**Genetically predicted DNA methylation biomarkers and** **epithelial ovarian cancer risk: data from nearly 63,000 women of European descent**

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**Abstract**

DNA methylation is instrumental to gene regulation. Global changes in the epigenetic landscape have been recognized as a hallmark of cancer. However, the role of DNA methylation in epithelial ovarian cancer is not fully established. High density geneticand DNA methylation data in white blood cells from the Framingham Heart Study (*N*=1,595) were used to build genetic models to predict DNA methylation levels. The prediction models were then applied to the summary statistics of a genome-wide association study (GWAS) of ovarian cancer including 22,406 EOC cases and 40,941 controls to investigate genetically predicted DNA methylation levels in association with EOC risk. Among 62,938 CpG sites (CpGs) investigated, genetically predicted methylation levels at 89 CpGs were significantly associated with EOC risk at a Bonferroni-corrected threshold of *P*<7.94×10-7. Of them, 87 are located at GWAS-identified EOC susceptibility regions and two reside in a genomic region not previously reported to be associated with EOC risk. Integrative analyses of genetic, methylation and gene expression data identified consistent directions of associations across 12 CpGs, five genes and EOC risk, suggesting that methylation at these 12 CpGs may influence EOC risk through regulating expression of these five genes, namely *MAPT*, *HOXB3*, *ABHD8*, *ARHGAP27* and *SKAP1*. We identified novel DNA methylation markers associated with EOC risk and propose that methylation at multiple CpGs may affect EOC risk through regulating gene expression.

**Introduction**

Ovarian cancer is one of the most deadly cancers among women in the United States (1) and around the world (2). Approximately 90% of ovarian neoplasms are epithelial ovarian cancer (EOC) (1), a heterogeneous disease that can be categorized into five major histotypes (1). Genetic factors have an important impact on EOC etiology and large-scale genome-wide association studies (GWAS) have identified 34 common risk loci for EOC to date (3). Of these, 27 are specific to the most common histotype, serous EOC (3). Yet known loci are estimated to account for only a small proportion (~6.4%) of EOC risk (3). Further, causal genes at most loci and the underlying pathogenic mechanisms remain to be identified.

In addition to genetic susceptibility, cancer initiation and progression are also influenced by epigenetics (4). The most extensively studied epigenetic marker is DNA methylation, which regulates chromatin structure (5) and gene expression (6). DNA methylation patterns are generally programmed during normal development (7) and abnormal methylation has been observed in multiple malignancies including EOC (8,9). Studies have identified multiple DNA methylation markers in tumor tissue samples as prognostic biomarker for EOC (10,11). Several studies have also investigated the potential of DNA methylation from white blood cells as early detection biomarkers for EOC and identified nearly 100 candidate CpGs for EOC risk (12-15). To date, only two CpGs, cg10061138 and cg10636246, were consistently observed across different studies (12-15). The lack of consistent findings may reflect the small sample sizes of prior works (200-400 cases), inadequate consideration of potential confounders and reverse causation.

DNA methylation is impacted by both environmental factors and genetic factors (6). High-throughput methylome profiling in both twin and familial studies has shown that methylation levels for a large number of CpGs are heritable (16,17). Furthermore, several studies (18,19) have revealed a large number of methylation quantitative trait loci (meQTL) in white blood cells. These results suggest that DNA methylation levels could be partially predicted by genetic variants. Indeed, meQTL single nucleotide polymorphisms (SNPs) appear to predict DNA methylation levels in white blood cells and the predicted methylation levels associate with disease risk (20,21). However, these studies only used single meQTL SNPs to predict methylation levels for each CpG site. The prediction accuracy is low because meQTL SNPs explain only a small proportion of variance. In the present study, we used a novel approach to overcome this limitation by building and validating statistical models to predict methylation levels based on multiple genetic variants in reference datasets. The prediction models were then applied to genetic data on 22,406 cases and 40,941 controls to test the hypothesis that genetically predicted DNA methylation associates with EOC risk. This approach could overcome the selection bias and reverse causations in conventional epidemiological studies of DNA methylation and disease because alleles are randomly assigned during gamete formation.

**Methods**

**Building DNA methylation prediction models using data from the Framingham Heart Study (FHS)**

Genome-wide DNA methylation and genotype data on white blood cell samples from individuals in the FHS Offspring Cohort were obtained from dbGaP (accession numbers phs000724 and phs000342, respectively). Detailed descriptions of the FHS Offspring Cohort have been previously reported (22). Genotyping was conducted using the Affymetrix 500K mapping array and imputation was performed with the1000 Genome Phase I (version 3) data as reference. Only SNPs with a minor allele frequency (MAF) of ≥0.05 and an imputation quality (R2) of ≥0.80 were used to build prediction models. Genome-wide DNA methylation profiling was generated using the Illumina HumanMethylation450 BeadChip. We used the R package “minfi” (23) to filter low quality methylation probes, evaluate cell type composition for each sample and estimate methylation beta-values. Methylation data were then quantile-normalized across samples, rank-normalized to remove potential outliers and then regressed on covariates including age, sex, cell type composition and top ten principal components (PCs) to eliminate potential experimental confounders and population structure. Finally, 1,595 unrelated individuals of European descent (883 females and 712 males, mean ± SD of age: 66.3 ± 9.0) with both genetic and DNA methylation data were included in prediction model building.

Using the elastic net method (α=0.50) implemented in the R package “glmnet” (24), we built a statistical model to predict methylation for each CpG site using SNPs within its 2 megabase (Mb) flanking region. For each model, we performed tenfold cross-validation as internal validation and calculated the squared value of the correlation coefficient between measured and predicted methylation levels, i.e. RFHS2, to estimate prediction performance.

**Evaluation of model performance using data from the Women’s Health Initiative (WHI)**

Using data on white blood cell samples from 883 independent healthy women of European descent from the WHI, we evaluated the performance of the established genetic prediction models. Data from the WHI samples were obtained from dbGaP (accession numbers phs001335, phs000675 and phs000315). Genotyping was conducted using the HumanOmniExpress and HumanOmni1-Quad array. The data were quality control (QC)-ed and imputed using similar criteria and procedures as those described for the FHS data. The Illumina HumanMethylation450 BeadChip was used to profile DNA methylation and data were then processed using the same pipeline as that for the FHS data. The prediction models established in FHS were applied to the genetic data in WHI to predict methylation levels at each CpG site for each sample. Then the predicted and measured methylation levels for each CpG site were compared by estimating the squared value of the Spearman correlation coefficient, i.e. RWHI2.

We used the following criteria to select prediction models for association analyses: 1) a prediction RFHS2 of ≥0.01 (correlation between measured and predicted methylation level of ≥0.10) in the FHS; 2) RWHI2 of ≥0.01 in the WHI; and 3) methylation probes on the HumanMethylation450K BeadChip not overlapping with any SNP included in the dbSNP database (25) (Build 151), considering that SNPs on the probes may have a potential impact on the methylation level estimation (19). In total, models for 63,000 CpGs met these requirements and were included in the downstream association analyses for EOC risk.

**Association between genetically predicted DNA methylation and EOC risk**

MetaXcan (26) was used to estimate the associations between genetically predicted methylation levels and EOC risk. The methodology of MetaXcan has been described elsewhere (26,27). Briefly, the following formula was used to evaluate the association Z-score:

$Z\_{m}≈ \sum\_{s\in Model\_{m}}^{}w\_{sm}\frac{\hat{σ}\_{s}}{\hat{σ}\_{m}} \frac{\hat{β}\_{s}}{se(\hat{β}\_{s})} $

In the formula, $w\_{sm}$ represents the weight of SNP $s$ on the methylation level of the CpG site $m$, estimated by the prediction model.$ \hat{σ}\_{s} $and $\hat{σ}\_{m}$ are the evaluated variances of SNP $s $and the predicted methylation level at CpG site$ m$, respectively. $\hat{β}\_{s}$ and $se(\hat{β}\_{s})$ represent the beta coefficient and standard error of SNP $s$ on EOC risk, respectively. For this study, the correlations between predicting SNPs for all CpGs were evaluated using the data from European participants in the 1000 Genomes Project Phase 3.

Beta coefficient $\hat{β}\_{s}$ and standard error $se(\hat{β}\_{s})$ for association between SNP s and EOC risk were obtained from the Ovarian Cancer Association Consortium (OCAC), which includes 22,406 EOC cases and 40,941 controls of European ancestry (3). Details of this consortium have been described elsewhere (3). For EOC patients, some may have had neo-adj chemotherapy before surgery. They were not included in sub-type analyses but included in the analyses for overall EOC risk (3). Cases were classified as one of five histotypes: high-grade serous (*N=*13,037), endometrioid (*n=*2,810), mucinous invasive (*N=*1,417), clear cell (*N=*1,366) or low-grade serous (*N=*1,012). In addition, there were 2,764 EOC cases that could not be categorized into any histotypes. Genotyping was conducted using OncoArray and other GWAS arrays, followed by imputation with the 1000 Genomes Project Phase 3 as reference. Association analyses were conducted within each dataset (different GWAS arrays) and the results were combined by a fixed-effect inverse-variance meta-analysis. Among the 751,157 SNPs included in the prediction models for 63,000 CpGs, summary statistics for associations between 751,031 (99.98%) SNPs and EOC risk were available from the OCAC. A total of 62,938 CpGs, corresponding to these 751,031 SNPs, were included in the final analyses. This study was approved by the OCAC Data Access Coordination Committee.

For risk analyses in OCAC, we used a Bonferroni-corrected threshold of *P*<7.94×10-7 (0.05/62,938) as a threshold for statistical significance in assessing the association between each of 62,938 CpGs and EOC risk. Associations of predicted methylation and EOC risk identified in the OCAC data were further evaluated using the summary statistics of two GWAS studies of ovarian cancer in the UK Biobank (28). However, the sample size of EOC cases is very small, with only 440 histologically diagnosed and 579 self-reported ovarian cancer cases among nearly 337,000 unrelated individuals of European descent. GWAS analyses were conducted using a linear regression model and the summary statistics data are available at <https://sites.google.com/broadinstitute.org/ukbbgwasresults/home>.

To estimate whether the identified associations of predicted methylation with EOC risk were independent of GWAS-identified EOC susceptibility variants, for each SNP included in the prediction model, we used GCTA-COJO (29) to evaluate the $\hat{β}\_{s}$ and $se(\hat{β}\_{s})$ with EOC risk after adjusting for the GWAS-identified variants for EOC. Then we re-conducted the MetaXcan analyses to investigate the associations of predicted methylation levels with EOC risk conditioning on the GWAS-identified EOC risk variants. We also performed stratification analyses by six EOC histotypes and estimated the heterogeneity across histotype groups by using Cochran’s Q test.

**Functional annotation of methylation markers**

Using ANNOVAR (30), all 62,938 investigated CpGs were classified into 11 functional categories: upstream, transcription start site upstream 1500bp (TSS1500), TSS200, 5’-untranslated region (UTR), exonic, intronic, 3’-UTR, downstream, intergenic, non-coding RNA (ncRNA) exonic and ncRNA intronic.

**Correlation analyses of DNA methylation with gene expression in white blood cells**

For those 89 CpGs with predicted methylation levels associated with EOC risk, we investigated methylation levels at CpGs in relation to expression levels of genes flanking these CpGs. Individual-level DNA methylation and gene expression data of white blood cell samples from the FHS Offspring Cohort were accessed from dbGaP (accession numbers phs000724 and phs000363). The detailed descriptions of the Offspring Cohort of the FHS, the DNA methylation data and gene expression data have been described previously (22,31). Totally, 1,367 unrelated participants with both methylation and gene expression data were included in correlation analyses. A threshold of *P*<0.05 was used to determine a nominally significant correlation between methylation level and gene expression level. In addition, we investigated whether methylation of those 89 EOC-associated-CpGs could regulate the expression of 19 homologous recombination (HR) genes (32,33) using data from the FHS.

**Association analyses of genetically predicted gene expression with EOC risk**

For genes with expression levels nominally correlated with methylation levels at CpGs that were associated with EOC, we further investigated whether genetically predicted gene expression levels were associated with EOC risk following methods described elsewhere (27). Briefly, genome-wide genetic and gene expression data on 6,124 different tissue samples donated by 369 participants of European ancestry included in the Genotype-Tissue Expression (GTEx) release 6 (34) were used to build genetic models for gene expression prediction by following the elastic net method (27). The models were then applied to the OCAC data to estimate the associations between genetically predicted gene expression levels and EOC risk by using MetaXcan (26). We used Bonferroni correction to declare statistically significant associations.

**Consistent directions of associations across methylation, gene expression and EOC risk**

To infer potential mechanisms underlying the identified associations between DNA methylation and EOC risk, we conducted an integrative analysis of the association results between predicted CpG methylation and EOC risk, correlations between CpG methylation and gene expression, and associations between gene expression and EOC risk. First, we examined whether the association directions among DNA methylation, gene expression and EOC risk were consistent. Then, we evaluated whether genetically predicted methylation might mediate associations between gene expression and EOC risk. Briefly, for each gene we used GCTA-COJO (35) to generate modified summary statistics of associations between SNPs in its expression prediction models and EOC risk after adjusting for SNPs included in the methylation prediction model of its corresponding CpG site. Finally, the prediction model of this gene was applied to the updated summary statistics using MetaXcan (26) to estimate the association between genetically predicted gene expression and EOC risk conditioning on the effects of the genetically predicted methylation level at corresponding CpG site.

**Results**

**DNA methylation prediction models**

**Figure 1** presents the overall workflow of this study. Data from the FHS Offspring Cohort were used to create methylation prediction models for 223,959 CpGs. Of these, 81,361 showed a prediction performance (RFHS2) of ≥0.01, representing at least 10% correlation between predicted and measured methylation levels. For these 81,361 CpGs, the numbers of SNPs in prediction models range from 1 to 276, with a median of 25. Applying these 81,361 models to genetic data from the WHI, 70,269 (86.4%) models showed a correlation coefficient between predicted and measured methylation levels (RWHI) of >10%. Among these 70,269 CpGs, methylation probes of 7,269 CpGs on the HumanMethylation450 BeadChip overlapped with SNPs, which may have affected the estimation of methylation levels (19), hence these CpGs were excluded. The remaining 63,000 CpGs were included in the downstream analyses.

**Associations of genetically predicted DNA methylation with EOC risk**

The prediction models were applied to the data from a GWAS of 22,406 EOC cases and 40,941 controls included in OCAC. Of 751,157 SNPs included in prediction models for the 63,000 CpGs, summary statistics of associations between 751,031 SNPs, corresponding to 62,938 CpGs, and EOC risk were available in OCAC. For these 62,938 CpGs, a high correlation of prediction performance between models based on FHS (RFHS2) and WHI (RWHI2) data was observed with a Pearson correlation coefficient of 0.95, indicating that for each of these CpGs, a same set of predicting SNPs could predict a very similar methylation level using either FHS or WHI data.

For most of these 62,938 CpGs, a large majority of predicting SNPs were available in OCAC (e.g., for 94% of the investigated CpGs, ≥95% of the SNPs in prediction models were available in OCAC). **Supplementary Figure 1** is the Manhattan plot presenting the associations between genetically predicted methylation levels and EOC risk. Among 62,938 CpGs investigated, 89 were significantly associated with EOC risk at a Bonferroni-corrected threshold of *P*<7.94×10-7 (**Table 1 and 2, Supplementary Table 1**). Among these 89 CpGs, a higher predicted methylation level was associated with an increased risk of EOC at 48 CpGs, and with a decreased EOC risk at the other 41 CpGs. These indicates that the methylation levels were predicted to be higher for 48 CpGs and lower for 41 CpGs among EOC cases than among controls. For these 89 CpGs, we also re-built the prediction models only using data of females (*N*=833) in FHS. A very high correlation was observed with a Pearson correlation coefficient of 0.99 between the prediction performance R2 values based on data of all FHS participants (*N*=1,595) and those based on data of only females (*N*=833). In the UK Biobank data, consistent associations were observed for 23 CpGs, including 12 at *P*<0.05 and 11 additional CpGs at *P*<0.10 (**Supplementary Table 2**). This relatively low replication rate is not unexpected considering the very limited statistical power of the UK Biobank data because of a very small number of cases (400~600 cases).

Among these 89 CpGs that were associated with EOC, two reside in a genomic region on chromosome 7 that has not yet been reported for EOC risk (500Kb away from any GWAS-identified EOC susceptibility variants) (**Table 1**). Given that there are no risk variants identified by previous GWAS on this chromosome, associations with EOC risk conditioning on proximally located risk variants could not be conducted. Among the remaining 87 CpGs located in nine previously identified EOC risk loci, no associations remained significant after an adjustment for all risk SNPs in the corresponding loci, suggesting that the associations of these 87 CpGs with EOC risk were all driven by known EOC risk SNPs in these loci (**Table 2** and **Supplementary Table 1**).

Stratification analyses by EOC histotypes revealed that all 89 CpGs were associated with both serous ovarian cancer and high-grade serous ovarian cancer, and fewer CpGs were associated with the other histotypes, including endometrioid ovarian cancer (cg25137403, cg14454907 and cg25708328), mucinous ovarian cancer (cg25137403, cg14454907, cg10086659 and cg25708328) and low-grade serous ovarian cancer (cg01572694) (**Supplementary Tables 3-4**). Fourteen of these 89 CpGs showed more significant associations with the serous and the high-grade serous ovarian cancers than with other histotypes, with a heterogeneity test *P*<5.62×10-4, a Bonferroni-corrected threshold (0.05/89) (**Supplementary Table 3**). Among these 89 CpGs, a significant correlation of methylation and gene expression was identified for 91 CpG-HR gene pairs, including 22 CpGs and 11 HR genes, at a Bonferroni-corrected threshold of *P*<2.96×10-5 (0.05/1,691) (**Supplementary Table 5**). Interestingly, methylation levels of three CpGs, i.e. cg13568213 (*9q34.2*), cg10900703 (*10p12.31*) and cg23659289 (*17q21.31*) showed a strong correlation with the expression level of the *ATM* gene,

**DNA methylation affecting EOC risk through regulating expression of neighbor gene**

For those 89 CpGs with predicted methylation levels associated with EOC risk, correlation analyses with gene expression were conducted for 63 pairs of CpG-gene, including 58 CpGs with 21 flanking genes that were annotated by ANNOVAR (30). Nominally significant correlations were observed for 26 CpG-gene pairs, including 26 CpGs and 12 genes, at *P*<0.05 (**Table 3, Supplementary Table 6**). Among them, the most significant correlation was observed between the increased methylation at the CpG cg19139618, located in the promoter region of the *SKAP1* gene, and the expression level of *SKAP1* with a *P* value of 2.98×10-15 (**Table 3**). In addition, increased methylation levels at two CpGs, cg10900703 and cg04231319 located in the introns of the *MLLT10* gene were significantly correlated with an increased expression of *MLLT10*, with *P* values of 2.79×10-11 and 1.36×10-5, respectively. For the two CpGs located in a putative novel locus, a higher methylation level for one of them, cg03634833, was correlated with a lower expression of the *ADAP1* gene in this locus with a *P* value of 2.99×10-3 (**Supplementary Table 6**). As expected, methylation levels at CpGs located at promoter regions (TSS1500 and TSS200) were more likely to be negatively correlated with expression of proximal genes. Nearly all CpGs located in downstream or 3’UTR showed a negative regulatory effect on expression of neighbor genes. For CpGs residing in intronic regions, both positive and negative correlations were observed.

For the 12 genes with expression levels correlated with DNA methylation, expression prediction models were built for seven genes with a prediction performance (R2) of ≥0.01 using GTEx data. Applying these seven models to the OCAC data, genetically predicted expression levels of three genes, namely *MAPT*, *HOXB3* and *ABHD8*, were significantly associated with EOC risk after Bonferroni correction (**Table 4**). At *17q21.31* and *17q21.32*, higher predicted expression levels of *MAPT* and *HOXB3* were associated with a decreased EOC risk with *P* values of 3.74×10-4 and 2.00×10-7, respectively. After adjusting for established EOC risk SNPs, the associations between these two genes and EOC risk disappeared. At *19p13.11*, an increased predicted expression level of *ABHD8* was associated with an increased EOC risk with a *P* value of 9.93×10-6. Conditioning on the EOC risk SNP in this locus, the association disappeared as well (**Table 4**). Of the five genes without prediction models, two were previously reported to be associated with EOC susceptibility, including *SKAP1* (36) and *ARHGAP27* (37).

We integrated the results of the association between DNA methylation and EOC risk, the correlation between DNA methylation and gene expression, and the association between gene expression and EOC risk. We identified consistent directions of associations across seven CpGs, including cg18878992, cg00480298, cg07368061, cg01572694, cg14285150, cg24672833 and cg17941109, three genes, including *MAPT*, *HOXB3* and *ABHD8*, and EOC risk (**Table 5**). The mechanism potentially underlying the associations of methylation at these seven CpGs and EOC risk may be their regulatory function on expression of these three genes. Among them, increased methylation at the CpG site cg14285150 was associated with an increased *HOXB3* expression (*P*=8.44×10-5) and decreased EOC risk (*P*=5.53×10-8). As expected, an increased expression of *HOXB3* was associated with a decreased EOC risk (*P*=2.00×10-7). Conditioning on SNPs included in the methylation prediction model for cg14285150, the association of *HOXB3* expression and EOC risk disappeared (*P*=0.51) (**Table 5**).

Expression prediction models could not be built for *SKAP1* at *17q21.32* and *ARHGAP27* at *17q21.31* in the present study, hence these two genes could not be investigated in association with EOC risk. However, higher expression levels of these two geneshave been previously reported to be associated with an increased risk of EOC (36,37). This is expected based on the association results of DNA methylation with EOC risk and DNA methylation with gene expression (**Table 5**). For example, a higher methylation at cg19139618 was associated with a lower expression of *SKAP1* (*P*=2.98×10-15) and lower EOC risk (*P*=7.08×10-7). Hence, the potential mechanism underlying the association between cg19139618 and EOC risk may be the down-regulation effects on *SKAP1* expression (**Table 5**).

**Discussion**

In this large study, we identified 89 CpGs that were significantly associated with EOC risk, including two CpGs located in a novel genomic region that have not yet been reported as a susceptibility locus for EOC. Integrating genetic, methylation and gene expression data suggested that methylation at 12 of 89 CpGs may exert their impacts on EOC risk through regulating expression of five genes. These results provide new insights into the regulatory pathways that connect genetics, epigenetics, gene expression and EOC risk.

We identified two methylation markers, cg18139273 and cg03634833, located at *7p22.3*, a novel genomic region that had not been reported as a risk locus for EOC. Both CpGs reside in the 3rd intron of the 1st transcript of the *ADAP1* gene, which encodes an ADP-ribosylation factor GTPase-activating protein (ArfGAP) with dual PH domains 1. ADAP1 functions as a scaffolding protein in several signal transduction pathways and is highly expressed in neurons, where it has roles in neuronal differentiation and neurodegeneration (38). This gene has also been reported to be involved in mitochondrial function (39) and is a target of the ErbB4 transcription factor in mammary epithelial cells (40). In the present study, we found that a higher methylation level at cg03634833 was significantly correlated with a lower *ADAP1* expression, which was associated with a non-significantly decreased EOC risk. Thus, methylation at cg03634833 might be associated with EOC risk through a regulatory function on *ADAP1* expression, or through other unidentified mechanisms.

Integrating results of association between DNA methylation and EOC risk, correlation between DNA methylation and gene expression, and association between gene expression and EOC risk, we observed consistent directions of associations across 12 CpGs, five genes and EOC risk. For the *MAPT* gene (*17q21.31*), increased methylation at two CpGs located in its exons, including cg18878992 and cg00480298, were associated with a decreased *MAPT* expression and increased EOC risk. For the other CpG site cg07368061, located at the 1st intron of *MAPT*, increased methylation was associated with a higher *MAPT* expression and lower EOC risk. As expected, an increased *MAPT* expression was associated with decreased EOC risk. The *MAPT* gene has been linked to multiple neurodegenerative disorders, including progressive supranuclear palsy (41), Parkinson’s disease (42,43) and Alzheimer's disease (42). In addition, a higher expression of a MAPT protein isoform (<70 kDa) was correlated with lower sensitivity to taxanes in breast cancer cells (44). Methylation of the microRNA (miRNA) *miR-34c-5p* was shown to regulate the *MAPT* expression, which was related to paclitaxel-resistance in gastric cancer cells (45).

Increased methylation of three CpGs in the 1st intron of the *HOXB3* gene (*17q21.32*), including cg01572694, cg14285150 and cg24672833, were associated with an increased expression of *HOXB3* and decreased EOC risk. As expected, an increased *HOXB3* expression was associated with decreased EOC risk. However, a previous study reported that the expression of *HOXB3* was up-regulated in EOC cell lines compared with normal samples (46). Nevertheless, this study only included five patients and the results have not been replicated by an independent study. On the other side, we investigated the genetically predicted methylation level in DNA from white blood cells, not in ovary or fallopian tube epithelial cells. It is possible that the correlation between methylation levels of these CpGs and *HOXB3* expression are different in ovary epithelial cells and white blood cells. For example, in the 5’UTR of *HOXB3*, higher methylation at the CpG cg12910797, was significantly associated with an increased EOC risk. While the increased methylation of this CpG was not correlated with the expression of *HOXB3* in white blood cells samples from the FHS (Spearman correlation coefficient r=-0.02; *P*=0.43). Higher methylation of this CpG was significantly correlated with a decreased *HOXB3* expression in ovarian serous cystadenocarcinoma samples from the Cancer Genome Atlas (TCGA) (Spearman correlation coefficient r=-0.27; *P*=**2.01×10-6**) (<http://gdac.broadinstitute.org/runs/analyses__2016_01_28/reports/cancer/OV-TP/Correlate_Methylation_vs_mRNA/nozzle.html>).

The higher methylation of the CpG site cg17941109, located at the 2nd intron of the *ABHD8* gene, was associated with a lower *ABHD8* expression and a lower EOC risk. This is consistent with the results of two recent studies showing that a higher expression level of this gene was associated with an increased risk of EOC (47,48). This gene is located at *19p13.11*, a susceptibility locus for both ovarian and breast cancers. Interestingly, in our unpublished data, the increased genetically predicted methylation level at cg17941109 was associated with decreased breast cancer risk and the genetically predicted expression of *ABHD8* was associated with an increased breast cancer risk. Increasing evidence also suggests that this protein family (ABHD) has a physiological significance in metabolism and disease (49).

For the *ARHGAP27* gene, increased methylation of two CpGs in the promoter region, cg16281322 and cg25708777, and one CpG in the 3’-UTR, cg07067577, were associated with lower expression level of *ARHGAP27* and lower EOC risk. For the *SKAP1* gene, a higher methylation at the CpG cg02957270, located at the promoter region, was associated with a higher expression level and increased EOC risk. Increased methylation of the other intronic CpG cg19139618 was associated with a lower *SKAP1* expression and a decreased EOC risk. In the present study, the associations of expression levels of these two genes and EOC risk could not be investigated because the prediction models for them could not be built. However, two large GWAS studies have identified these two genes as EOC susceptibility genes with solid experimental evidence (36,37). Differential expression analyses showed significantly higher expression of *ARHGAP27* in ovarian cancer than in normal cells (37). It is suggested that the *ARHGAP27* gene mayplay a role in carcinogenesis through the dysregulation of Rho/Rac/Cdc42-like GTPases (50). The expression of *SKAP1* was significantly greater in ovarian cancer cells when compared to primary human ovarian surface epithelial cells (36)*.* Our study is the first to suggest that these two genes may be associated with EOC risk through methylation regulation.

Several epidemiological studies have investigated the associations of CpG methylation and EOC risk in white blood cells and tumor tissue samples (12-15). Approximately 100 CpGs have been identified to be associated with EOC risk. However, only two CpGs, cg10061138 and cg10636246, showed consistent association directions in two or more studies. In the present study, the prediction models could not be built for these two CpGs; hence neither could be investigated in association with EOC risk. Among the remaining 98 reported CpGs, reliable prediction models were only built for 20 of them and only two, cg19399532 and cg21870884, could be replicated at *P*<0.10 with the same association directions as previously reported. Such a low replication rate is not unexpected because of several potential limitations in traditional epidemiological studies, including possible false associations because of small sample size, lack of validation in other studies, potential confounders and reverse causation.

The methodology of this study is similar with that of transcriptome-wide association studies (TWAS), in which gene expression prediction models were established and applied to GWAS data to investigate genetically predicted gene expression in association with various diseases and traits. Of the five genes identified in the present study, expression levels of two genes, ***HOXB3* and *ABHD8*, were significantly associated with EOC risk at the Bonferroni-corrected threshold (P<2.2×10-6) in our previous TWAS study for EOC** (51)**.** The *MAPT* gene showed an association with EOC at *P*=3.74×10-4 in the TWAS, however the association didn’t reach the Bonferroni-corrected threshold. For *ARHGAP27* and *SKAP1*, gene expression prediction model could not be built, and they were not investigated in the TWAS. Expression levels of these two genes were reported to be associated with EOC (36,37)**.** Some genes identified in TWAS were not tested in the present study because the methylation prediction model could not be built for CpGs flanking them. In addition, except DNA methylation, there are other biological processes that regulate gene expression. Further, the regulation of DNA methylation on gene expression differs according to the locations of the CpGs. Therefore, integrating the results of methylation and gene expression analyses may help to understand the biological basis of EOC.

It would be ideal to build methylation prediction models using data from normal ovary or fallopian tube epithelial cells. However, it is almost impossible to collect tissue samples from a large population of healthy women. However, as demonstrated by multiple studies, the large majority of the meQTLs identified in white blood cells were consistently detected across different tissue types (26,52,53). These results indicate that the genetically determined methylation at many CpGs are predictable and consistent among different tissues. Hence, it is reasonable to build methylation prediction models using data from white blood cell samples and then investigate predicted DNA methylation in association with EOC. It will be promising to validate the findings in the present study by directly measuring methylation levels in pre-diagnosis blood samples in prospective studies to overcome reverse causation; however, the majority of the samples included in the present study were collected after cancer diagnosis. It is possible that DNA methylation regulation on gene expression differs across tissues. In the present study, data in white blood cell samples were used, which is another limitation. In the association analysis of predicted gene expression with EOC risk, the models were built using data from a limited sample size of GTEx, thus the number of genes evaluated in our study was small. More consistent associations across methylation, gene expression and EOC risk could be identified with a larger sample size to build gene expression prediction models.

Strengths of this study include the large number of samples in the reference dataset used in model building and that the model performance was evaluated in an independent dataset. Using genetic variants as study instruments, we can effectively overcome many limitations commonly encountered in conventional epidemiologic studies. In addition, this is the largest study of DNA methylation with EOC risk and a very stringent criterion was used, providing high statistical power to identify reliable associations between genetically predicted methylation and EOC risk. Finally, the integrative analyses of genetic, DNA methylation and gene expression data led to identification of consistent evidence to support the hypothesis that DNA methylation could impact EOC risk through regulating gene expression.

In summary, in the largest study conducted to date investigating DNA methylation in association with EOC risk to date, we identified multiple CpGs that were significantly associated with EOC risk and proposed that several CpGs may affect EOC risk through regulating expression of five genes. Our study demonstrates the feasibility of integrating multi-omics data to identify novel biomarkers for EOC risk and brings new insights into the etiology of this malignancy.

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**Tables**

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| **Table 1. Two novel methylation-EOC associations for two CpGs located at a genomic region not yet reported for EOC risk** |
| CpG | Chr | Position | Closest gene | Classification | RFHS2 a | Histotype | Z score | OR (95% CI) b | P value |
| cg18139273 | 7 | 962,582 | *ADAP1* | Intronic | 0.01 | Overall | -4.95 | 0.51 (0.39-0.66) | 7.25×10-7 |
| Serous c | -4.87 | 0.46 (0.34-0.63) | 1.13×10-6 |
| High-grade serous | -4.83 | 0.46 (0.33-0.63) | 1.39×10-6 |
| Endometrioid  | -1.78 | 0.59 (0.33-1.06) | 0.08 |
| Mucinous | -0.99 | 0.67 (0.30-1.49) | 0.32 |
| Clear cell | -1.87 | 0.46 (0.21-1.04) | 0.06 |
| Low-grade serous | -0.97 | 0.62 (0.24-1.63) | 0.33 |
| cg03634833 | 7 | 965,534 | *ADAP1* | Intronic | 0.09 | Overall | -5.00 | 0.84 (0.79-0.90) | 5.81×10-7 |
| Serous c | -4.85 | 0.83 (0.77-0.89) | 1.21×10-6 |
| High-grade serous | -4.85 | 0.82 (0.76-0.89) | 1.25×10-6 |
| Endometrioid  | -2.21 | 0.83 (0.71-0.98) | 0.03 |
| Mucinous | -1.40 | 0.87 (0.71-1.06) | 0.16 |
| Clear cell | -1.76 | 0.84 (0.69-1.02) | 0.08 |
| Low-grade serous | -0.87 | 0.91 (0.73-1.13) | 0.39 |
| a Correlation between predicted and measured methylation levels. b OR, odds ratio per standard deviation increase in genetically predicted methylation level; CI, confidence interval. c Including high-grade serous and low-grade serous ovarian cancers. |

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| **Table 2. Selected a seven methylation-EOC associations driven by previously identified EOC risk SNPs** |
| CpG | Chr | Position | Closest gene | Classification | Z score | OR (95% CI) b | P value | RFHS2 c | EOC risk SNPs  | Distance to the risk SNPs (kb) | P value adjusted for the risk SNPs |
| cg25137403 | 2 | 177,022,172 | *HOXD4*; *HOXD3* | Intergenic | 7.51 | 1.24 (1.18-1.32) | 5.96×10-14 | 0.15 | rs6755777; rs711830 | 21;15 | 0.09 |
| cg26405475 | 3 | 156,324,038 | *SSR3*; *TIPARP-AS1* | Intergenic | -9.45 | 0.69 (0.64-0.74) | 3.42×10-21 | 0.07 | rs62274041 | 111 | 0.34 |
| cg08478672 | 8 | 129,374,295 | *MIR1208*; *LINC00824* | Intergenic | 5.08 | 1.29 (1.17-1.42) | 3.81×10-7 | 0.06 | rs1400482 | 167 | 0.05 |
| cg14653977 | 9 | 136,038,692 | *GBGT1* | Intronic | 5.99 | 1.75 (1.46-2.09) | 2.04×10-9 | 0.03 | 9:136138765 d | 100 | 0.09 |
| cg04231319 | 10 | 21,824,447 | *MLLT10* | Intronic | -5.72 | 0.88 (0.84-0.92) | 1.05×10-8 | 0.19 | rs144962376 | 54 | 0.94 |
| cg07067577 | 17 | 43,506,829 | *ARHGAP27* | 3'UTR | -7.49 | 0.73 (0.67-0.79) | 6.86×10-14 | 0.07 | rs1879586 | 60 | 0.01 |
| cg21956434 | 19 | 17,377,697 | *BABAM1* | TSS1500 | 7.07 | 1.13 (1.09-1.17) | 1.53×10-12 | 0.34 | rs4808075 | 12 | 0.39 |
| a Selected from 87 CpG-EOC associations. For each locus, only the most significantly associated CpG was presented.Complete list of results for all CpG-EOC associations is available in **Supplementary Table 1**. b OR, odds ratio per standard deviation increase in genetically predicted methylation level; CI, confidence interval. c Correlation between predicted and measured methylation levels. d GRCh37 position. |

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| **Table 3. Selected a correlations between methylation levels at 26 CpGs and expression levels of 12 genes, data from the Framingham Heart Study** |
| CpG | Chr | Position | Classification | Closest gene | Rho | P value |
| cg25137403 | 2 | 177,022,172 | Downstream | *HOXD4* | -0.06 | 0.02 |
| cg22211092 | 3 | 156,361,584 | Downstream | *SSR3* | 0.09 | 9.43×10-4 |
| cg03634833 | 7 | 965,534 | Intronic | *ADAP1* | -0.08 | 2.99×10-3 |
| cg14653977 | 9 | 136,038,692 | Intronic | *GBGT1* | -0.06 | 0.02 |
| cg24267699 | 9 | 136,151,359 | TSS1500 | *ABO* | -0.09 | 8.07×10-4 |
| cg10900703 | 10 | 21,824,407 | Intronic | *MLLT10* | 0.18 | 2.79×10-11 |
| cg23659289 | 17 | 43,472,725 | 3'UTR | *ARHGAP27* | -0.19 | 9.89×10-13 |
| cg07368061 | 17 | 44,090,862 | Intronic | *MAPT* | 0.08 | 2.02×10-3 |
| cg19139618 | 17 | 46,504,791 | Intronic | *SKAP1* | -0.21 | 2.98×10-15 |
| cg14285150 | 17 | 46,659,019 | Intronic | *HOXB3* | 0.11 | 8.44×10-5 |
| cg22311200 | 17 | 46,695,514 | Downstream | *HOXB8* | 0.08 | 2.59×10-3 |
| cg17941109 | 19 | 17,407,198 | Intronic | *ABHD8* | -0.06 | 0.03 |
| a Selected from correlations between 26 CpGs and 12 genes. For each gene, only the most significantly correlated CpG was presented. Complete list of results for all CpG-EOC associations is available in **Supplementary Table 6**.  |

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| **Table 4. Three genes with genetically predicted expression levels associated with EOC risk** |
| Region | Gene | Type | Z score | P value | Adjusted a P value | R2 b |
| *17q21.31* | *MAPT* | Protein | -3.56 | 3.74×10-4 | 0.40 | 0.08 |
| *17q21.32* | *HOXB3* | Protein | -5.20 | 2.00×10-7 | 0.71 | 0.12 |
| *19p13.11* | *ABHD8* | Protein | 4.42 | 9.93×10-6 | 0.59 | 0.23 |
| a Adjusting for the EOC risk SNPs in the corresponding locus. b Correlation between predicted and measured gene expression levels.  |

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| **Table 5. Consistent directions of associations across CpG methylation, gene expression and EOC risk for 12 CpGs and five genes** |
| CpG | Chr | Position | Gene | Classification | CpG Vs. EOC risk | CpG Vs. Gex b | Gex b Vs. EOC risk | Adjuested a Gex Vs. EOC risk  |
| Dir b | P value | Dir b | P value | Dir b | P value | Dir | P value |
| cg18878992 | 17 | 43,974,344 | *MAPT* | 5'UTR | + | 8.85×10-13 | - | 2.64×10-3 | - | 3.74×10-4 | - | 0.48 |
| cg00480298 | 17 | 44,068,857 | *MAPT* | Exonic | + | 6.39×10-9 | - | 3.98×10-3 | - | 3.74×10-4 | - | 0.65 |
| cg07368061 | 17 | 44,090,862 | *MAPT* | Intronic | - | 4.26×10-13 | + | 2.02×10-3 | - | 3.74×10-4 | - | 1.00 |
| cg01572694 | 17 | 46,657,555 | *HOXB3* | Intronic | - | 5.52×10-9 | + | 7.49×10-3 | - | 2.00×10-7 | - | 0.82 |
| cg14285150 | 17 | 46,659,019 | *HOXB3* | Intronic | - | 5.53×10-8 | + | 8.44×10-5 | - | 2.00×10-7 | - | 0.51 |
| cg24672833 | 17 | 46,659,318 | *HOXB3* | Intronic | - | 9.00×10-8 | + | 5.51×10-3 | - | 2.00×10-7 | - | 0.41 |
| cg17941109 | 19 | 17,407,198 | *ABHD8* | Intronic | - | 2.88×10-9 | - | 0.03 | + | 9.93×10-6 | - | 0.57 |
| cg19139618 | 17 | 46,504,791 | *SKAP1* | Intronic | - | 7.08×10-7 | - | 2.98×10-15 | + | NA c |
| cg02957270 | 17 | 46,508,097 | *SKAP1* | TSS1500 | + | 4.40×10-12 | + | 0.01 | + |
| cg07067577 | 17 | 43,506,829 | *ARHGAP27* | 3'UTR | - | 6.86×10-14 | - | 1.20×10-3 | + | NA c |
| cg16281322 | 17 | 43,510,478 | *ARHGAP27* | TSS200 | - | 6.82×10-13 | - | 1.14×10-9 | + |
| cg25708777 | 17 | 43,510,841 | *ARHGAP27* | TSS1500 | - | 4.61×10-13 | - | 4.11×10-8 | + |
| a Adjusting for all the predicting SNPs included in prediction models of corresponding CpGs b Dir, direction of association/correlation; Gex, gene expression c *SKAP1* and *ARHGAP27* are previously identified EOC-susceptibility genes.  |

**Figure 1. Study design flow chart**

**Figure 1**. **The overall workflow of this study**

Build DNA methylation prediction models using FHS data

External validation using WHI data

Association analyses of genetically predicted DNA methylation levels with EOC risk using OCAC data

Correlation between methylation and gene expression using FHS data

Genetically predicted gene expression and EOC risk using GTEx and OCAC data

Functional annotation for EOC-associated CpGs