Imaging, Diagnosis, Prognosis

Systematic CpG Islands Methylation Profiling of Genes in the Wnt Pathway in Epithelial Ovarian Cancer Identifies Biomarkers of Progression-Free Survival

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Note:
Supplementary data for this article are available at Clinical Cancer Research.

Introduction

Epithelial ovarian cancer (EOC) is the most lethal of all the gynecologic cancers accounting for 52% of all gynecologic cancer-related deaths (1). Patients with advanced disease have surgical cytoreduction followed by plati-

num-based chemotherapy, but there is a low 5-year survival rate of less than 30% (1–5). There is a need to identify key pathways in ovarian cancer whose activity is associated with patient survival to assist our understanding of the molecular basis of disease progression and to identify biomarkers of sufficient discriminatory prognostic and predictive power to be of clinical value. The established prognostic factors of EOC at clinical presentation before primary chemotherapy are residual disease and patient age, as well as histology, stage and grade of tumor (5–7); however, these clinical factors do not provide insights into biological mechanisms for the clinical progression of tumors. Although many individual biomarkers in EOC have been investigated (8–14), currently only 2 serum biomarkers CA125 and HE4 have been approved by the FDA for monitoring patients with EOC (15). Identifying robust biomarkers of patient survival following conventional treatment of EOC will also provide a solid basis for characterizing potential predictive biomarkers to novel treatment strategies and hence for stratification of patients for targeted care in clinical practice.

Abstract

Purpose: Wnt pathways control key biological processes that potentially impact on tumor progression and patient survival. We aimed to evaluate DNA methylation at promoter CpG islands (CGI) of Wnt pathway genes in ovarian tumors at presentation and identify biomarkers of patient progression-free survival (PFS).

Experimental Design: Epithelial ovarian tumors (screening study n = 120, validation study n = 61), prospectively collected through a cohort study, were analyzed by differential methylation hybridization at 302 loci spanning 189 promoter CGIs at 137 genes in Wnt pathways. The association of methylation and PFS was examined by Cox proportional hazards model.

Results: DNA methylation is associated with PFS at 20 of 302 loci (P < 0.05, n = 111), with 5 loci significant at false discovery rate (FDR) less than 10%. A total of 11 of 20 loci retain significance in an independent validation cohort (n = 48, P ≤ 0.05, FDR ≤ 10%), and 7 of these loci, at FZD4, DVL1, NFATC3, ROCK1, LRP5, AXIN1, and NKD1 genes, are independent from clinical parameters (adjusted P < 0.05). Increased methylation at these loci associates with increased hazard of disease progression. A multivariate Cox model incorporates only NKD1 and DVL1, identifying two groups differing in PFS [HR = 2.09; 95% CI (1.39–3.15); permutation test P < 0.005]. Methylation at DVL1 and NFATC3 show significant association with response. Consistent with their epigenetic regulation, reduced expression of ROCK1, NKD1 and FZD4 is an indicator of early-disease relapse in an independent ovarian tumor cohort (n = 311, adjusted P < 0.05).

Conclusion: The data highlight the importance of epigenetic regulation of multiple promoter CGIs of Wnt pathway genes in ovarian cancer and identify methylation at NKD1 and DVL1 as independent predictors of PFS. Clin Cancer Res; 17(12): 4052–62. ©2011 AACR.
Aberrant DNA methylation frequently occurs in cancer, particularly at regions generally unmethylated in normal cells, known as CpG islands (CGIs). CGIs often colocalize with the promoters of genes and promoter hypermethylation is associated with repression of gene transcription (16). Several studies have shown that CGI methylation has potential as a biomarker for monitoring tumor progression (17) and is associated with platinum-based chemoresistance in EOC (18, 19). 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.

The Wnt pathways control a variety of biological processes including cell proliferation, differentiation, and migration and have a crucial role in development and tissue homeostasis (20, 21). Wnt signaling is transduced through binding of Wnt molecules to the transmembrane frizzled receptors proteins and activation of downstream signaling pathways depending on the interaction between Wnt molecules and their specific receptors. In the "canonical" pathway, binding of Wnt molecules to frizzled/LRP transmembrane receptor complex results in disheveled phosphorylation and inhibition of Axin, Gsk3b, and APC complex, subsequently allowing β-catenin to accumulate in the nucleus and interact with transcription factors of the T-cell–specific transcription factor/lymphoid enhancer binding factor family (22–24). Although mutations of APC and β-catenin are frequently observed in colorectal cancer (25, 26), they are rare in EOC (24, 27), with the exception of endometrioid subtypes (28). Among numerous noncanonical pathways, Wnt/planar cell polarity signaling and Wnt/Ca2+ signaling are involved in tumorigenesis (29–31).

In cancers in which Wnt pathway gene mutations are rare, the Wnt pathway can become disregulated through epigenetic mechanisms, such as promoter hypermethylation (32–34). The Wnt pathway extracellular inhibitors, such as SFRPs family and WIF1, are frequently silenced by promoter hypermethylation in cancers including EOC (33, 35–40). However, promoter methylation of the Wnt pathway has not been comprehensively studied in EOC. Our aim was to systematically profile DNA methylation at promoter CGIs of Wnt pathway genes and evaluate their role in tumor progression by assessing their association with patient progression-free survival (PFS) and overall survival (OS) in EOC in a prospective cohort study.

**Materials and Methods**

**Patients**

Tumor biopsies were prospectively collected in an ongoing Scottish Gynaecology Clinical Trial Group (SGCTG)/National Cancer Research Institute (NCRI) cohort study since 1997. Tumors included were restricted to those from patients with confirmed EOC treated with cytoreductive surgery and platinum-based chemotherapy, excluding mucinous and clear cell tumors. These latter histologic tumor types were excluded due to their different clinical outcome from more common serous and endometrioid EOC (41, 42). Macroscopically normal ovarian tissue taken adjacent to the tumor and from tissue adjacent to benign ovarian cysts and tumors were also collected. The information about progression and survival status of patients is obtained monthly for the first 2 years after chemotherapy, subsequently 6 monthly for 5 years and annually thereafter. PFS was defined as the time from first-line chemotherapy to progressive disease or early death due to EOC or other causes. OS was defined as the time from first-line chemotherapy to death due to EOC or other causes. Response was measured by Response Evaluation Criteria in Solid Tumors (RECIST) 1.0 criteria. The primary objective of the cohort study is to prospectively examine the association of DNA methylation with PFS. Secondary objectives are to examine for association of DNA methylation with OS and response to treatment. The study is approved by the MREC (multicentre research ethics committees) for Scotland (reference number 01/165). Genomic DNA was extracted for methylation analysis as previously described (43).

**Differential methylation hybridization**

Samples were assayed in duplicates by differential methylation hybridization (DMH), as previously described (44). Briefly, DNA was digested with MseI, ligated to an end-linker, and divided into 2 aliquots. One aliquot was mock treated, the other aliquot was digested with the methylation-sensitive restriction enzyme MsrBC which cuts (G/A)mCN40–3,000(G/A)mC (45, 46). PCR amplification was conducted with primers binding to the end linkers, the amplicons were then labeled with Cy3 or Cy5 and hybridized to the custom-designed 60 mer microarrays fabricated by using Agilent SurePrint Tehcnology (Agilent).
Labeling of DNA, array hybridization and image scanning was done by Oxford Gene Technology (Oxford, UK) according to the standard Agilent aCGH protocol. The raw signal intensities were extracted by Agilent feature extraction (v9.5.3.1). Probes with high background noise (about 3%) were treated as missing data points and imputed by KNN algorithm ($k = 10$; refs. 47, 48). Probes with high ($>65,000$, signal saturation) and low ($<$mean + 2 SD of negative controls) signal intensities were removed from the analysis. Within-array normalization was done by using MLDA package (49) tailored for DMH datasets. We removed samples in which more than 10% of the probes had poor quality on both duplicate arrays from the analysis. The DMH data preprocessing procedure is detailed in Supplementary Method 1. DMH ratio is the ratio of the signals from McrBC mock digested and MseI digested samples. The DMH dataset is available at GEO (accession ID: GSE23240).

**Target selection on Agilent custom-designed microarray**

Genes involved in the Wnt signaling pathway were collected from Kyoto Encyclopedia of Genes and Genomes (KEGG; entry ID: hsa04310; ref. 50). Promoter CGIs within 2 kb of the transcription start site of the genes were obtained from UCSC (University of California, Santa Cruz) database (51) and from a genome-wide prediction of CGIs (52) as a supplementary set, both of which fulfill the mathematical model proposed by Gardiner-Garden to define a CGI: CG content greater than 50%, length of sequence is more than 200 bp and ratio of observed to expected CpGs is larger than 0.6 (53). The genome positions of targets are specified by Human Mar. 2006 (NCBI36/hg18) assembly. Out of 148 genes in the Wnt pathway, 137 (92.6%) had a CpG island within 2 kb of the promoter and these are represented by 302 MseI fragments (130–6,000 bp) analyzed by DMH.

**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 the cancer genome atlas (TCGA) data portal (http://cancergenome.nih.gov/datasetportal), consisting of 311 high-grade serous tumors.

The expression microarray data have been preprocessed and normalized across the samples, and methylation data have been summarized as β value which was calculated as $M/(M + U)$, in which $M$ is the signals of methylation bead type and $U$ is the signals of unmethylation bead type of the targeted CpG site (see http://tcga-data.nci.nih.gov/docs/TCGA_Data_Primer.pdf and Supplementary Method 2 for details).

**Statistical analysis**

All statistical analyses were done in R (version 2.10.1) and SPSS (version 18.0).

**Sample size and statistical power estimation**

The initial screening set consisted of DMH data from 120 tumors with 111 subsequently shown to have suitable data quality and 102 patients had disease progression (see Results). To estimate approximate statistical power of this screening set prior to analysis, we assumed 5% of the loci examined in the Wnt pathway were true positives and split patients into 2 groups based on the median methylation level at each locus (in the subsequent analysis methylation levels have been treated as continuous variables, meaning we are underestimating the study power). With a HR at 1.75 and false discovery rate (FDR; ref. 54) less than 50%, we estimated the average power of the screening study to be 80% (Supplementary Method 3).

The subsequent validation study was analyzed by DMH a year later. Calculation of the statistical power for the sample size planned ($n = 50$) assumed the true positive loci rate was 40% (following on from the FDR rate observed in the screening set at $P < 0.05$) and by using a FDR of 10%. Sample size calculations were carried out by using the observed HRs per unit change and observed variable variances from the screening data set. Testing was 1-sided as associations of interest will be in the same direction as in the screening set. The validation power achieved for the largest observed effect size in the screening set was 88%, but only 30% for the median observed effect size. Among the 5 loci with FDR less than 10% from the screening set, the median power was 70% (Supplementary Method 3).

**Determination of methylation frequency in EOC**

Methylation of loci for estimating frequency of methylation in EOC were defined by DMH ratios of multiple probes targeting the same locus being significantly greater than unmethylated controls from chromosome 16 and the mitochondrial genome. The unmethylated controls were selected from the mitochondrial genome and low CG density region from chromosome 16 (59,000,000–61,000,000) that lacks McrBC recognition site (49). In average, each MseI fragment was covered by 12 probes and by at least 7 probes. To examine what the percentage of tumors in SGCTG cohort are methylated at the loci we examined, 1-sided Mann–Whitney $U$ test was used to compare the methylation levels of multiple probes targeting the same MseI fragment to those of unmethylated controls ($n = 93$) designed on the microarray. The significance level was set at $P < 0.0003$ on the basis of Bonferroni correction.

**Survival analysis**

The workflow of the survival analysis is illustrated in Supplementary Fig. S1. Cox proportional hazard model was used to examine for association between methylation and patient survival. 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 used as a continuous variable in the Cox model. The association between methylation and PFS was examined by
univariate Cox model first, and then multivariate analysis was carried out to evaluate the independence of loci identified from clinical parameters. The variables applied for adjustment in multivariate analysis included histologic type, International Federation of Gynecology and Obstetrics stage, grade, and age. The significance of estimated HRs was calculated by using the score test.

To determine the best predictors of PFS in patients with late-stage (stages III and IV) ovarian cancer, a multivariate Cox model was constructed by using the forward stepwise method based on likelihood ratio statistics with a probability of 0.05 for entry and 0.10 for removal (Supplementary Method 4). Among the variables including clinical parameters and validated, independent methylation markers, only 2 methylation markers meet the entry criteria, thus selected into the model in this study (see Results). Subsequently, a methylation index (MI) was calculated by using the selected covariates from this model. Leave-one-out cross validation (LOOCV) was done to evaluate the predictive value of this multivariate Cox model (Supplementary Method 5).

Kaplan–Meier curves were used to show the PFS in the patients with high/low methylation at promoter CGIs, and those with high/low MI. Because average methylation frequency of the Wnt pathway genes observed is approximately 25%, the third quartile was used as the cutpoint to define high/low methylation. Kaplan–Meier survival curves of 2 groups were compared by the log-rank test (2-sided). The significance of log-rank test of 2 groups with high/low MI determined by the third quartile was assessed by a permutation test applied to the entire variable selection and model fitting process.

The correlation between response and promoter methylation in the Wnt pathway was tested by logistic regression. This is restricted to patients with measurable disease at baseline. Patients were classified as responders (complete or partial response) or nonresponders (stable disease or progressive disease) according to RECIST 1.0 criteria.

External validation of prognostic value of biomarkers identified from SGCTG cohort was done in TCGA cohort by using methylation level (β value)/expression as a continuous variable in univariate analysis, then multivariate analysis was conducted to adjust the HR of disease progression/increase in expression by clinical parameters (stage, grade, and age) in this cohort. The direct association between methylation and expression was evaluated by Spearman correlation.

Results

Patient characteristics

Epithelial ovarian tumors prospectively collected through a cohort study, were analyzed by DMH at 302 Wnt pathway associated loci, as defined by KEGG (entry ID: hsa04310; ref. 50). Mucinous and clear cell cancers were excluded due to their different clinical outcome from more common serous and endometrioid EOC (37, 38). In an initial screening stage, from 120 ovarian tumor samples analyzed by DMH, 4 samples were excluded from subsequent analysis due to poor quality of signal intensities in more than 10% of probes in the duplicates and 5 samples were excluded as methylation controls did not reach acceptance criteria. Therefore, 111 tumors remained in the analysis. Assuming a HR of 1.75 and FDR (54) less than 50%, then the estimated average power of the screening study is 0.8 (Supplementary Method 2). In a subsequent validation stage, 59 further tumors collected through the same protocol were examined. Eleven samples were excluded due to not reaching acceptance criteria, so 48 tumors remained in the analysis. Among the loci with FDR less than 10% from the screening set the median power of the validation study was about 70%. The genes in the Wnt pathway frequently methylated (>5%, i.e., methylated in more than 5% of tumors) in ovarian cancer and unmethylated in PBMC are shown in Supplementary Table S1. Following the REMARK recommendations (55), we present full details of clinical parameters in Supplementary Table S2 and their relationship to patient outcome in Supplementary Tables S3 and S4.

Methylation of Wnt pathway loci in EOC associates with PFS

To evaluate the association between methylation at promoter CGIs of the Wnt pathway and tumor progression, we used average DMH ratios as a continuous variable in the univariate Cox model with PFS as the endpoint (see Methods). In the screening study, we identified methylation of 20 of 302 loci at promoter CGIs of 17 genes that may be associated with PFS (P < 0.05), with 5 loci at genes FZD4, DVL1, CCND1, CCND3, and NKD1 significant at FDR less than 10% after multiple test correction (see univariate survival analysis in Table 1). In an independent patient cohort (n = 48), 11 of 20 loci identified in the screening stage were still prognostic, P ≤ 0.05 and FDR ≤ 10% (Table 2).

The OR of 11 loci identified as having P ≤ 0.05 and FDR ≤ 10% in univariate analysis from the validation study were adjusted by age, stage, grade, and histologic type and the patients were stratified into 3 groups who either received platinum alone, combination of platinum and taxane, or other platinum-based treatment. Hypermethylation at 7 loci was associated with increased hazard of disease progression independent from clinical parameters: CGIs at FZD4, DVL1, AXIN1, and LRP5 (adjusted P < 0.05) and CGIs at NKD1, ROCK1, and NFATC3 (adjusted 0.05 < P < 0.06; see multivariate survival analysis in Table 3 and Fig. 1A–G). Of the 7 loci, patients with increased methylation level at gene DVL1, FZD4, and NKD1 also had higher risk of death (P < 0.05, FDR ≤ 20%; Table 4).

DNA methylation at the 6 loci associated with poor PFS were assessed for any relationship with response of the patients to first-line platinum-based chemotherapy. To maximize the numbers of patients with response data, this was examined in the screening and validation sets combined, therefore, the average DMH ratio of each locus was transformed to a Z score (standardized average DMH ratio of each locus within each study). Increased methylation at
Table 1. Univariate PFS analysis of screening set: loci with $P < 0.05$

<table>
<thead>
<tr>
<th>Genes</th>
<th>Univariate PFS analysis (n = 111)</th>
<th>$HR^a$ 95% CI</th>
<th>$P^b$</th>
<th>FDR$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>FZD4</td>
<td>123.3 (8.9–1,701.30)</td>
<td>&lt;0.001***</td>
<td>&lt;0.1*</td>
<td></td>
</tr>
<tr>
<td>CCND1$^\dagger$</td>
<td>3.9 (1.8–8.3)</td>
<td>&lt;0.001***</td>
<td>&lt;0.1*</td>
<td></td>
</tr>
<tr>
<td>NKD1</td>
<td>31.4 (3.7–268.3)</td>
<td>0.002**</td>
<td>&lt;0.1*</td>
<td></td>
</tr>
<tr>
<td>TAF8$</td>
<td>$CCND3</td>
<td>0.5 (0.3–0.8)</td>
<td>0.002**</td>
<td>&lt;0.1*</td>
</tr>
<tr>
<td>DVL1</td>
<td>12.1 (2.6–55.5)</td>
<td>0.001**</td>
<td>&lt;0.1*</td>
<td></td>
</tr>
<tr>
<td>FRAT1</td>
<td>0.3 (0.1–0.7)</td>
<td>0.005**</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>ROCK1</td>
<td>2263.1</td>
<td>0.008**</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>PPP2R2B</td>
<td>34.0 (2.4–476.6)</td>
<td>0.009**</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>CTBP1$^\dagger$</td>
<td>2.3 (1.2–4.3)</td>
<td>0.015*</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>CCND1$^\dagger$</td>
<td>48.6 (2.1–1,108.6)</td>
<td>0.015*</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>NKD2</td>
<td>10.6 (1.5–74.1)</td>
<td>0.017*</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>FZD9</td>
<td>7.4 (1.4–41.0)</td>
<td>0.021*</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>EEFSEC$</td>
<td>$RUVBL1</td>
<td>2.4 (1.2–5.2)</td>
<td>0.021*</td>
<td>0.5</td>
</tr>
<tr>
<td>AXIN1</td>
<td>24.5 (1.4–423.3)</td>
<td>0.028*</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>C4orf42$</td>
<td>$CTBP1$^\dagger$</td>
<td>1.9 (1.3–3.3)</td>
<td>0.030*</td>
<td>0.6</td>
</tr>
<tr>
<td>WNT4</td>
<td>41.1 (1.3–1,282.7)</td>
<td>0.034*</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>LRP5</td>
<td>5.7 (1.3–30.2)</td>
<td>0.041*</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>NFATC3</td>
<td>6.4 (1.3–57.6)</td>
<td>0.042*</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>FRAT1</td>
<td>0.7 (0.6–1.0)</td>
<td>0.040*</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>SFRP5</td>
<td>51.2 (1.2–2,886.6)</td>
<td>0.045*</td>
<td>0.6</td>
<td></td>
</tr>
</tbody>
</table>

$^a$HR per unit increase in DMH ratio (continuous variable) estimated from Cox proportional hazard regression model.
$^b$CI of the estimated HR.
$^c$P value of score test (2-sided).
$^d$Multiple test correction based on 302 loci.
$^e$FDR < 0.1; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.
$^f$Different loci within the promoter region of CCND1/CTBP1 were identified as fulfilling the definition of a CGI.

DVL1 and NFATC3 was correlated with poor response. The OR of the patients with progressive or stable disease ($n = 29$) to the patients with partial or complete response ($n = 54$) in every unit increase of methylation Z score is 1.7 (95% CI: 1.1–2.8, $P = 0.026$, FDR < 10%) and 1.6 (95% CI: 1.0–2.6, $P = 0.032$, FDR < 10%) for DVL1 and NFATC3, respectively.

Wnt pathway ML in the late-stage ovarian tumors

To identify the best methylation predictors of PFS in the ovarian tumors from both screening and validation sets, the average DMH ratio of each locus was transformed to a Z score. We excluded 10 stage I tumors from 159 tumors because these may be biologically distinct entities and patients with early-stage ovarian cancer have much better clinical outcome than patients with late-stage cancer. From the Z scores of 150 patients with late-stage EOC (stages III and IV), we constructed a multivariate Cox model, of which the covariates were selected by likelihood ratio forward stepwise algorithm from clinical parameters (age, stage, grade, and histologic type) and promoter methylation at FZD4, DVL1, NKD1, ROCK1, LRP5, AXIN1, and NFATC3 (see Supplementary Method 4). The final model includes DVL1 ($HR = 1.24$; 95% CI: 1.05–1.46; $P = 0.01$) and NKD1 ($HR = 1.28$; 95% CI: 1.04–1.57; $P = 0.02$) as the 2 best predictors. The HR represents the relative risk per unit increase in Z score. The remaining variables including clinical parameters and 5 methylation biomarkers were not selected into the model due to not meeting the entry criteria in forward stepwise method (see Methods), that is, the association between PFS and methylation at FZD4, ROCK1, LRP5, AXIN1, and NFATC3, and other conventional prognostic factors: grade, stage, age, and histologic type do not provide additional prognostic information beyond that provided by DVL1 and NKD1. A MI calculated from this model ($MI = 0.25 \times Z_{NKD1} + 0.22 \times Z_{DVL1}$. $Z$ denotes Z score) can identify 2 distinct prognostic groups by using the third quartile of the index as the cutoff ($HR = 2.09$; 95% CI: 0.6–7.0).

Table 2. Univariate PFS analysis in the validation set of loci with $P < 0.05$ in screening set

<table>
<thead>
<tr>
<th>Genes</th>
<th>Univariate PFS analysis (n = 48)</th>
<th>$HR^a$ 95% CI</th>
<th>$P^b$</th>
<th>FDR$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>FZD4</td>
<td>2.5 (0.9–6.6)</td>
<td>0.034*</td>
<td>&lt;0.1*</td>
<td></td>
</tr>
<tr>
<td>CCND1$^\dagger$</td>
<td>1.4 (0.6–3.4)</td>
<td>0.225</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>NKD1</td>
<td>2.2 (0.9–5.2)</td>
<td>0.039*</td>
<td>&lt;0.1*</td>
<td></td>
</tr>
<tr>
<td>TAF8$</td>
<td>$CCND3</td>
<td>1.3 (0.9–1.8)</td>
<td>0.929</td>
<td>1</td>
</tr>
<tr>
<td>DVL1</td>
<td>4.3 (1.2–15.0)</td>
<td>0.010*</td>
<td>&lt;0.1*</td>
<td></td>
</tr>
<tr>
<td>FRAT1</td>
<td>1.2 (0.7–2.1)</td>
<td>0.787</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>ROCK1</td>
<td>4.3 (1.0–19.4)</td>
<td>0.027*</td>
<td>&lt;0.1*</td>
<td></td>
</tr>
<tr>
<td>PPP2R2B</td>
<td>1.7 (0.1–27.6)</td>
<td>0.357</td>
<td>0.4</td>
<td></td>
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<tr>
<td>CTBP1$^\dagger$</td>
<td>1.3 (0.7–2.4)</td>
<td>0.176</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>CCND1$^\dagger$</td>
<td>3596.2</td>
<td>0.001*</td>
<td>&lt;0.1*</td>
<td></td>
</tr>
</tbody>
</table>

$^a$HR per unit increase in DMH ratio (continuous variable) estimated from Cox proportional hazard regression model.
$^b$CI of the estimated HR.
$^c$P value of score test (1-sided).
$^d$Multiple test correction based on 20 loci.
$^e$FDR < 0.1; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.
$^f$Different loci within the promoter region of CCND1/CTBP1 were identified as fulfilling the definition of a CGI.
Methylation of Wnt Pathway Loci in EOC Associates with PFS

Expression of genes associated with methylated loci and PFS

Promoter hypermethylation is frequently associated with gene inactivation (16); therefore, we further examined the association between expression and PFS of the 7 linked candidate genes as well as direct correlation between expression and methylation. This was examined in the TCGA cohort (n = 311). Among the 7 genes, NKD1 is not present on the Affymetrix HGU133A expression microarray and lacks coverage of CpG sites on HumanMethylation2 BeadChip. For the remaining 6 genes, low expression of FZD4, DVL1, and ROCK1 indicated a higher risk of recurrent/progressed disease (FZD4: HR = 0.8; 95% CI: 0.7–0.9; adjusted P = 0.002; DVL1: HR = 0.8; 95% CI: 0.6–1.0; adjusted P = 0.035; ROCK1: HR = 0.7; 95% CI: 0.5–1.0; adjusted P = 0.045), independent from stage, grade, and age (see univariate and multivariate PFS analysis in expression in Table 5). The reduced expression of FZD4, DVL1, and ROCK1 correlating with increased hazard of disease progression is consistent with promoter methylation leading to the downregulation of the genes, although direct inverse association between expression and methylation was only found at DVL1 (r = –0.13, P = 0.023) and ROCK1 (r = –0.25, P < 0.001), but not at FZD4 (r = 0.02, P = 0.766) by Spearman correlation (see correlation between methylation and expression in Table 5). Again, consistent with the methylation data is that decreased expression of DVL1 was related to poor response to platinum-based chemotherapy in the TCGA cohort [PD + SD (n = 36) vs. PR + CR (n = 217), OR = 0.5, 95% CI: 0.3–0.9, P = 0.035].

Discussion

We examined DNA methylation at CGIs at the promoter regions of Wnt pathway genes, as defined by KEGG (entry ID: hsa04310; ref. 45), in 159 EOCs. Using average DMH ratio as a continuous variable we identified 7 loci (FZD4, DVL1, ROCK1, NFACT3, AXIN1, LRP5, and NKD1) of which methylation was significantly associated with PFS in evaluation and validation tumor cohorts and are independent from known clinical prognostic features.

As with any microarray experiment, systematic bias can be introduced from any of the multiple steps of DMH: such as, DNA preparation, hybridization and image scan. In particular, DMH based on McrBC restriction enzyme, involves extra steps of digestion, ligation, and PCR amplification. Therefore, the reproducibility of technical duplicates was evaluated by using R² (coefficient of determination, ranging from 0 to 1) to estimate the variations introduced in DMH assays. After background correction, with array normalization and feature selection, the average R² of duplicate arrays in the screening set (n = 111) and in the following validation set (n = 48) was very close to 1, 0.92 (n = 111; mean ± SD: 0.92 ± 0.04)

Table 3. Multivariate PFS analysis of loci significantly associated with PFS in univariate analysis

<table>
<thead>
<tr>
<th>Genes</th>
<th>Multivariate PFS analysis (n = 111)</th>
<th>Adjusted HR 95% CI</th>
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<tbody>
<tr>
<td>FZD4</td>
<td>64.5 (3.4–1,243.8) 0.006*</td>
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</tr>
<tr>
<td>NKD1</td>
<td>10.2 (0.9–113.4) 0.059†</td>
<td></td>
</tr>
<tr>
<td>DVL1</td>
<td>8.3 (1.7–39.1) 0.008*</td>
<td></td>
</tr>
<tr>
<td>ROCK1</td>
<td>285.4 (1.0–82,977.6) 0.051†</td>
<td></td>
</tr>
<tr>
<td>CCND1</td>
<td>4.6 (0.5–37.9) 0.161</td>
<td></td>
</tr>
<tr>
<td>EEFSEC/RUVBL1</td>
<td>1.7 (0.8–3.7) 0.184</td>
<td></td>
</tr>
<tr>
<td>AXIN1</td>
<td>44.9 (2.0–1,004.6) 0.016*</td>
<td></td>
</tr>
<tr>
<td>WNT4</td>
<td>15.6 (0.4–670.8) 0.152</td>
<td></td>
</tr>
<tr>
<td>LRP5</td>
<td>6.8 (1.3–36.6) 0.026*</td>
<td></td>
</tr>
<tr>
<td>NFACT3</td>
<td>6.6 (1.0–44.9) 0.052†</td>
<td></td>
</tr>
<tr>
<td>SFRP5</td>
<td>26.8 (0.4–1,774.0) 0.124</td>
<td></td>
</tr>
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1.39–3.15; log-rank test, P = 2.42 × 10⁻⁴; permutation test, P < 0.005; Fig. 1H). LOOCV was used to predict the multivariate Cox model in 150 late-stage EOCs (see Supplementary Fig. S2).

External validation of the biomarkers from Wnt pathways in TCGA study

To validate in a further independent tumor set the prognostic value of the 7 validated methylation biomarkers that are independent from clinical parameters (at genes FZD4, DVL1, NKD1, ROCK1, AXIN1, LRP5, and NFACT3), we analyzed associations between PFS and methylation at these loci in data from a large (n = 111) independent cohort of high-grade serous ovarian tumors (see Method and Supplementary Method 6) available through TCGA Pilot study established by the National Cancer Institute (NCI) and National Human Genome Research Institute (NHGRI; unpublished). The methylation data was generated by using a different method from DMH: analysis of bisulfite modified DNA by Illumina HumanMethylation27 BeadChip. Individual CpG sites, either within exactly the same genomic location or within the same promoter CGI of the genes identified in our study, are present on the HumanMethylation27 BeadChip, except at NKD1. Among the 6 loci examined, methylation of DVL1, ROCK1, LRP5, and AXIN1 were still prognostic in this independent patient cohort (1-sided P < 0.05, FDR < 0.10%, n = 311), and methylation of FZD4 shows marginal correlation with PFS in this patient cohort (1-sided P = 0.06, FDR < 10%; see univariate PFS analysis in methylation in Table 5).
Figure 1. Kaplan–Meier plots of PFS. High/low methylation at A, FZD4 CGI; B, NKD1 CGI; C, DVL1 CGI; D, ROCK1 CGI; E, AXIN1 CGI; F, LRP5 CGI; G, NFATC3 CGI. Combined analysis of the evaluation and validation set shows that patients with hypermethylation at these loci have increased HR of disease progression (P < 0.05). The cutoff was determined by the third quartile in 156 patients (n = 159, 3 patients have PFS missing). Time is from the patients received the first line of chemotherapy. H, Kaplan–Meier plots of PFS in the patients with high/low MI estimated from the multivariate Cox model including DVL1 and NKD1 in late-stage ovarian cancer. The cutoff was determined by the third quartile in 147 patients (n = 150, 3 patients have PFS missing). Time is from the patients received the first line of chemotherapy.
We further examined 2 loci of SFRP5 with response to platinum-based chemotherapy (40). Because it has been shown that transcriptional silencing of SFRP5 is associated with promoter methylation (40), we further examined if expression of SFRP5 was correlated with response to first-line platinum-chemotherapy in the TCGA dataset, but no significant association was observed. However, we noted response was measured by both the reduction of tumor size and CA125 concentration in the previous study, whereas in our study response was measured by RECIST 1.0 criteria based on the shrinkage of tumor size. Furthermore, different composition of tumor stage and histology type in the cohort studies might also cause the differences. We observe that the prognostic value of FZD4, DVL1, and ROCK1 at 2 molecular levels, increased methylation and decreased expression, are correlated with increased hazard of disease progression in EOC. This indicates expression of these 3 genes might be tightly regulated by promoter methylation. The interaction between individual Wnts and their specific receptors is thought to dictate the type of downstream signaling pathways that are activated. Accordingly, the Wnts have historically been divided into 2 classes: those that signal through the “canonical” (β-catenin dependent) or the “noncanonical” (β-catenin independent) signaling pathway (21). FZD4 is a member of the frizzled receptor family and plays a crucial role in corpus luteum formation and function in mouse ovary (20). FZD4 is required along with LRP5 and the absence of ROR2, for Wnt-5a dependent β-catenin transcriptional activation (21). Three disheveled genes, DVL1, DVL2, and DVL3 have been identified in human. The Wnt3a-sensitive canonical pathway is particularly sensitive to knockdown of either Del1 or Del3 (56). Thus, promoter CGI methylation of DVL1 and FZD4 leading to downregulation of the expression of these 2 genes could suppress the “canonical” signaling pathways in EOC, while potentially activating the noncanonical pathways.

Although the majority of the samples from SGCTG cohort are serous ovarian tumors, a small number of tumors were endometrioid (n = 12). It has been reported that 16% to 38% of ovarian endometrioid cases are deregulated in the Wnt pathway because of activating mutation of β-catenin, which is a key player in the Wnt canonical pathway (57). Given the prognostic value of 5 of 7 independent methylation biomarkers found in SGCTG cohort have been confirmed in the TCGA cohort, which only contain high-grade serous ovarian tumors, we would argue that the presence of β-catenin mutation in endometrial cancers is unlikely to be a major confounding factor.

Further investigation is required for the loci that did not show a direct correlation between expression and methylation. As well as the limitations of microarray coverage, limited statistical power, and potential lack of appropriate transcription factors at the unmethylated promoters, these analyses are mainly confounded by the tumor samples in the TCGA cohort not being microdissected, which could influence TCGA expression data due to expression of the genes in normal infiltrating tissues. It is also possible that the biomarkers identified are heterogeneous methylation.
occurs in a defined region usually in or near promoter mutation, cancer-specific hypermethylation generally biomarkers, DNA methylation is amplifiable and can be clinical biomarker (62–65). Compared with protein-based number of advantages of using DNA methylation as a  

Table 5. Survival analysis in TCGA expression and methylation dataset (n = 311, disease progression/relapse = 184) of genes in the Wnt pathway associated with CGI loci independently associated with PFS

<table>
<thead>
<tr>
<th>Genes</th>
<th>Univariate PFS analysis (methylation)</th>
<th>Correlation between methylation and expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR 95% CI</td>
<td>P (1-sided)</td>
</tr>
<tr>
<td>FZD4</td>
<td>2357.6 (0.1 × 10^7–3.3 × 10^7)</td>
<td>0.056</td>
</tr>
<tr>
<td>NKD1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DVL1</td>
<td>29.5 (5.1–172.1)</td>
<td>8.382 × 10^-5***</td>
</tr>
<tr>
<td>ROCK1</td>
<td>463.70.4 (0.2 × 10^5–9.1 × 10^9)</td>
<td>0.042*</td>
</tr>
<tr>
<td>AXIN1</td>
<td>45.1 (1.7–1.187)</td>
<td>0.011*</td>
</tr>
<tr>
<td>LRP5</td>
<td>64.4 (0.6–6697)</td>
<td>0.039*</td>
</tr>
<tr>
<td>Nfatc3</td>
<td>0.3 (0.1–0.8)</td>
<td>0.495</td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th>Genes</th>
<th>Univariate PFS analysis (expression)</th>
<th>Multivariate PFS analysis (expression)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR 95% CI</td>
<td>P (2-sided)</td>
</tr>
<tr>
<td>FZD4</td>
<td>0.8 (0.7–0.9)</td>
<td>0.003**</td>
</tr>
<tr>
<td>NKD1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DVL1</td>
<td>0.8 (0.6–1.0)</td>
<td>0.038*</td>
</tr>
<tr>
<td>ROCK1</td>
<td>0.7 (0.5–1.0)</td>
<td>0.04*</td>
</tr>
<tr>
<td>AXIN1</td>
<td>1 (0.7–1.3)</td>
<td>0.833</td>
</tr>
<tr>
<td>LRP5</td>
<td>0.9 (0.7–1.0)</td>
<td>0.115</td>
</tr>
<tr>
<td>Nfatc3</td>
<td>1 (0.8–1.3)</td>
<td>0.887</td>
</tr>
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</table>

- **HR per unit increase in expression estimated from Cox proportional hazard regression model.**
- **CI of the estimated HR.**
- **P value of score test.**
- **Multiple test correction based on 6 loci.**
- **P value adjusted by stage, grade, and age. Age was used as a continuous variable; stage and grade were used as categorical variables.**
- **FDR ≤ 0.1; 95% FDR ≤ 0.01; *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.**

in the tumor tissue, that is, differential methylation of those biomarkers only exist in a subset of tumor (or normal infiltrating) cells, therefore, the changes of expression of those genes in a subpopulation are masked by the general expression levels in the entire tumor. A further limitation of the study is the low average power of the analysis in the validation set (see Materials and Methods). However, 11 of 20 independent biomarkers identified in the first screening set have been validated in the second patient cohort. Nevertheless, those loci that have not been validated need further investigation in a larger sample set.

Although multiple types of prognostic biomarkers show promise in ovarian cancer, such as serum CA125 and HE4 (15), plasma lysophosphatidic acid (58), mRNA expression profiling (59, 60) and microRNAs (61), there are a number of advantages of using DNA methylation as a clinical biomarker (62–65). Compared with protein-based biomarkers, DNA methylation is amplifiable and can be detected easily by PCR-based methods. Contrary to gene mutation, cancer-specific hypermethylation generally occurs in a defined region usually in or near promoter region of the genes. In addition, the advantages of DNA methylation over gene or microRNA expression are (i) it is fairly stable in vivo (heritable in cell division and do not fluctuate in response to short-term stimuli) and ex vivo (can survive routine processing for histopathology), (ii) DNA methylation is readily detected in tumor DNA from body fluids, such as plasma, and (iii) it is less influenced by normal cell contamination.

In conclusion, we have shown that methylation of multiple promoter CGIs in the Wnt pathway is frequently observed in EOC and identified methylation of key loci as significantly associated with PFS (CGIs at FZD4, DVL1, NKD1, ROCK1, AXIN1, LRP5, and Nfatc3) that are independent from clinical parameters. We have used these data to construct a multivariate Cox model that incorporates 2 independent CGIs at NKD1 and DVL1, which can identify 2 groups of patients with distinct PFS. Methylation changes at the Wnt pathway will be relevant for patient stratification in future clinical trials of ovarian cancer, particularly for novel drugs targeting the Wnt pathway (66).
Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

Acknowledgment

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References


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Systematic CpG Islands Methylation Profiling of Genes in the Wnt Pathway in Epithelial Ovarian Cancer Identifies Biomarkers of Progression-Free Survival


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