Predictive Biomarkers and Personalized Medicine

Promoter CpG Island Methylation of Genes in Key Cancer Pathways Associates with Clinical Outcome in High-Grade Serous Ovarian Cancer

Wei Dai¹, Constanze Zeller¹, Nahal Masrour¹, Nadeem Siddiqui³, James Paul⁴, and Robert Brown¹,²

Abstract

Purpose: We aimed to identify DNA methylation biomarkers of progression-free survival (PFS) to platinum-based chemotherapy in high-grade serous ovarian cancer (HGSOC) within biologically relevant ovarian cancer-associated pathways.

Experimental Design: Association with PFS of CpG island (CGI) promoter DNA methylation at genes in the pathways Akt/mTOR, p53, redox, and homologous recombination DNA repair was sought with PFS as the primary objective in a prospectively collected ovarian cancer cohort (n = 150). Significant loci were validated for associations between PFS, methylation, and gene expression in an independent The Cancer Genome Atlas (TCGA) data set of HGSOC (n = 311).

Results: DNA methylation at 29 CGI loci linked to 28 genes was significantly associated with PFS, independent from conventional clinical prognostic factors (adjusted P < 0.05). Of 17 out of the 28 genes represented in the TCGA data set, methylation of VEGFB, VEGFA, HDAC11, FANCA, E2F1, GPX4, PRDX2, RAD54L, and RECQL4 was prognostic in this independent patient cohort (one-sided P < 0.05, false discovery rate < 10%). A multivariate Cox model was constructed, with clinical parameters (age, stage, grade, and histologic type) and significant loci. The final model included NDK1, VEGFB, and PRDX2 as the three best predictors of PFS (P = 6.62 × 10⁻⁶, permutation test P < 0.05). Focussing only on known VEGFs in the TCGA cohort showed that methylation at promoters of VEGFA, VEGFB, and VEGFC was significantly associated with PFS.

Conclusions: A three loci model of DNA methylation could identify two distinct prognostic groups of patients with ovarian cancer (PFS: HR = 2.29, P = 3.34 × 10⁻⁵; overall survival: HR = 1.87, P = 0.007) and patients more likely to have poor response to chemotherapy (OR = 3.45, P = 0.012). Clin Cancer Res; 1–10. ©2013 AACR.

Introduction

There are an ever-increasing number of emerging novel agents being examined in clinical trials of ovarian cancer (1). However, debulking surgery with platinum-based chemotherapy remains the cornerstone of treatment at first presentation. Initial response rates are generally good (>75%), but patients relapse and will eventually develop resistant disease leading to treatment failure. Length of progression-free survival (PFS) of patients from primary presentation is an indication of whether patients will respond to second-line platinum-based chemotherapy (2). If robust biomarkers of poor PFS to platinum-based chemotherapy can be identified, then poor prognosis patients can potentially be stratified for novel treatment strategies. This may become particularly relevant for molecular targeted therapies used in the maintenance setting, where those patients with high risk of relapsing earlier can be identified. DNA methylation has many advantages as a biomarker: its relative stability (in vivo and in vitro), functional links to gene expression, and potential to be detected in cell-free DNA from body fluids (3–5).

Targeted molecular therapies being clinically evaluated in ovarian cancer include angiogenesis inhibitors. Angiogenesis has been shown to be a crucial requirement for metastatic ovarian cancer and the development of ascites (6). Clinical trials using bevacizumab, a humanized monoclonal antibody targeting the proangiogenic VEGFA, in combination with conventional chemotherapy have shown that

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Debulking surgery with platinum-based chemotherapy remains the primary treatment for high-grade serous ovarian cancer. Independent biomarkers of progression-free survival (PFS) to platinum-based chemotherapy could aid in identifying patients prone to early relapse who could particularly benefit from novel treatments. We have evaluated DNA methylation at promoter CpG islands of genes in multiple key pathways implicated in epithelial ovarian cancer, including Akt/mTOR, p53, Redox, and homologous recombination DNA repair-associated pathways. We have identified nine loci whose methylation is associated with PFS independent from conventional clinical parameters. Together with our previous study of the Wnt pathway, 3 loci (NKD1, VEGFB, and PRDX2), when combined in a multivariate Cox model, are strong predictors of PFS independent from known clinical factors (PFS: HR = 2.3, P = 3.3 × 10^-3; overall survival: HR = 1.9, P = 0.007). These loci have the potential to aid in stratifying patients for targeted therapy in ovarian cancer.

**Translational Relevance**

bevacizumab in combination with first-line chemotherapy has improved PFS times in patients with late-stage ovarian cancer (7, 8). Prolonged PFS times have also been observed using bevacizumab in addition to chemotherapy in recurrent chemoresistant ovarian cancer (9, 10). However, preselection of patient subgroups based on their molecular and histologic subtypes may be required for patients to optimally benefit from targeted agents beyond that achieved with conventional therapies.

In this study, we aimed at identifying the prognostic value of DNA methylation at CpG islands at the promoter of genes associated with known pathways involved in ovarian cancer development and progression. These are mainly genes and pathways as defined in Kyoto Encyclopedia of Genes and Genomes (KEGG) and included Akt/mTOR, p53, BRCA1/2, Redox, and homologous recombination-associated pathways. Previously we have shown that multiple CpG islands associated with the Wnt pathway significantly associate with PFS independently from clinical parameters (11). Together with the present study, we show the potential of DNA methylation biomarkers to be used for patient stratification for targeted care in clinical practice.

**Materials and Methods**

**Patients**

Tumor biopsies were prospectively collected in an ongoing Scottish Gynaecology Clinical Trial Group (SGCTG)/National Cancer Research Institute (London, United Kingdom) cohort study. Primary tumors included in the current study were restricted to those from patients with confirmed epithelial ovarian cancer (EOC) excluding clear cell and mucinous tumors, and treated with cytoreductive surgery followed by platinum-based chemotherapy. Biopsies of tumor were obtained at initial laparotomy or laparoscopic biopsy at the same time as diagnostic biopsy. All samples analyzed were collected before chemotherapy.

The primary endpoint of this study is to systematically examine any association between promoter methylation and PFS, defined as the time from the start of first-line chemotherapy to progressive disease or early death due to EOC or other causes. The secondary endpoints are the association of promoter methylation with response to platinum-based chemotherapy measured by Response Evaluation Criteria in Solid Tumors (RECIST) 1.0 criteria and overall survival (OS). Progression and survival status was assessed 2 monthly for the first 2 years posttreatment and subsequently 6 monthly to 5 years and annually thereafter as defined in the protocol. The study has been approved by MREC for Scotland (reference number 01/165). Genomic DNA was extracted from fresh-frozen tumors for methylation analysis as previously described (12).

One hundred and seventy-nine EOC were used in this study. Twenty samples that were excluded from subsequent analysis due to poor quality of signal intensities in more than 10% of probes in the duplicates or methylation controls did not reach acceptance criteria. Nine samples with ovarian tumors only in the ovaries (stage I) were further excluded. Therefore, the analysis was focused on 150 stage III and IV ovarian tumors. In a subsequent validation stage, data from 311 high-grade late-stage serous tumor samples profiled by The Cancer Genome Atlas (TCGA) project on HumanMethylation27 BeadChip using Illumina Infinium assay were analyzed. Full details of clinical parameters are shown in Supplementary Table S1. Throughout this study, we have followed the REMARK recommendations (13).

**Design of Agilent customized promoter CpG island microarray**

Genes involved in Akt/mTOR pathway, p53 pathway, BRCA1/2 pathway, redox pathway, homologous recombination pathways were mainly collected from KEGG. Promoter CGIs of those genes were identified as previously described (11). The genomic locations of the targets are specified by Human Mar. 2006 (NCBI36/hg18) assembly. In total, 51 genes represented by 78 loci in the Akt/mTOR pathway, 68 genes by 140 loci in the p53 pathway, 64 genes by 101 loci in the BRCA1/2 pathway, 48 genes by 63 loci in the Redox pathway, and 35 genes by 45 loci in homologous recombination were examined in this study. A full list of genes and the genomic location of the promoter regions targeted on the array are in Supplementary Table S2.

The 60mer-oligos/probes targeting those regions were mainly selected from Oligome (Oxford Genome Technology). The selected probes were uploaded to eArray (Agilent Technologies), and the 60-mer microarray was fabricated using Agilent SurePrint Technology. The customized array was further evaluated in differential methylation hybridization (DMH) assay using 0% and 100% methylated samples (Millipore). Only 3% of probes were identified as noninformative either due to lack of McrBC recognition sites within the loci or having low log2-transformed DMH.
ratios in 100% methylated samples compared with 0% methylated sample (Supplementary Fig. S1).

**Differential methylation hybridization**

Methylation levels of our targets were measured by DMH in duplicates, and microarray data preprocessing was done as previously described (11). In brief, DNA was digested with MseI, ligated to an end-linker, and divided into two aliquots. One aliquot was mock treated, the other aliquot was digested with the methylation-sensitive restriction enzyme, McrBC (14, 15), followed by PCR amplification. The amplicons labeled with Cy3 or Cy5 were then competitively hybridized to the customized microarrays. Labeling of DNA, array hybridization, and image scanning were done according to the standard Agilent aCGH protocol. DMH ratio is the ratio of the signals from McrBC mock digested and McrBC-digested samples. The DMH dataset is available at Gene Expression Omnibus (accession ID: GSE23240). The quality of DMH assay was assessed as previously described (11).

**The Cancer Genome Atlas dataset**

The level 2 expression dataset on Affymetrix HGU133A microarrays and level 3 methylation dataset on Illumina HumanMethylation27 Beadchip of serous tumors were obtained from TCGA data portal (16). We limited the analysis in late-stage tumors with methylation and expression data, therefore, 311 high-grade serous ovarian cancers (HGSOC) were included in the study.

The expression microarray data have been preprocessed and normalized across the samples, and methylation data have been summarized as \( \beta \) value, which was calculated as \( M/(M+U) \), where \( M \) is the signal of methylation bead type and \( U \) is the signal of unmethylation bead type of the targeted CpG site. The poor quality probes have been excluded by TCGA (16).

**Bisulphite pyrosequencing**

Bisulphite pyrosequencing was conducted in a panel of \( n = 142 \) HGSOC from the SGCTG cohort to validate the prognostic value of promoter methylation at VEGFA, VEGFB, and VEGFC as previously described (11). In brief, 1 \( \mu \)g of genomic DNA was bisulfite modified using the EpiTect Bisulfite Kit (Qiagen) according to the manufacturer’s instructions. Pyrosequencing primer sets and PCR conditions are listed in Supplementary Table S3. The methylation was quantified as the percentage of methylated cytosine over the sum of methylated and unmethylated cytosines using Pyro Q-CpG software (Biostage). The methylation level of the three genes in each sample was calculated by using the average percentages of methylation across all targeted CpG sites in duplicates, respectively, and subsequently, was used as a continuous variable in the Cox model in the survival analysis.

**Statistical power estimation**

The initial screening set consisted of DMH data from 150 tumors, with 133 (89%) patients having disease progression. To estimate the approximate statistical power of this screening set before analysis, we assumed that 5% of the loci examined in each pathway were true positives and split patients into two groups based on the upper quartile of methylation level at each locus following what we have observed in previous methylation profiling study (11). With a HR at 2 and false discovery rate (FDR; ref. 17) less than 10%, we estimated the average power of the screening study to be 75% (Supplementary Methods S1). In the subsequent analysis, methylation levels have been treated as a continuous variable, meaning we are underestimating the study power.

**Survival analysis**

All the survival analysis was done in R (version 2.10.1) using survival package. The DMH ratios of multiple probes targeting the same locus (MseI fragment) were averaged. The mean value of methylation at the locus in duplicates was then standardized to Z score \([Z \sim \mathcal{N}(0, 1)]\). The Z scores were used as a continuous variable in the Cox model. The proportional hazards were examined before the association between methylation and PFS was examined by univariate Cox model. The HR was then adjusted by conventional prognostic factors [Federation Internationale des Gynecologistes et Obstetristes (FIGO) stage, grade, histology, and age] in multivariate Cox model. The significance of estimated HRs was calculated by Score test in univariate analysis, and Wald test in multivariate analysis. External validation of prognostic value of biomarkers identified from SGCTG cohort was done in TCGA cohort using methylation level (\( \beta \) value) as a continuous variable in univariate analysis.

To determine the best predictors of PFS in patients with late-stage (stage III and IV) ovarian cancer, a multivariate Cox model was constructed using the forward stepwise method based on likelihood ratio statistics with a probability of 0.05 for entry and 0.10 for removal. Among the variables including clinical parameters and validated, independent methylation markers identified in current study and previous study (11), only three methylation markers meet the entry criteria, thus selected into the model in this study (see Results). Subsequently, a methylation index (MI) was calculated using the selected covariates from this model. Permutation test involved in the same process as our modeling procedure including feature selection in the univariate and multivariate analysis adjusted by clinical parameters as well as model construction in the SGCTG cohort was conducted 100 times to evaluate the significance of the final multivariate Cox model.

**Logistic regression analysis**

The correlation between response and promoter methylation in AKT/mTOR pathway, p53 pathway, BRCA1/2 pathway, Redox pathway, and HR pathway was tested by logistic regression. Patients were classified as responders (complete or partial response) or nonresponders (stable disease or progressive disease or not evaluable response generally due to the poor physical condition of the patients) according to RECIST 1.0 criteria. The analysis was restricted
to patients with measurable disease at baseline level. Methylation level was used as a continuous variable as well as a categorical variable in SGCTG cohort and as a categorical variable in TCGA cohort, where the categorical variable was used, the top 20% of the patients with high methylation level at the biomarker examined were categorized into the "high-methylation group," otherwise, they were included in the "low-methylation group."

We constructed a multivariate logistic regression model incorporating multiple methylation biomarkers selected by forward stepwise likelihood ratio algorithm in SGCTG cohort. The prediction value of these biomarkers was further evaluated in TCGA cohort (Supplementary Methods S2).

**Results**

**DNA methylation and association with PFS**

We have systematically profiled CpG island (CGI) promoter DNA methylation at genes in pathways implicated in ovarian cancer, including p53, AKT, redox, and DNA repair pathways (Table 1). Association with PFS was sought in 150 stage III/IV ovarian tumors prospectively collected through a cohort study (SGCTG cohort). Mucinous and clear cell cancers were excluded because of their different clinical outcomes from more common serous and endometrioid EOC (18, 19). Thirty-eight loci were identified as significantly associated with PFS ($P < 0.01$ and FDR $< 10\%$), see univariate PFS analysis in SGCTG cohort in Table 1 for a summary and univariate PFS analysis in Supplementary Table S4 for details).

The HRs of 38 loci identified as having $P < 0.01$ and FDR $< 10\%$ in univariate analysis were adjusted by age, stage, grade, and histologic type, and the patients were stratified into three groups who either received platinum alone ($n = 42$), combination of platinum and taxane ($n = 85$), or other platinum-based treatment ($n = 16$). We found that hypermethylation at 29 loci linked to 28 genes was associated with increased hazard of disease progression independent from conventional clinical prognostic factors: CGIs at DNA methylation and association with PFS

**Table 1.** A summary of the PFS analysis in five key signaling pathways

<table>
<thead>
<tr>
<th>Pathway/family</th>
<th>Source</th>
<th>Total # genes</th>
<th>Total # CGIs</th>
<th># Loci (130-6,000 bp)</th>
<th># Loci Univariate PFS analysis $P &lt; 0.01$ and FDR $&lt; 10%$</th>
<th># Loci Multivariate PFS analysis $P &lt; 0.05$</th>
<th># Validated loci/# loci on the array</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akt/mtOR</td>
<td>hsa04150$^a$</td>
<td>51</td>
<td>51</td>
<td>78</td>
<td>6</td>
<td>4</td>
<td>2/3</td>
<td>VEGFA, VEGFB$^a$</td>
</tr>
<tr>
<td>p53</td>
<td>hsa04115$^a$</td>
<td>68</td>
<td>87</td>
<td>140</td>
<td>14</td>
<td>10</td>
<td>1/4</td>
<td>SESN2</td>
</tr>
<tr>
<td>BRCA1/2</td>
<td>Biocompare$^b$</td>
<td>64</td>
<td>72</td>
<td>101</td>
<td>10</td>
<td>7</td>
<td>3/5</td>
<td>E2F1, FANCA, HDAC11</td>
</tr>
<tr>
<td>Redox</td>
<td>Manually curated</td>
<td>48</td>
<td>44</td>
<td>63</td>
<td>5</td>
<td>5</td>
<td>2/3</td>
<td>PRDX2, GPX4$^c$</td>
</tr>
<tr>
<td>HR</td>
<td>ko03440$^d$</td>
<td>35</td>
<td>31</td>
<td>45</td>
<td>3</td>
<td>3</td>
<td>2/2</td>
<td>RECLQ4, RAD54L$^e$</td>
</tr>
</tbody>
</table>

$^a$KEGG.


$^c$The locus is significantly correlated with response measured by RECIST criteria (version 1.0; logistic regression, $P < 0.05$).
cohort data. Covariates were selected by likelihood ratio forward stepwise algorithm from clinical parameters (age, stage, grade, and histologic type) and promoter methylation at VEGFA, VEGFB, HDAC11, FANCA, E2F1, GPX4, PRDX2, RAD54L, and RECQL4 identified in the SGCTG cohort and validated in TCGA cohort, as well as methylation at 7 gene promoters (FZD4, DVL1, NKD1, ROCK1, LRPS, AXIN1, and NFATC3) that have been shown to be significantly associated with PFS in SGCTG cohort in a previous study of promoter CGI methylation profiling in the Wnt pathway in EOCs using identical platforms and statistical analysis plan (11). The final model included NKD1 [HR = 1.26; 95% confidence interval (CI), 1.02–1.45, P = 0.025], VEGFB [HR = 1.22; 95% CI, 1.04–1.44, P = 0.015], and PRDX2 (HR = 1.22; 95% CI, 1.02–1.45, P = 0.029) as the three best predictors of PFS (P = 6.62 × 10^-5; permutation test P = 0.05). The HR represents the relative risk per unit increase in Z score. The remaining variables including clinical parameters and remaining 14 methylation biomarkers were not selected into the model because they did not provide additional prognostic information beyond that provided by NKD1, VEGFB, and PRDX2. A MI calculated from this model \((\text{MI} = 0.23 \times Z_{\text{NKD1}} + 0.2 \times Z_{\text{VEGFB}} + 0.2 \times Z_{\text{PRDX2}}, Z\) denotes Z score) could identify two distinct prognostic groups using the third quartile of the index as the cutoff (PFS: HR = 2.29; 95% CI, 1.53–3.42, log-rank test P = 3.34 × 10^-5; OS: HR = 1.87; 95% CI, 1.18–2.97, log-rank test P = 0.007; Fig. 1). The patients with increased MI were also more likely to have poor response to chemotherapy (OR = 3.45; 95% CI, 1.31–9.08, P = 0.012; PR+CR vs. PD+SD+NE; 30% vs. 70%).

Subsequently, we further evaluated the multivariate model incorporating VEGFB and PRDX2 in terms of association with PFS in the TCGA cohort data (the locus linked to NKD1 is absent on Illumina BeadChip and so NKD1 could not be included in this model). Consistent with the result found in SGCTG cohort, the association between MI estimated from this model and progression/relapse-free survival, as well as response to chemotherapy remained significant in this independent patient cohort (PFS: log-rank test P = 0.009; response: logistic regression analysis adjusted OR = 4.11; 95% CI, 1.21–13.96, P = 0.024). However, the association with OS did not stand in this cohort (OS: log-rank test P = 0.291). By combining with the expression dataset from TCGA, patients with higher methylation of VEGFB had increased expression of VEGFB, whereas patients with higher methylation of PRDX2 had reduced expression of PRDX2 (Fig. 2).

Surgical debulk status is correlated with survival in advanced ovarian cancer (20). In the SGCTG and TCGA cohorts studied in the present study, patients with any microscopic tumor after surgery have shorter OS and PFS than those without any detectable tumor (SGCTG: HR = 1.98; 95% CI, 1.1–3.58, P = 0.023; TCGA cohort: HR = 1.67; 95% CI, 1.06–2.65, P = 0.029). We therefore further assessed the association between PFS and the MI estimated from our study adjusted by debulk status by forcing both variables into the multivariate Cox model. The MI remained significant after adjustment (SGCTG: adjust HR = 2.56; 95% CI, 1.54–4.26, 4.11; 95% CI, 1.17–7.06, P = 0.021, n = 228), showing that the MI has independent prognostic value from surgical outcome.

**DNA methylation correlates with response to platinum-based chemotherapy**

DNA methylation at the 29 loci associated with poor PFS was assessed for any relationship with patients’ response to first-line platinum-based chemotherapy. Increased methylation at VEGFB, RRM2, CD82, TR211T, GPX4, RAD54L, and EME2 was associated with poor response in SGCTG cohort (Supplementary Table S6). Out of these 7 biomarkers, methylation of VEGFB and GPX4 are the most significant and independent biomarkers of response identified by
Figure 2. Methylation and expression of VEGFB, PRDX2, VEGFA, and VEGFC in TCGA cohort. The top 20% of the patients with high methylation level at the biomarker examined were categorized into the “high-methylation group,” otherwise, they were included in the “low-methylation group.” A, left, methylation level of candidate biomarker and (B, right) expression level of candidate biomarker. Mann–Whitney U test (two sided) was used to examine the significant difference between two groups. *, P < 0.05.
multivariate logistic regression analysis using forward stepwise likelihood ratio algorithm (Supplementary Methods S2). Seventy-eight percent (29/37) patients with increased methylation either at VEGFB or at GPX4 had poor response to chemotherapy (OR = 6.18; 95% CI, 2.47–15.43, \( P = 0.0001 \)). Consistently, patients with methylation either at VEGFB or GPX4 from TCGA cohort were more likely to have poor response to chemotherapy (OR = 1.99; 95% CI, 0.93–4.25, \( P = 0.078 \)). This trend became clearer after correction for batch effect (adjusted OR = 3.56; 95% CI, 1.09–11.62, \( P = 0.036 \); Supplementary Methods S2).

**Association between disease progression and methylation at VEGFs in EOCs**

Antiangiogenesis treatment has shown clinical benefit to patients with ovarian cancer when combined with chemotherapy (7, 8). The VEGFs are prime regulators of pathologic angiogenesis. Given that two biomarkers identified in this study are associated with VEGFA and VEGFB, we systematically examined methylation at promoter regions and expression of VEGFs in the TCGA cohort for correlation with progression/relapse-free survival and response to chemotherapy where the majority of the patients received platinum-based chemotherapy. Expression data showed that patients with increased methylation at VEGFB and VEGFA had elevated expression level, whereas patients with increased methylation at VEGFC had reduced expression of this gene (Fig. 2). Among 5 members of VEGFs including VEGFA, VEGFB, VEGFC, VEGFD, and PIGF, we found that methylation at the promoter region of VEGFA, VEGFB, and VEGFC was significantly relevant to disease progression (TCGA cohort in Table 2).

Methylation of VEGFA, VEGFB, and VEGFC was then quantified by bisulfite pyrosequencing in the SGCTG cohort. Consistent with previous findings, patients with increased methylation of VEGFB and decreased methylation of VEGFC have increased the risk of tumor progression (one-sided \( P < 0.05 \); SGCTG cohort in Table 2). Methylation at VEGFA shows the trend to be correlated with PFS in this analysis, though the methylation level at promoter region of this gene is extremely low (about 1%).

Methylation of VEGFB is a strong predictor of response to chemotherapy in both SGCTG and TCGA cohort (SGCTG cohort: OR = 5.92; 95% CI, 1.85–18.94, \( P = 0.003 \); TCGA cohort: OR = 2.13; 95% CI, 0.97–4.68, \( P = 0.059 \)). Expression level of VEGFB also showed a significant association with response in TCGA cohort (OR = 2.57; 95% CI, 1.22–5.43, \( P = 0.013 \); Table 3).

**Discussion**

Novel biomarkers of disease progression and response to chemotherapy are needed to guide current treatment strategies thereby potentially optimizing patient benefit and clinical trial design. We have systematically profiled CGI DNA methylation at genes in pathways previously implicated in ovarian cancer development and progression and identified validated association of methylation at multiple CGI with clinical outcomes in HGSOC. Aberrant DNA methylation frequently occurs in cancer, particularly at CGIs, which are generally unmethylated in normal cells. CGIs often colocalize with the promoters of genes, and promoter hypermethylation is associated with repression of gene transcription (21). Several studies have shown that CGI methylation has potential as a biomarker for monitoring tumor progression (22, 23) and is associated with platinum-based chemoresistance in EOC (24, 25). However, many of these studies are either limited by small sample size or lack of validation of the methylation biomarker as an independent prognostic marker. By building on our previously reported DNA methylation model derived from loci at genes in the Wnt pathway (11), we present here, a novel multivariate Cox model including three DNA methylation-dependent loci (NKD1, VEGFB, PRDX2) that can separate patient subgroups with late-stage ovarian cancer with distinct PFS and OS, and shows improved prognostic value when compared with conventional clinical parameters. Alongside our previously identified Wnt-associated NKD1 locus, this model includes loci from further cancer-related pathways VEGFB and PRDX2. Furthermore, MI estimated from this model is associated with response to the first-line platinum-based chemotherapy, suggesting these pathways might be involved in the chemosensitivity in EOCs.

**Table 2. Association between PFS and methylation of VEGFA, VEGFB, and VEGFC**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Genomic location</th>
<th>HR (95% CI)</th>
<th>Two-sided P</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGFA</td>
<td>chr6: 43845984-43845985</td>
<td>1.35 (0.99–1.84)</td>
<td>0.056</td>
<td>311</td>
</tr>
<tr>
<td>VEGFB</td>
<td>chr11: 63758874-63758875</td>
<td>1.95 (1.12–3.42)</td>
<td>0.018</td>
<td>311</td>
</tr>
<tr>
<td>VEGFC</td>
<td>chr4: 177951450-177951451</td>
<td>0.92 (0.86–0.99)</td>
<td>0.021</td>
<td>311</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genes</th>
<th>Genomic location</th>
<th>HR (95% CI)</th>
<th>One-sided P</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGFA</td>
<td>chr6: 43845984-43845985</td>
<td>1.17 (0.94–1.44)</td>
<td>0.08</td>
<td>141</td>
</tr>
<tr>
<td>VEGFB</td>
<td>chr11: 63758262-63758287</td>
<td>1.12 (0.99–1.28)</td>
<td>0.044</td>
<td>126</td>
</tr>
<tr>
<td>VEGFC</td>
<td>chr4: 177951442-177951520</td>
<td>0.99 (0.981.00)</td>
<td>0.03</td>
<td>142</td>
</tr>
</tbody>
</table>
The identification of VEGFA, VEGFB, and VEGFC as associated with clinical outcome in patients with ovarian cancer is particularly of interest given the clinical trials showing improvement of PFS in patients with ovarian cancer treated with the VEGF-targeted agent bevacizumab in addition to standard chemotherapy (carboplatin and paclitaxel). It has been proposed that bevacizumab activity might be limited to a small proportion of patients (26), and hence it is important to identify patient subsets likely to benefit from bevacizumab. Thus the prognostic and predictive value of DNA methylation at VEGF loci in such bevacizumab trials now needs to be assessed.

In contrast with VEGFA, which has a defined role in angiogenesis and has recently been shown to fulfil a direct, tumor-promoting effect (27), the function of VEGFB remains largely elusive (28). A number of studies have reported conflicting observations and relatively little is known about the role of VEGFB in tumorigenesis and progression (29). In brain and retinal neuron apoptosis models, VEGFB can potently inhibit apoptosis and promote survival (30). Although the role of VEGFB may largely depend on tumor context, an antiapoptotic role for VEGFB could provide a rational for increased methylation of VEGFB and increased VEGFB expression being associated with worse PFS and poor response to platinum-based chemotherapy in ovarian cancer. Although speculative, VEGFB methylation may indicate a subset of patients, which may particularly benefit from bevacizumab treatment in not only abrogating tumor angiogenesis but also targeting increased tumor growth, while being more resistant to platinum-induced DNA apoptosis.

We initially identified two out of five VEGF-associated genes, including VEGFA and VEGFB, as having prognostic value in high-grade serous carcinoma. Systematic analysis of VEGF family members from the TCGA dataset showed that methylation of VEGFC was further associated with PFS of patients with ovarian cancer. These observations highlight the prognostic significance of VEGF biomarkers for ovarian cancer. Furthermore, methylation of VEGFB, in particular, emerged as being a predictor of response in two independent patient cohorts. It is interesting that patients with increased VEGFB methylation at the promoter region have elevated VEGFB expression in the TCGA cohort. We looked for transcription factor binding around the PFS-associated CpG site and found a consensus-binding site for the transcription factor ZEB1 (AREB6/TCF8) close to this genomic site. ZEB1 is a negative transcriptional regulator of angiogenesis (31). It has been reported that increased methylation close to the ZEB transcription-binding site impairs ZEB transcription factor binding at TP73 thereby upregulating gene expression in ovarian cancer cell lines (32); however, it remains unclear whether this is also the case at VEGFB.

In contrast with VEGFB where increased methylation increases the risk of disease progression, methylation at the

### Table 3. Association between response and methylation/expression of VEGFA, VEGFB, and VEGFC

#### TCGA Cohort (HumanMethylation27)

<table>
<thead>
<tr>
<th>Genes</th>
<th>Methylation level&lt;sup&gt;a&lt;/sup&gt;</th>
<th>OR (95% CI)</th>
<th>P</th>
<th># PR: CR</th>
<th># PD: SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGFA</td>
<td>High methylation</td>
<td>1.61 (0.66–3.92)</td>
<td>0.29</td>
<td>24</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Low methylation</td>
<td>1</td>
<td></td>
<td>150</td>
<td>31</td>
</tr>
<tr>
<td>VEGFB</td>
<td>High methylation</td>
<td>2.13 (0.97–4.68)</td>
<td>0.059</td>
<td>30</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Low methylation</td>
<td>1</td>
<td></td>
<td>144</td>
<td>27</td>
</tr>
<tr>
<td>VEGFC</td>
<td>High methylation</td>
<td>1.03 (0.43–2.43)</td>
<td>0.955</td>
<td>35</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Low methylation</td>
<td>1</td>
<td></td>
<td>139</td>
<td>31</td>
</tr>
</tbody>
</table>

#### TCGA Cohort (Affymetrix HGU133A)

<table>
<thead>
<tr>
<th>Genes</th>
<th>Expression level&lt;sup&gt;b&lt;/sup&gt;</th>
<th>OR (95% CI)</th>
<th>P</th>
<th># PR: CR</th>
<th># PD: SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGFA</td>
<td>High expression</td>
<td>1.15 (0.48–2.72)</td>
<td>0.759</td>
<td>32</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Low expression</td>
<td>1</td>
<td></td>
<td>142</td>
<td>31</td>
</tr>
<tr>
<td>VEGFB</td>
<td>High expression</td>
<td>2.57 (1.22–5.43)</td>
<td>0.013</td>
<td>34</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Low expression</td>
<td>1</td>
<td></td>
<td>140</td>
<td>24</td>
</tr>
<tr>
<td>VEGFC</td>
<td>High expression</td>
<td>2.2 (0.73–6.61)</td>
<td>0.159</td>
<td>139</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Low expression</td>
<td>1</td>
<td></td>
<td>35</td>
<td>4</td>
</tr>
</tbody>
</table>

<sup>a</sup>Twenty percent of the patients with highest level of methylation were categorized in the "high-methylation group," otherwise, they were included in the "low-methylation group."

<sup>b</sup>If expression was positively correlated with methylation, 20% of the patients with highest level of expression were categorized in the "high-expression group," otherwise, they were included in the "low-expression group."

If expression was reversely correlated with expression, 20% of the patients with lowest level of expression were categorized in the "low-expression group," otherwise, they were in the "high-expression group."
VEGFC locus reduces risk of progression. In tumors, VEGFC fulfills a role in promoting lymphangiogenesis and metastasis via the lymphatic system (33). In ovarian cancer, VEGFC protein expression and serum levels have been correlated with lymphatic metastasis, and high VEGFC correlates with poor prognosis (34, 35). It has been suggested that induction of VEGFC by the transcription factor LEDGF/p75 could be a potential strategy in tumors to escape VEGFA-targeted therapy and to sustain tumor progression (36). In this respect, it remains to be seen whether patients with increased risk of progression due to low VEGFC methylation and high VEGFC expression may benefit from targeted therapies such as antiangiogenesis agents.

Taken together, by using systematic profiling of DNA methylation at CGI promoters of pathways relevant to ovarian carcinogenesis, we have identified three DNA methylation biomarkers (NKD1, VEGFB, PRDX2) that give rise to a MI capable of predicting PFS in patients with ovarian cancer independently from known clinical prognostic features. These biomarkers could aid in the identification of patients with suboptimal benefit from standard platinum-based chemotherapy. Patients with an increased MI may especially benefit from being stratified for more targeted therapies. In addition, methylation at individual VEGF family members associates with differential risk of disease progression and further evaluation of the predictive value of methylation and expression at VEGFB and VEGFC as biomarkers for patient’s response to targeted therapies such as bevacizumab is warranted.

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

Authors’ Contributions
Conception and design: N. Siddiqui, J. Paul, R. Brown
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): W. Dai, C. Zeller, N. Siddiqui, J. Paul, R. Brown
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): W. Dai, J. Paul, R. Brown
Writing, review, and/or revision of the manuscript: W. Dai, C. Zeller, N. Siddiqui, J. Paul, R. Brown
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N. Masrour
Study supervision: R. Brown

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References
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