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Prognostic and diagnostic significance of DNA methylation patterns in high grade serous ovarian cancer

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ABSTRACT

Objective. Altered DNA methylation patterns hold promise as cancer biomarkers. In this study we selected a panel of genes which are commonly methylated in a variety of cancers to evaluate their potential application as biomarkers for prognosis and diagnosis in high grade serous ovarian carcinoma (HGSOC); the most common and lethal subtype of ovarian cancer.

Methods. The methylation patterns of 10 genes (*BRCA1*, *EN1*, *DLEC1*, *HOXA9*, *RASSF1A*, *GATA4*, *GATA5*, *HSULF1*, *CDH1*, *SFN*) were examined and compared in a cohort of 80 primary HGSOC and 12 benign ovarian surface epithelium (OSE) samples using methylation-specific headloop suppression PCR.

Results. The genes were variably methylated in primary HGSOC, with *HOXA9* methylation observed in 95% of cases. Most genes were rarely methylated in benign OSE, with the exception of *SFN* which was methylated in all HGSOC and benign OSE samples examined. Methylation of *DLEC1* was associated with disease recurrence, independent of tumor stage and suboptimal surgical debulking (HR 3.5 (95% CI:1.10–11.07), $p = 0.033$). A combination of the methylation status of *HOXA9* and *EN1* could discriminate HGSOC from benign OSE with a sensitivity of 98.8% and a specificity of 91.7%, which increased to 100% sensitivity with no loss of specificity when pre-operative CA125 levels were also incorporated.

Conclusions. This study provides further evidence to support the feasibility of detecting altered DNA methylation patterns as a potential diagnostic and prognostic approach for HGSOC.

Introduction

Ovarian cancer is asymptomatic in its early stages, and the typical late diagnosis leads to a poor outcome for patients. It is the sixth leading cause of cancer-related mortality in women and the most common cause of death from cancers of the female genital tract [1]. Epithelial ovarian cancer (90% of ovarian cancer) is diagnosed at an advanced stage (FIGO III and IV) in 75% of all cases, where the disease has spread throughout the abdomen. Patients with advanced stage disease have a 5-year survival of only 30% in contrast to early-stage disease (confined to the ovaries), where 5-year survival exceeds 80% [1].

Ovarian cancer is a heterogeneous disease both histologically and in patterns of disease progression. Epithelial ovarian cancer (EOC) is composed of four major histologic subtypes: endometrioid, mucinous, clear cell and high-grade serous ovarian cancer (HGSOC). These subtypes appear to arise via different molecular/genetic pathways, with endometrioid and mucinous carcinomas typically developing more slowly with a multistep progression to invasive cancer, while HGSOC develops rapidly and, as yet, does not have a clearly defined precursor lesion. This latter subtype is typically diagnosed in late stage, and is mainly responsible for the high lethality rate of ovarian cancer. It is also the subtype with the highest prevalence, estimated at ~70% of all cases [2].

The ability to accurately detect early stage HGSOC, ideally as a pre-invasive stage, would greatly improve ovarian cancer survival. Given the low prevalence of ovarian cancer in the general population (30–50 cases/100,000 women), successful screening tests would require an extremely high sensitivity and specificity [3]. The

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glycoprotein CA125, the best-characterized serum biomarker for EOC, is used for post-operative monitoring of disease burden and distinguishing malignant from benign pelvic masses, but alone has insufficient specificity and sensitivity for population-based screening [4]. Ultrasound-based screening has resulted in a positive predictive value of 9.4%, while a screening algorithm with CA125 and ultrasound imaging has achieved a positive predictive value of 19% in a clinical trial [3], indicating the importance of combining EOC prognostic biomarkers for improved screening. A better understanding of the molecular pathogenesis of EOC is likely to aid identification of new biomarkers for the early detection of the disease.

During the development of cancer, cells undergo profound alterations in the patterns of DNA methylation, with functional consequences in the activity of key genes intimately involved in the carcinogenic process [4]. Hypermethylation of CpG islands in gene promoter regions has been observed as a frequent mechanism associated with inactivation of tumor suppressor genes which contributes to oncogenic transformation. As aberrant methylation is thought to be one of the earliest molecular changes in carcinogenesis, the detection of alterations in DNA methylation patterns has potential applicability to the detection of early-stage or pre-malignant disease [5,6]. Specific methylated DNA markers can be detected in the serum, plasma and peritoneal fluid of ovarian cancer patients [7]. However, the ratio of tumor-derived DNA to non-tumor DNA in body fluids such as plasma may be low, and highly sensitive PCR assays need to be designed to avoid detecting what may be a large excess of closely related sequence. The headloop suppression PCR method offers such an approach [8], and hence is particularly suited to evaluating methylation status of selected genes in clinical samples.

A number of studies have identified methylation changes in ovarian cancer, mostly using a candidate gene approach based on commonly methylated tumor suppressor genes (reviewed in [5]). Many of these studies are limited by small sample size, and often combine different EOC subtypes. As part of the goal of developing a methylation-based diagnostic test for HGSOC, this study examined the methylation patterns of selected genes known to be frequently methylated in diverse cancers, and shown to be methylated in ovarian cancer in at least one previously published study. Here we describe a panel of genes that show differential methylation between HGSOC and benign ovarian surface epithelium (OSE), and when incorporated into a model with pre-operative serum CA125 measurements provide excellent discrimination between HGSOC and benign OSE. These data provide a rational basis for further developing this approach as a potential screening tool for the detection of HGSOC.

Materials and methods

Patient cohort

The cohort comprised 80 ovarian cancer patients undergoing primary laparotomy at the Gynecological Cancer Centre, Royal Hospital for Women, Sydney, Australia between 1991 and 2007. All patients were diagnosed with high grade (2–3) serous ovarian cancer (HGSOC), and were treated post-operatively with standard chemotherapeutic regimens. Clinical (age, menopausal status, pre-operative CA125 levels, residual disease following surgery), pathological (histopathologic diagnosis, tumor grade, Federation International Gynecological Oncologists (FIGO) stage) and outcome (disease recurrence, death from ovarian cancer) data were collected on each patient.

Benign ovarian surface epithelial cell (OSE) scrapings from 12 women with non-diseased ovaries who had undergone surgery for benign gynecological conditions or endometrial cancer were collected into RNAprotect (Qiagen, Hilden, Germany) and stored at 4 °C prior to processing for DNA extraction. CA125 levels from control patients were obtained retrospectively by assaying frozen plasma. Fresh-frozen ovarian carcinoma tissue samples were collected immediately after surgical resection, snap frozen in liquid nitrogen and stored at −80 °C until use. Histological classification and tumor grade in each sample were confirmed by a gynecological pathologist (JPS). The percentage of tumor cell content of each specimen was determined to be at least 70%. All experimental procedures were approved by the Human Research Ethics Committee of the Sydney South Eastern Area Health Service (00/115) with informed consent from each patient.

Bisulfite treatment of DNA

Genomic DNA was extracted from tumor samples and benign OSE samples using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Genomic DNA was treated with sodium bisulfite as described previously [9]. Modified DNA was re-suspended in sterile water and stored at −20 °C until used.

Methylation analysis

Mapping of methylated promoter CpG residues

Bisulfite-treated-specific, methylation-unspecific primers were designed in CpG dense regions of each candidate gene promoter around the transcriptional start site, as determined using Genome Browser (www.genome.ucsc.edu), and were used to amplify bisulfite-treated DNA. PCR products were purified and directly sequenced using a BigDye terminator v3.1 cycle sequencing kit (PerkinElmer Waltham, MA USA) on an ABI Prism 3100 Genetic Analyser (Applied Biosystems, Foster City, USA). Methylation-specific headloop suppression primers and probe for each gene were then designed (Supplementary Table 1).

Methylation specific headloop suppression PCR assays

High sensitivity and specificity headloop suppression PCR assays [8] were developed as previously described [10] to examine DNA methylation of the CpG island associated with each candidate gene promoter based on frequently methylated CpG sites in cancer cell lines. Real-time PCR was carried out using an ABI Prism HT7900 sequence detection system (Applied Biosystems, Foster City, USA) under standard thermocycling. Bisulphite-treated CpGenome universal methylated DNA (Chemicon International, Temecula, USA) and reference genomic DNA (Roche Mannheim, Germany) were used as positive and negative control DNA, respectively. For genes where the negative control was shown to be fully (*SFN*) or partially (*GATA4*, *CDH1*, *HSULF1*) methylated as determined by sequencing of bisulfite-treated DNA, DNA from ovarian cancer cell lines with no gene methylation, as determined by sequencing, was used as non-methylated controls. The sensitivity of the assays ranged from 10 to 50 pg of methylated DNA, with specificity of a ratio of methylated allele in a background of unmethylated alleles ranging from 1:1000 to 1:4000 (Supplementary Table 1). Headloop suppression PCRs were performed in triplicate for each gene and considered positive when at least 2 replicates exhibited amplication with a cycling threshold (Ct) of <45.

Statistical analysis

Candidate gene methylation and dichotomized clinicopathological variables (FIGO stage, grade, age, menopausal status, optimal surgical debulking, chemotherapy regime and preoperative CA-125 levels) were correlated to patient outcome using comprehensive clinical follow-up data for each patient. Variables were compared to length of survival as defined from the date of initial diagnosis to the date of patient death or, in the case of surviving patients, their most recent follow-up date (whereupon they were censored). Potential predictors of overall survival (defined as death due to ovarian cancer) were

evaluated by Kaplan–Meier analysis and Cox proportional hazards models. Sensitivity and specificity of gene methylation discrimination of HGSOC and benign OSE were determined by comparing methylation frequencies for single and combinations of genes in cancers and controls. A p-value of <0.05 was considered statistically significant. Association of methylation of each gene with clinicopathological variables (age at diagnosis, FIGO stage, optimal residual disease, presence of ascites, menopausal status, pre-operative CA125 levels) was determined using a Chi-square test or Fischer's exact test as appropriate with dichotomized data, or using logistic regression for continuous variables. All statistical analyses were performed using STATA9 software (StataCorp, College Station, TX USA) and R [11].

Results

Methylation patterns in HGSOC

Eight genes, all candidate tumor suppressor genes and previously reported to be methylated at a high frequency in ovarian cancer (EOC) and/or HGSOC specifically (HGSOC) in at least one published study, were selected for methylation analysis (Table 1): *DLEC1*, *HOXA9*, *RASSF1A*, *GATA4*, *GATA5*, *SFN*, *CDH1*, *HSULF1*. Although not known to be methylated at high frequency in HGSOC, *BRCA1* was also selected, since loss of expression of BRCA1 by mutation or methylation has been shown to play a key role in ovarian and breast cancer development [12]. Lastly, *EN1*, recently identified as being located in a genomic region frequently hypermethylated in colon and prostate cancer [10] was selected in order to characterise methylation frequency in ovarian cancer.

Headloop suppression PCR assays were used to determine the methylation patterns in DNA isolated from 80 primary HGSOC patients with complete clinicopathological information (Table 2) and 12 benign OSE samples. Results from the headloop suppression PCR assays demonstrated that the genes were predominantly unmethylated in benign OSE, with the marked exception of *SFN* which was methylated in all samples tested (Fig. 1A). The frequency of methylation of the other genes in HGSOC varied widely ranging from very low (8% *DLEC1*) to very high methylation (95% *HOXA9*, 80% *EN1*) (Fig. 1A). Correction for multiple testing revealed that individual gene methylation was not associated with any of the clinicopathological variables tested (Supplementary Table 2).

To examine the methylation profiles in tumors from the HGSOC patient cohort, gene methylation data were tabulated (Supplementary Table 3). Fig. 1B displays the number of genes methylated in each sample and the proportion of the samples harboring these methylation events (Fig. 1B). HGSOC samples had increased methylated loci compared to control samples, with seventy-nine (98.8%) tumor samples showing methylation of at least one gene, 95% of the samples showing at least 2 methylated genes, 83.3% at least 3 methylated genes and 65% at least 4 methylated genes.

Table 1
Genes selected for methylation analysis in HGSOC cohort by headloop PCR.

Gene name	Chromosome location	Methylation in EOC	Methylation in HGSOC	Assay	Reference
BRCA1	17q21	12% (12/98)	9.4% (6/64)	Southern blot and MSP	(15)
CDH1	16q22.1	42% (34/80)		MSP	(27)
DLEC1[a]	3p22-p21.3	–	54% (7/13)[a]	MSP	(14)
EN1	2q13-q21	–	–	HL	(28)
GATA4	8p23.1-p22	60% (9/15)	0% (0/6)	MSP	(16)
GATA5	20p13.33	33% (5/15)	17% (1/6)	MSP	(16)
HOXA9	7p15-p14	51% (26/51)	21% (4/19)	MSP	(17)
HSULF1	8q13.2-q13.3	100% (16/16)		Bisulphite Sequencing	(29)
RASSF1A	3p21.3	49% (23/47)	42% (8/19)	MSP	(17)
SFN	1p36.11	–	26.3% (5/19)	MSP	(18)

[a] DLEC1 methylation was reported in HGSOC samples previously selected for having repressed DLEC1 gene expression.

Table 2
Clinicopathologic characteristics of high grade serous ovarian cancer patients and benign ovarian surface epithelium controls.

Characteristics	No. of patients	%	No. of patients	%
	High grade ovarian cancer (n = 80)		Benign ovarian surface epithelium (n = 12)	
Age (years)				
≤60	47	58.75	10	83.33
>60	33	41.25	2	16.67
Median	59		43.2	
Mean	60.19		46.08	
SD (range)	11.78	(24.0–85.3)	11.12	(34.0–71.2)
FIGO stage				
I/II	12	15		
III/IV	68	85		
Tumor grade				
G2	26	32.5		
G3	54	67.5		
Surgical debulking (n = 79)				
Optimal ≤1 cm	60	75.95		
Suboptimal >1 cm	19	24.05		
Presence of ascites				
No	29	36.25		
Yes	51	63.75		
Menopausal status				
Pre/Peri	17	21.25	9	75
Post	63	78.75	3	25
CA125 (n = 77)			(n = 11)	
≤200 U/ml	14	18.18	11	100
>200 U/ml	63	81.82	0	0
Median (U/ml)	755		16	
Mean (U/ml)	2456		41.82	
SD (range)	5715	(7–36100)	43.99	(9–126)
Adjuvant chemotherapy				
P only	24	30		
P + T	45	56.25		
P + C	10	12.5		
Refused chemo	1	1.25		
Outcome				
Complete response	64	80		
Progressive disease	16	20		
Recurrence[a] (n = 64)	54	84.38		
Death related to ovarian cancer	37	46.25		
Survival time to cancer death (n = 37)				
Mean (months)	32.32			
SD (range)	15.11	(9.2–76.7)		

C = cyclophosphamide; P = platinum; T = taxatere.
[a] In patients with complete response to treatment (n = 64).

Methylation and patient outcome

To examine associations between patterns of methylation and patient outcome, Kaplan–Meier curves were generated to examine the impact of gene methylation on patient survival. In contrast to clinicopathological features known to be associated with outcome i.e. FIGO stage, residual disease and ascites (Table 3, Fig. 2A–C), there was no association between the number of methylated genes and patient outcome suggesting that accumulation of extra gene methylation is not a significant determinant of survival (Fig. 2D). Furthermore, no significant associations were observed between individual gene methylation and outcome except for *DLEC1* (Fig. 2E), where the minority of patients with methylated DNA showed decreased overall survival ($p = 0.021$). Multivariate Cox proportional hazard modeling showed that this association was independent of FIGO stage, adjuvant chemotherapy and surgical debulking (HR 3.5 (95% CI:1.10–11.07), $p = 0.033$), establishing its status as an independent predictor of

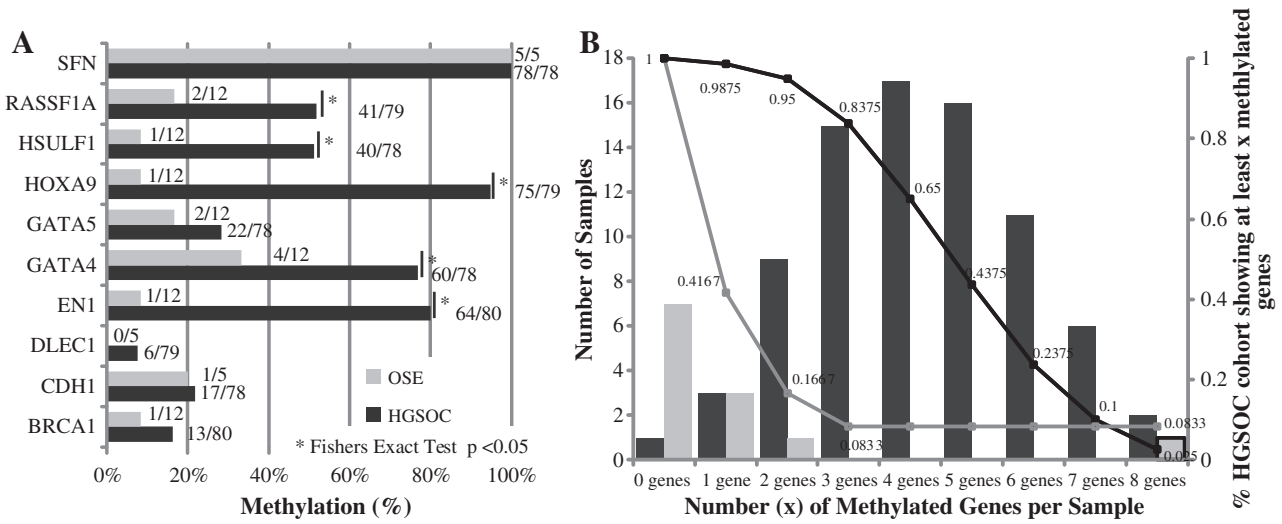

Fig. 1. (A) Methylation proportions of gene candidates in HGSOC and benign OSE. Headloop suppression PCR was used to assess methylation of 10 genes in a cohort of ovarian cancers and benign OSE. The proportion of the cohort showing methylation is plotted for cancers (black bars) relative to benign OSE (gray bars). Fisher's Exact Test was used to evaluate whether methylation frequency was significantly different in cancers compared to benign OSE. (B) Distribution of gene methylation in the HGSOC and control cohorts. For each total number of methylated genes identified (x), the number of HGSOC (dark gray bars) and control (light gray bars) samples demonstrating that extent of methylation is displayed (left y-axis). The cumulative proportion of HGSOC or control samples harboring at least x number of methylated genes is also displayed (black line/right y-axis).

Table 3
Analysis of gene methylation with recurrence-free and overall survival in HGSOC.

	Univariate analysis			
	Overall survival		Recurrence-free survival	
Variable	HR (95% CI)	p-value	HR (95% CI)	p-value
Age	0.99 (0.96–1.02)	0.38	0.99 (0.97–1.01)	0.456
FIGO Stage	**5.37 (1.28–22.57)**	**0.022**	**5.87 (2.29–15.02)**	**0.000**
Surgical debulking	**2.67 (1.32–5.41)**	**0.006**	**6.31 (2.86–13.91)**	**0.000**
Adjuvant Chemotherapy				
Platinum/ cyclophosphamide vs. other	**2.24 (1.04–4.82)**	**0.038**	1.052 (0.49–2.26)	0.987
Menopausal status	1.16 (0.53–2.54)	0.716	0.78 (0.38–1.61)	0.500
Preoperative CA125	0.98 (0.49–1.94)	0.948	1.25 (0.71–2.19)	0.436
Ascites	**2.91 (1.27–6.69)**	**0.012**	**2.51 (1.35–4.66)**	**0.004**
Methylation				
BRCA1	0.52 (0.20–1.35)	0.178	0.59 (0.28–1.27)	0.176
CDH1	1.21 (0.53–2.78)	0.648	1.13 (0.56–2.27)	0.735
DLEC1	**3.67 (1.22–11.03)**	**0.021**	1.30 (0.46–3.65)	0.618
EN1	0.6 (0.30–1.22)	0.163	0.72 (0.39–1.36)	0.310
GATA4	1.54 (0.70–3.41)	0.286	0.97 (0.53–1.78)	0.929
GATA5	0.99 (0.44–2.19)	0.973	0.80 (0.43–1.50)	0.492
HOXA9	2.92 (0.40–21.46)	0.292	1.16 (0.41–3.23)	0.780
HSULF1	1.14 (0.59–2.20)	0.705	1.32 (0.77–2.27)	0.316
RASSF1A	0.97 (0.50–1.87)	0.920	1.20 (0.70–2.06)	0.516
	Multivariate analysis			
	Overall survival		Recurrence-free survival	
Variable	HR (95% CI)	p-value	HR (95% CI)	p-value
FIGO Stage				
I/II vs III/IV	2.50 (0.53–11.77)	0.247	**4.33 (1.55–12.10)**	**0.005**
Surgical Debulking				
≤1 vs. >1 cm	**2.46 (1.17–5.16)**	**0.018**	**4.92 (2.24–10.79)**	**0.000**
Adjuvant Chemotherapy				
Platinum/ cyclophosphamide vs. other	1.97 (0.86–4.53)	0.112		
Methylation status				
DLEC1	**3.50 (1.10–11.07)**	**0.033**		
Ascites				
No vs. yes	1.90 (0.78–4.66)	0.160	1.44 (0.73–2.85)	0.296

Bold refers to results with p-value<0.05.

patient outcome. These data support the role of *DLEC1* as a potential tumor suppressor in ovarian cancer [13].

Methylation patterns and diagnosis

In order to investigate the ability of gene methylation to distinguish carcinoma from benign tissue, we sought to determine which gene combinations could discriminate between HGSOC and benign OSE (Table 4). *HOXA9* methylation alone provided the best discrimination and could predict HGSOC at a sensitivity of 95.0% and specificity of 91.7%. The combination of two genes (*HOXA9* and *EN1*) improved sensitivity to 98.8% with no loss in specificity. Incorporating additional genes that on their own significantly distinguished HGSOC from control samples did not improve sensitivity/specificity. Additionally, incorporation of *BRCA1* methylation, to include a degree of ovarian cancer specificity, did not improve the ability of *HOXA9*/*EN1* methylation to distinguish between HGSOC and benign OSE.

To assess if gene methylation could be used to improve existing molecular biomarkers, we investigated discrimination properties of pre-operative serum CA125 levels in our cohort (Table 4). In this data set, CA125 by itself shows a sensitivity of 81.8% and a specificity of 100%. However, combining CA125 levels with methylation status of *HOXA9* and *EN1* increased sensitivity to 100%. Furthermore, this level of discrimination was conserved when the analysis was confined to the earlier stage (FIGO 1–2) tumors in the cohort (n = 12). This indicates that these methylation changes are likely to occur early in HGSOC development.

Discussion

In this study we examined the methylation patterns of commonly methylated candidate tumor suppressor genes in a large, well annotated cohort of HGSOC patients, with the aim of determining whether differentiated patterns can serve as potential markers of clinical benefit in disease diagnosis and prognosis. The restricted set of genes examined was selected on the basis that they were known to be methylated in other cancer types [5]. However, future studies expanding this work would be expected to include a broader

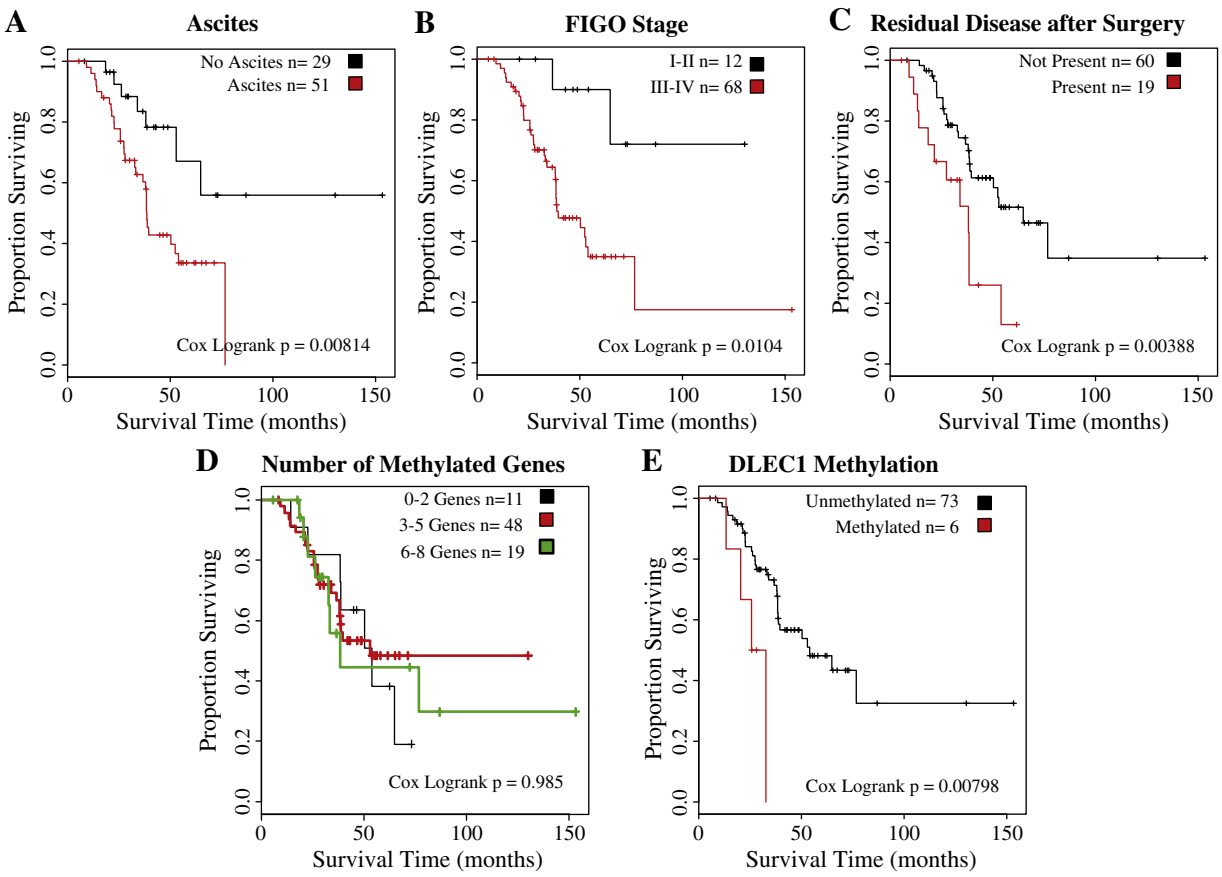

Fig. 2. Kaplan–Meier curves of clinicopathological variables and gene methylation and their effect on patient outcome. Evaluation of the impact of ascites (A), stage (B), residual disease (C), number of methylated genes (D) and DLEC1 methylation (E) on overall survival. A Cox proportional hazards model was fitted to the data and a significant effect on survival was determined by a log-rank p-value of <0.05.

spectrum of genes arising from more direct interrogation of the ovarian cancer methylome.

BRCA1, GATA5 and *RASSF1A* showed comparable levels of methylation to previously published reports [14–16] for the frequency of methylation of these genes in ovarian cancer. However, in some cases our data differed from that in the literature; for example *GATA4, HOXA9* and *SFN* were methylated at frequencies considerably higher than reported in previous studies [15–17]. These observed differences are likely due to the increased sensitivity of the headloop suppression PCR assay in clinical material as well as the larger cohort size studied. It is also possible that these differences reflect heterogeneity within tumor samples themselves. Interestingly, while *SFN* was 100% methylated in HGSOC, it was also methylated in all the control benign OSE samples examined, thus eliminating it is as a useful diagnostic marker.

One of the major findings of this study was the very high frequency of methylation of two homeobox genes, *HOXA9* and *EN1*. *HOXA9* alone, methylated in almost all (>95%) of the primary tumors tested, also showed the strongest discrimination between HGSOC and benign OSE, rendering it one of the most frequently methylated gene yet identified in HGSOC. A previous study has demonstrated that *HOXA9* is methylated in a small proportion (4/19) of HGSOC [16]. Due to the small sample size employed in both studies, further studies using a larger cohort are necessary to validate our findings. Regardless, our data suggest that *HOXA9* gene methylation is a prominent feature of HGSOC.

Aberrant silencing of *HOXA9* by DNA methylation has previously been demonstrated in epithelial ovarian cancer [16] and may have biological consequences in the development of HGSOC. Recent evidence, including the presence of serous tubule intraepithelial lesions in the fimbrial end of the fallopian tube in *BRCA* cases and identical *p53* mutations in fallopian tube neoplasia and serous ovarian tumors, suggest that HGSOC originate from the fallopian tube [18–20]. *HOX* genes are involved in vertebrate axial patterning during development, with *HOXA9* regulating serous differentiation of the Müllerian ducts to fallopian tubes [21–23]. Thus HOXA9 methylation in ovarian cancer may reflect a loss of transcriptional plasticity during the development of disease and a shift towards de-differentiation/high-grade classification of epithelial cells [24]. Indeed the entire *HOXA1 − A13* cluster is suppressed in prostate cancer, by long range epigenetic silencing (LRES), resulting in reduced plasticity of this region in the cancer cells [24].

Table 4
Discrimination between HGSOC and controls in study cohort by different models.

	Cases (n = 80)	Controls (n = 12)	Sensitivity	Specificity
HOXA9	75	1	0.950	0.917
HOXA9 and EN1	79	1	0.988	0.917
HOXA9 and EN1 and RASSF1A and GATA4 and HSULF1[a]	79	4	0.988	0.667
HOX and EN1 and BRCA1	79	1	0.988	0.917
CA125 > 200	63	0	0.818	1
HOX and EN1 and CA125 > 200	80	1	1	0.917
HOX and EN1 and BRCA and CA125 > 200	80	1	1	0.917

[a] Genes that singularly significantly differentiate between HGSOC and controls.

The *EN1* gene is best characterized in its involvement in central nervous system pattern formation [25], however perturbation in cancer has also been observed. While *EN1* has been demonstrated to be frequently methylated and associated with LRES in prostate cancer [10], and colorectal cancer [26], it has not been previously implicated in ovarian cancer development. As a homeobox gene, linked to development of stem cells in the ovary of *Drosophila* [27], *EN1* repression may also be a crucial step in HGSOC carcinogenesis.

While the methylation of many of these genes is associated with the presence of disease, only *DLEC1* was associated with poor patient outcome in this cohort. Our data demonstrate that *DLEC1* methylation, despite the low frequency observed in this HGSOC cohort, is an independent marker for poor outcome, similar to its role in non-small cell lung carcinoma [28]. Due to its observed role in suppressing colony formation of ovarian cancer cell lines [13], loss of DLEC1 expression in ovarian cancer may contribute to increased cell proliferation and disseminated disease, which are commonly associated with reduced survival. The relative paucity of biomarkers of poorer outcome in HGSOC suggests that this finding may have potential clinical utility whereby early identification of patients with this aberration may influence treatment decisions. However, *DLEC1* methylation was only apparent in a minority of patients i.e. 6 of 79 (7.6%) and these data need to be validated in a large independent cohort of ovarian cancer patients.

Gene methylation profiles of HGSOC tissues revealed that a model comprised of *HOXA9* and *EN1* achieved a high level of sensitivity and specificity in distinguishing ovarian cancer from benign ovaries in this dataset. In addition, our study demonstrates that combining gene methylation with pre-operative CA125 levels increased the sensitivity of the assay to 100%, albeit in the relatively small population available for this study. That this level of discrimination was conserved even in early stage cases, where the disease is confined to the ovaries and/or pelvic regions and not yet spread to the peritoneal cavity (although the smaller numbers indicates potential for significant error), indicates that comparing methylation profiles may be useful for the development of a diagnostic test for early stage disease and urgently needs replication in a large, well characterized population of cases and matched controls. Indeed, The Cancer Genome Atlas Network recently compared DNA methylation profiles of 489 HGSOC tumors and normal controls, focusing on those associated with decreased gene expression [29]. These large cohort studies will be helpful in providing independent validation sets for potential biomarker studies.

While the discrimination parameters reported here fall short of the current estimate that a suitable screening test for ovarian cancer would require a sensitivity of at least 75% and a specificity of more than 99.6% [30], it is promising that an improvement in the test is observed on addition of CA125 data to the gene methylation data. This suggests that in combination with CA125, a patient gene methylation signature is likely to perform better than CA125 alone. The next challenge in the development of a potential diagnostic test involves the need to translate these findings to blood, a more suitable and less invasive source for biomarker examination and quantitation. There have been reports of the detection of cancer-specific methylation patterns in ovarian cancer patient plasma and peritoneal fluid [7] so this development appears feasible. Furthermore, our use of highly sensitive and specific headloop suppression PCR assays for the detection of DNA methylation in clinical samples should be directly applicable to plasma as an approach for screening. Our findings provide encouraging support for the further development of methylation signatures for new diagnostic approaches.

Supplementary materials related to this article can be found online at doi:10.1016/j.ygyno.2011.11.026.

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Conflict of interest statement

The authors declare no conflict of interest or relevant financial relationships.

References

[1] Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. CA Cancer J Clin 2010;60:277–300.
[2] Landen Jr CN, Birrer MJ, Sood AK. Early events in the pathogenesis of epithelial ovarian cancer. J Clin Oncol 2008;26:995–1005.
[3] Havrilesky LJ, Whitehead CM, Rubatt JM, Cheek RL, Groelke J, He Q, et al. Evaluation of biomarker panels for early stage ovarian cancer detection and monitoring for disease recurrence. Gynecol Oncol 2008;110:374–82.
[4] Bast Jr RC, Badgwell D, Lu Z, Marquez R, Rosen D, Liu J, et al. New tumor markers: CA125 and beyond. Int J Gynecol Cancer 2005;15(Suppl. 3):274–81.
[5] Barton CA, Hacker NF, Clark SJ, O'Brien PM. DNA methylation changes in ovarian cancer: Implications for early diagnosis, prognosis and treatment. Gynecol Oncol 2008;109:129–39.
[6] Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet 2002;3:415–28.
[7] Ibanez de Caceres I, Battagli C, Esteller M, Herman JG, Dulaimi E, Edelson MI, et al. Tumor cell-specific BRCA1 and RASSF1A hypermethylation in serum, plasma, and peritoneal fluid from ovarian cancer patients. Cancer Res 2004;64:6476–81.
[8] Rand KN, Ho T, Qu W, Mitchell SM, White R, Clark SJ, et al. Headloop suppression PCR and its application to selective amplification of methylated DNA sequences. Nucleic Acids Res 2005;33:e127.
[9] Clark SJ, Statham A, Stirzaker C, Molloy PL, Frommer M. DNA methylation: bisulphite modification and analysis. Nat Protoc 2006;1:2353–64.
[10] Devaney J, Stirzaker C, Qu W, Song JZ, Statham AL, Patterson KI, et al. Epigenetic deregulation across 2q14.2 differentiates normal from prostate cancer and provides a regional panel of novel DNA methylation cancer biomarkers. Cancer Epidemiol Biomarkers Prev 2010;20:148–59.
[11] R_DevelopmentCoreTeam. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2010.
[12] Bianco T, Chenevix-Trench G, Walsh DC, Cooper JE, Dobrovic A. Tumour-specific distribution of BRCA1 promoter region methylation supports a pathogenetic role in breast and ovarian cancer. Carcinogenesis 2000;21:147–51.
[13] Kwong J, Lee JY, Wong KK, Zhou X, Wong DT, Lo KW, et al. Candidate tumor-suppressor gene DLEC1 is frequently downregulated by promoter hypermethylation and histone hypoacetylation in human epithelial ovarian cancer. Neoplasia 2006;8:268–78.
[14] Baldwin RL, Nemeth E, Tran H, Shvartsman H, Cass I, Narod S, et al. BRCA1 promoter region hypermethylation in ovarian carcinoma: a population-based study. Cancer Res 2000;60:5329–33.
[15] Wakana K, Akiyama Y, Aso T, Yuasa Y. Involvement of GATA-4/-5 transcription factors in ovarian carcinogenesis. Cancer Lett 2006;241:281–8.
[16] Wu Q, Lothe RA, Ahlquist T, Silins I, Trope CG, Micci F, et al. DNA methylation profiling of ovarian carcinomas and their in vitro models identifies HOXA9, HOXB5, SCGB3A1, and CRABP1 as novel targets. Mol Cancer 2007;6:45.
[17] Kaneuchi M, Sasaki M, Tanaka Y, Shiina H, Verma M, Ebina Y, et al. Expression and methylation status of 14-3-3 sigma gene can characterize the different histological features of ovarian cancer. Biochem Biophys Res Commun 2004;316:1156–62.
[18] Jarboe EA, Folkins AK, Drapkin R, Ince TA, Agoston ES, Crum CP. Tubal and ovarian pathways to pelvic epithelial cancer: a pathological perspective. Histopathology 2008;53:127–38.
[19] Levanon K, Crum C, Drapkin R. New insights into the pathogenesis of serous ovarian cancer and its clinical impact. J Clin Oncol 2008;26:5284–93.
[20] Levanon K, Ng V, Piao HY, Zhang Y, Chang MC, Roh MH, et al. Primary ex vivo cultures of human fallopian tube epithelium as a model for serous ovarian carcinogenesis. Oncogene 2010;29:1103–13.
[21] Du H, Taylor HS. Molecular regulation of mullerian development by Hox genes. Ann N Y Acad Sci 2004;1034:152–65.
[22] Taylor HS, Vanden Heuvel GB, Igarashi P. A conserved Hox axis in the mouse and human female reproductive system: late establishment and persistent adult expression of the Hoxa cluster genes. Biol Reprod 1997;57:1338–45.
[23] Cheng W, Liu J, Yoshida H, Rosen D, Naora H. Lineage infidelity of epithelial ovarian cancers is controlled by HOX genes that specify regional identity in the reproductive tract. Nat Med 2005;11:531–7.
[24] Coolen MW, Stirzaker C, Song JZ, Statham AL, Kassir Z, Moreno CS, et al. Consolidation of the cancer genome into domains of repressive chromatin by long-range epigenetic silencing (LRES) reduces transcriptional plasticity. Nat Cell Biol 2010;12:235–46.
[25] Herrup K, Murcia C, Gulden F, Kuemerle B, Bilovocky N. The genetics of early cerebellar development: networks not pathways. Prog Brain Res 2005;148:21–7.

[26] Mayor R, Casadome L, Azuara D, Moreno V, Clark SJ, Capella G, et al. Long-range epigenetic silencing at 2q14.2 affects most human colorectal cancers and may have application as a non-invasive biomarker of disease. Br J Cancer 2009;100: 1534–9.
[27] Bolivar J, Pearson J, Lopez-Onieva L, Gonzalez-Reyes A. Genetic dissection of a stem cell niche: the case of the Drosophila ovary. Dev Dyn 2006;235:2969–79.
[28] Seng TJ, Currey N, Cooper WA, Lee CS, Chan C, Horvath L, et al. DLEC1 and MLH1 promoter methylation are associated with poor prognosis in non-small cell lung carcinoma. Br J Cancer 2008;99:375–82.
[29] TCGA. Integrated genomic analyses of ovarian carcinoma. Nature 2011;474:609–15.
[30] Clarke-Pearson DL. Clinical practice. Screening for ovarian cancer. N Engl J Med 2009;361:170–7.