frequency. (C) Restored gene expression in ovarian cells after epigenetic drug treatments. Expression of each gene examined was restored in at least one of the ovarian cell lines. (D) Network analysis showing the closest functions for high methylated genes. The weight of the edge indicates the probability of genes to connect components (contained gene or proteins) in the functional pathways. Red squares represent proteins involved in signaling pathway and blue circles represent genes. The blue gradient indicates methylation level.
Figure 3. Analysis of ZIC family methylation patterns. (A) Methylation profiles of CpG islands located at regions around ZIC1, ZIC4, ZIC2 and ZIC5 on the genome browser. The left panel shows Chr3: 148,584,017 ~14,869,093 and contains nine CGIs (CGI 1~9); right panel shows Chr13: 99,401,912 ~99,450,871 and contains eight CGIs (CGI 10~17). Around ZIC1 and ZIC4, the region presents high methylation in malignant samples. However, some malignant samples showed low methylation (middle of the genome browser). (B) Estimating correlations between CGIs. High concordance of methylated CGI at chromosome 3 but not at chromosome 13. CGI 4 is highly concordant with CGI 1, 2, 5 and 6, and the CGIs related to exon loci. (C) Validation of methylation levels of ZIC1 promoter used bisulfite pyrosequencing. Validated regions were located 439 to 496 base pair upstream from the TSS (transcriptional orientation indicated by the arrow). The red boxes show that methylation levels of 86 malignant samples are higher (p < 0.001 by Mann-Whitney U test) than 33 non-malignant samples (containing 15 benign, 6 normal tissues and 14 normal paraffin tissues) and squares indicate outliers. (D) Area under the receiver operating curves (AUC) was used to estimate the accuracy of predicting malignant samples associated by methylation levels. The average of seven CG sites accurately distinguished 80% of malignant samples using a 41.7% methylation cutoff value.
independent risk factors for poor PFS (CpG site 6, HR = 2.37, 95% CI = 1.15 to 4.87, p = 0.019; CpG site 7, HR = 2.45, 95% CI = 1.17 to 5.13, p = 0.017; Table 1; Table S4). In addition, a correlation (p < 0.05) between hypermethylated ZIC1 and early disease recurrence was observed (Table S5). Kaplan-Meier survival analysis showed that lower PFS probability correlated (p < 0.01 for CpG sites 6 and 7) with high ZIC1 methylation (Fig. 5C).

Discussion

In the current study, we used MethylCap-seq to carry out an unbiased, genome-wide DNA methylation analysis of 101 ovarian tissues. We identified 577 differentially methylated regions or DMRs that distinguished, with 87% accuracy, malignant from non-malignant (benign and normal) samples. We further
identified the most significant DMRs (n = 57) that could, with 89% accuracy, classify the malignant samples, representing potential OC DNA methylation biomarkers. Although we and others have conducted genome-wide methylation studies on advanced stage samples to select methylation biomarkers,25,26 many of the resulting candidate genes have neither been validated using quantifiable methods nor verified in an independent patient cohort.27 The present study is the first to perform next generation sequencing on ovarian tumors and validate the methylome findings in patient samples.

Based on 577 DMRs, we further conducted survival analysis to select outcome-associated markers. The association of high frequency HM-DMRs with poor outcome supports the notion of a CGI methylator phenotype (CIMP) in OC. CIMP has been reported to describe a subset of colorectal tumors with concordant, cancer specific methylation of a group of genes.28 This form of epigenetic instability has also been reported in OC and other human neoplasms,29-34 but the mechanism is unclear. Aberrant methylation patterns have been associated with increased or altered activity of DNA methyltransferases (DNMT1, DNMT3a or DNMT3b). DNMT3b overexpression has been reported in OC cells, and overexpression of DNMT3a was reported in sporadic OC, although the relationship between gene hypermethylation and altered DNMT levels in OC cells is not straightforward.35 A “poor prognosis-CIMP” has also been reported in human neoplasms,38-42 and the current study shows a relationship between a high frequency HM-DMRs and poor PFS across all stages of OC (Fig. 2B), and a trend for high frequency HM-DMRs and poor PFS of early stage disease (Fig. S4). Thus, the 53 DMRs identified in this study could serve as potential markers for evaluating OC outcome, but this would need to be examined prospectively.

The zinc-finger domains in ZIC proteins bind with GLI proteins at Gli-binding sequence (GBS) to regulate gene transcription.43 ZICs have been shown to be primarily involved in embryonic development,44 but a recent paper reported a role for ZIC2 in OC.45 In addition, GLI proteins have been shown to regulate several critical functions during embryonic development and activate downstream targets of the Hh signaling pathway in tumorigenesis, and Gli1 and Gli2 are the prime transcriptional effectors of Hh signaling in cancer.46,47 In OC, expression of Gli1/Gli2 has been reported, indicating an activated Hh pathway.48 Gli2 inhibition resensitized drug resistant OC cells to taxanes, and Gli1 was recently shown to play a role in OC cisplatin sensitivity.49 Our results support a role for the GLI family in drug resistance, through hypermethylation-mediated silencing of ZIC1, a putative tumor suppressor.50 SKOV3 and Cp70 were more responsive to cyclopamine-induced decreases in Gli2 expression compared with the less drug resistant A2780 and TOV112D cell lines (Fig. S6). Similarly, ZIC1 overexpression suppressed Gli2 in SKOV3 and Cp70 cells (Fig. 4C). We suggest that high expression of Gli2 combined with low ZIC1 expression may contribute to drug resistance in OC.

Our comprehensive methylome analysis has identified functional DNA methylation biomarkers predictive of OC clinical outcome. Our findings underscore the importance of DNA methylation in OC, support the notion that therapies targeting specific pathways altered by this epigenetic mark may hold promise in OC, and as a whole the methylome data may represent a
resource for continued development of epigenetic-based therapies for this devastating disease. We have shown that ZIC1 and ZIC4, related to the hedgehog pathway, a key signaling pathway in OC, are DNA methylation prognostic biomarkers and that loss of negative Hh signaling regulation may contribute to disease progression.

Materials and Methods

Samples. Samples from 86 malignant (75 cases for genome-wide methylation analysis), 20 benign and 6 normal epithelial ovarian tissues were collected during surgery, frozen immediately in liquid nitrogen and stored at −80°C or in liquid nitrogen until analysis. An additional 14 normal paraffin tissues were used to confirm methylation levels. Specimens were obtained from patients between 1991 and 2010 and acquired from Tri-Service General Hospital (Taipei, Taiwan). This study was conducted in accordance with the Helsinki human subjects’ doctrine and was approved by the Institutional Review Boards of the Tri-Service General Hospital. Informed written consent was obtained from each patient. The inclusion and exclusion criteria have been previously described. The histological diagnosis of epithelial OC included Stage I, II, III and IV according to the International Federation of Gynecology and Obstetrics (FIGO). Normal epithelial ovarian tissue was collected by scraping the ovarian epithelium from patients diagnosed with uterine leiomyoma. All clinical information was recorded and reviewed from the patient hospital records. Patient demographics for genome-wide methylation analysis are listed in Table S1.

DNA isolation and MethylCap-seq assay. Genomic DNA was extracted from ovarian tissue or cell lines using the QIAamp DNA Mini Kit (QIAGEN GmbH). Methylated DNA was enriched from 2 μg genomic DNA using the MethylMiner Methylated DNA Enrichment Kit (Invitrogen) following the manufacturer’s instructions. Briefly, genomic DNA was sonicated to ~200-bp, captured by MBD proteins and precipitated using 1 M salt buffer. Enriched methylated DNA was used to generate libraries for sequencing following the standard protocols from Illumina. MethylCap-seq libraries were sequenced using the Illumina Genome Analyzer IIx System. Image analysis and base calling were performed using the standard Illumina pipeline. Unique reads (up to 36-bp reads) were mapped to the human reference genome (hg18) using the ELAND algorithm, with up to two base-pair mismatches (see Table S6).
AVG_{R,G} = \frac{\sum_i M_{R,G}}{S_R}, \quad R = (b_{1,0}, b_{1,1}, \ldots, b_{1,n}) \tag{2}

AVG_{R,G} means the average methylation level of group G at the R region. \( M_{R,G} \) is the methylation levels of each sample of group G at the R region. Then, we identified statistical significance of different methylation at the R region using two-tailed and paired t-test (p ≤ 0.001). Differentially methylated loci were analyzed between malignant and normal ovarian tissues.

Cell and culture conditions. Ovarian cancer cells (A2780, Cp70, SKOV3 and TOV112D) and immortalized normal ovarian cells (IOSE) were cultured at 37°C in an atmosphere of 5% (v/v) CO₂ in air. The culture conditions for A2780 and CP70 cells have been described in detail. SKOV3 and TOV112D, IOSE cells were cultured in RPMI 1640 with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1% MEM NEAA, 1 mM MEM sodium pyruvate solution and 10 mM HEPES. TOV112D, IOSE were cultured in 1:1 (v/v) ratio of MCDB-105/Medium-199 supplemented with 15% (v/v) fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin. Epidermal growth factor (EGF) (5 μg/ml, 1 mg/ml) and hydrocortisone (200 μg, 1 mg/ml) in 500 L were added to the culture medium for IOSE cells. Growth media were cell culture were purchased from Gibco (Invitrogen Corporation).

RNA extraction and QRT-PCR analysis of ovarian cell lines. Total RNA was isolated from cultured cells with the PureLink RNA Mini Kit (Ambion, Life Technologies) and reverse transcribed by using SuperScript® III Reverse Transcriptase (Invitrogen). Real-time PCR (qRT-PCR) was performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems). The ΔCt and ΔΔCt was calculated using the housekeeping gene GAPDH and experimental condition controls as a reference, respectively. PCR reactions were performed in triplicate (see Table S7 for PCR primer sequences). Epigenetic regulation of gene expression was examined by using ovarian cell lines treated with the DNA demethylating agent 5-aza-deoxycytidine (5-aza-dc, 5 μM or 10 μM; Sigma-Aldrich) alone or in combination with Trichostatin A (TSA, 0.3 μM; Sigma-Aldrich), a histone deacetylase inhibitor (HDACI). The transcriptional correlation between ZIC1 and GLI3 examined by treating cells with the Hedgehog signaling inhibitor cyclophamide (8 μM; LC Laboratories) alone or in combination with 5-aza-dc (5 μM) for 3 d.

Cell transfection and viability assay. Ovarian cells (1.5 × 10⁶) were transfected with pCMV6-ZIC1, pCMV6-ZIC4 and pCMV6 vector (OriGene) using Amaxa™ Cell Line Nucleofector® Kit (Lonza), cultured for 24 h in a 6-well plate, trypsinized, counted and used in the assays described below. Exogenous ZIC1 or ZIC4 was confirmed by qRT-PCR. The MTS assay was used to determine cell viability, as per the manufacturer’s directions (Promega). Briefly, 2,000 cells were seeded in 96-well cell-culture plates in triplicate under experimental condition. After 0, 24, 48, 72 h incubation, CellTiter 96 Aqueous One Solution reagent (Promega) was added for 90 min and absorbance was measured at 490 nm.

Migration and invasion assay. For cell migration and invasion assay, 2 × 10⁵ cells were seeded in BD BioCoat™ Control and Growth Factor Reduced BD Matrigel™ Invasion Chamber containing a 8.0 μm PET membrane insert (BD Bioscience). To determine the number of cells migrating through the pores or invading through the matrigel, after 22 h, inserts were washed, fixed using paraformaldehyde, and then incubated with 1 μg/mL Hoechst 333042 (Life Technologies) for 15 min at 37°C. The stained cells were counted; 6–8 random images (× 100) per well were obtained.

Survival analysis. Progression free survival (PFS) was defined as the interval from primary surgery or first line chemotherapy to recurrence. Sixty-six patients were included in the PFS analysis (nine patients were eliminated due to persistent disease). Persistent disease was based on increased serum CA125 titers (or plateaued > 35 U/mL) after chemotherapy. Recurrent disease (before follow-up) was observed in 32 (48%) patients and considered censored data for PFS. For methylation levels at each locus, we identified the 60th percentile as a cutoff value to dichotomize high and low methylation for each DMR. The PFS analysis used Kaplan-Meier survival curve and COX regression models. Univariate and multivariate Cox regression analysis were used to calculate hazard ratios (HR) and 95% confidence intervals (CI) for evaluating risks of disease progression associated with DNA methylation level. The multivariate Cox proportional hazards model was performed to determine the independent prognostic value of age, DNA methylation status, stage, grade, and histological subtype.

Statistical analysis. To evaluate the accuracy of the two groups (malignant and non-malignant samples), we calculated the optimal cutoff value using the most significant area under the receiver operating characteristic (ROC) curve (AUC). The relationship between gene expression and methylation was assessed by Pearson correlation coefficient. P-values less than 0.05 were considered statistically significant (assigned *p < 0.05, **p < 0.01, ***p < 0.001). Two-tailed t-test was used to compare continuous variables in different treatment and control groups. Methylation levels in malignant and non-malignant samples were determined using bisulfite pyrosequencing and compared by two-tailed Mann-Whitney U test. All analyses were performed using the statistical package in R (R version 2.14.0) or MedCalc (version 9.0).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Supplemental Materials
Supplemental materials may be found here: www.landesbioscience.com/journals/epigenetics/article/24816
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