

体を添加し、IL-6 の作用を中和した状態で、同様に Th17 分化を評価した。また、野生型マウスに、PD-1 欠損 RAG2 欠損マウスの骨髄および野生型 CD4 陽性 T 細胞を移植し、非リンパ球でのみ PD-1 を欠損するマウスを作製、ここに結核死菌と自己ペプチド(MOG35-55)を投与して、実験的自己免疫性脳脊髄炎を誘導した。病勢を観察し、定法によって点数化して評価した。

(倫理面への配慮)

本研究は、京都大学医学研究科の定める動物実験実施要綱、および京都大学動物実験指針を遵守して行った。また、PD-1 欠損マウスは、組み換え DNA 技術を用いて作出されており、事前に、京都大学組み換え DNA 実験委員会の承認を得たうえで行った。

C. 研究結果

昨年度までに、PD-1 欠損マウスに結核死菌(250 μ g)を投与すると、同時に投与した脳由来ペプチドに対し、炎症性サイトカインである IL-17 を産生する T 細胞が多く産生され、野生型コントロールマウスに比較してはるかに強い自己免疫性脳脊髄炎が惹起されること、PD-1 欠損マウスの抗原提示細胞、特に、CD11b を表現するマクロファージは、野生型に比べ、Th-17 の分化に必須の IL-6 を多く産生することを見出している。この IL-6 と Th17 分化亢進の関係を調べるため、PD-1 欠損脾臓細胞を用いて抗原特異的 T 細胞を分化させる培養系において、IL-6 と、そのレセプターの結合を阻害する抗体を作用させた。この処置は、Th17 分化を阻害抗体の濃度依存性に抑制したため、PD-1 欠損マクロファージからの IL-6 産生が Th17 分化亢進に直接かかわっていることが明らかになった。また、骨髄移植法によって、マクロファージなど、非リンパ球系

細胞でのみ PD-1 を欠損するマウスを作製した。このようなマウスでは、PD-1 を発現するコントロールマウスに比較して、実験的脳脊髄炎の早期発症および重症化が観察された。

D. 考察

PD-1 欠損下では、マクロファージから通常より多くの IL-6 産生が起こり、自己反応性 T 細胞に作用して Th-17 細胞を分化させる可能性が示唆された。IL-6 は、PD-1 欠損マウスと野生型マウスで結核菌刺激後初期に差が見られた唯一のサイトカインであり、PD-1 抗体治療開始時の血清 IL-6 を測定すれば、免疫増強療法の副作用としての自己免疫疾患発症を予測できる可能性がある。

E. 結論

結核菌など、抗原提示細胞からの IL-6 を誘導する活性をもつ病原体、およびカビなどの環境因子は、潜在的に自己反応性 Th-17 の分化も起こす可能性を持っている。ここに PD-1 阻害を行うと、自己反応性 Th-17 の活性化を誘導し、思わぬ自己免疫病を発症する可能性がある。よって、患者における、不顕性感染などの微生物学的因子は、PD-1 抗体療法の際に慎重にモニタリングされる必要がある。

F. 健康危険情報

なし

G. 研究発表

1. 論文発表

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2. 学会発表

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H. 知的財産権の出願・登録状況(予定を含む)

1. 特許取得 なし
2. 実用新案登録 なし
3. その他 なし

厚生労働科学研究費補助金（難病・がん等の疾患分野の医療の実用化研究事業）
分担研究報告書

がん免疫逃避機構を標的にした次世代型免疫治療の臨床応用と新規バイオマーカーの探索に関する研究

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A. 研究目的

末梢血腫瘍細胞(CTC)の検出は乳癌患者を中心に診断のみならず治療効果判定や予後因子としてその有用性が文献報告されている。しかし卵巣癌における末梢血腫瘍細胞の検出については、その診断法や臨床的な意義について未だ不明である。今回われわれはテロメラーゼ特異的アデノウイルスを用いた卵巣癌の新規診断法について検討を行った。

B. 研究方法

卵巣癌患者 15 例(漿液性腺癌 7 例、粘液性腺癌 4 例、明細胞癌 2 例、類内膜腺癌 2 例)を対象に術前に末梢血 7.5ml を採取し、hTERT promoter および green fluorescence protein (GFP) 遺伝子を組み込んだテロメラーゼ特異的増殖型アデノウイルス OBP-401 を用いて、GFP 陽性細胞の蛍光顕微鏡下確認から、末梢血腫瘍細胞(CTC)の検出を行った。なお本検討は倫理委員会承認を受け、実施にあたっては被験者全員に事前に文書同意取得した。

C. 研究結果

D. 1. 上皮性卵巣癌の術前血液 15 例中 10 例 (67%) に GFP 陽性細胞が検出されたが、健常人では 1 例も GFP 陽性細胞が検出され

なかった。2.GFP 陽性細胞の検出率は病理組織型、FIGO 進行期、最大腫瘍径、腹水の有無、および腹膜播種と有意な関連性を認めなかった。3. GFP 陽性細胞は CA125 陰性例 3 例中 1 例、CA199 陰性例 10 例中 6 例で検出可能であった。4. 免疫組織染色を用いた検討において GFP 陰性と判定された全症例で原発巣に hTERT の発現をみとめた。

E. 結論

術前末梢血を用いたテロメラーゼ特異的増殖型アデノウイルスによる CTC の検出は、一定の臨床条件を有する卵巣癌患者の有用な術前直接診断法となりうると考えられ、特に計画的妊孕能温存治療、低侵襲治療、再発卵巣癌の早期診断等への応用が期待される。

F. 研究発表

論文発表

準備中

G. 知的所有権の取得状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

Ⅲ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍;なし 雑誌:

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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IV. 研究成果の刊行物・別刷

Epigenetic determinants of ovarian clear cell carcinoma biology

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Targeted approaches have revealed frequent epigenetic alterations in ovarian cancer, but the scope and relation of these changes to histologic subtype of disease is unclear. Genome-wide methylation and expression data for 14 clear cell carcinoma (CCC), 32 non-CCC and four corresponding normal cell lines were generated to determine how methylation profiles differ between cells of different histological derivations of ovarian cancer. Consensus clustering showed that CCC is epigenetically distinct. Inverse relationships between expression and methylation in CCC were identified, suggesting functional regulation by methylation, and included 22 hypomethylated (UM) genes and 276 hypermethylated (HM) genes. Categorical and pathway analyses indicated that the CCC-specific UM genes were involved in response to stress and many contain hepatocyte nuclear factor (HNF) 1-binding sites, while the CCC-specific HM genes included members of the estrogen receptor alpha (ERalpha) network and genes involved in tumor development. We independently validated the methylation status of 17 of these pathway-specific genes, and confirmed increased expression of HNF1 network genes and repression of ERalpha pathway genes in CCC cell lines and primary cancer tissues relative to non-CCC specimens. Treatment of three CCC cell lines with the demethylating agent Decitabine significantly induced expression for all five genes analyzed. Coordinate changes in pathway expression were confirmed using two primary ovarian cancer datasets ($p < 0.0001$ for both). Our results suggest that methylation regulates specific pathways and biological functions in CCC, with hypomethylation influencing the characteristic biology of the disease while hypermethylation contributes to the carcinogenic process.

Ovarian cancer is the fifth most common cause of cancer death in women¹ and has the worst mortality rate of all gynecologic cancers.² Epithelial ovarian cancers are a heterogeneous disease with distinct clinicopathological and molecular features³ and are classified pathologically into four major histologic subtypes based entirely on tumor cell morphologic criteria: serous adenocarcinoma (SAC), mucinous adenocarcinoma (MAC), endometrioid adenocarcinoma (EAC) and clear cell carcinoma (CCC). Because these histologic subtypes of ovarian cancer have unique clinical characteristics and behavior, histology-specific biomarkers and individualized

therapeutic strategies are needed that improve therapeutic options and outcomes. Ovarian CCC is distinct from the other major histologic types of epithelial ovarian cancer^{4,5} and often arises from endometriosis. The clinical outcome of advanced-stage CCC is generally poor and this may be related to the relative resistance to conventional platinum- or taxane-based chemotherapies.⁶⁻⁸ Although the different histologic subtypes of ovarian cancer have been associated with genetic defects, such as *TP53* mutations in high-grade SAC, *PTEN* and *CTNNB1* mutations in EAC and *KRAS* mutations in MAC, the molecular features of CCC have remained elusive.⁹ Recent genome-wide technologies have revealed frequent *ARID1A* mutations^{10,11} and overexpression of *HNF1B*¹² in ovarian CCC. We previously identified an ovarian CCC-specific gene signature characterized by activation of the *HNF1B* pathway. Furthermore, some of these signature genes are regulated by DNA methylation.¹³

DNA methylation is an epigenetic mechanism of gene regulation that plays a crucial role in many biological processes including X-chromosome inactivation, genomic imprinting, aging and cancer.¹⁴ In cancer cells, global DNA hypomethylation and aberrant hypermethylation of tumor suppressor genes are well-established mechanisms that contribute to cancer cell transformation.¹⁵ Analysis of individual genes has led to a large number now reported as targets of DNA methylation in ovarian cancer.^{1,16} However, genome-wide methylation profiles may be more instructive because the

Key words: ovarian clear cell carcinoma, HNF1, ERalpha, methylation, epigenetic characterization

Additional Supporting Information may be found in the online version of this article

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What's new?

Ovarian cancer has several subtypes, and although different genetic mutations have been associated with particular subtypes, the molecular characteristics of ovarian clear cell carcinoma remain hazy. Aberrant DNA methylation can turn cells cancerous, and this study compared patterns of gene methylation in ovarian clear cell carcinomas, other ovarian cancer cells, and normal cells. They found that the clear cell carcinomas could indeed be identified by their distinctive pattern of DNA methylation. They found that this methylation pattern increased expression of certain stress response genes, while other genes, with tumor suppressive functions, were stifled.

methylation status of any particular single gene has not been shown to be predictive, diagnostic or prognostic for this disease.^{15,17} Higher resolution elucidation of DNA methylation profiles may lead to the discovery of biomarkers for diagnosis, improved methods of classification and disease monitoring as well as to a better understanding of cancer biology. Although several comprehensive DNA methylation analyses have been reported in ovarian cancer, limitations have included a relatively small number of samples, lack of focus on histological subtypes and in the limited number and selection of genes analyzed.^{14,17-20} Only four genes (*14-3-3 sigma* as *SNF*, *PYCARD*, *WT1* and *HNF1B*) have been reported as aberrantly methylated in CCC.^{1,13,16,21} Therefore, epigenetic features that more fully characterize histological subtypes including CCC have yet to be identified.

In this study, our initial objective was to determine if epigenomic patterns could distinguish the various histologic subtypes of ovarian cancer, including CCC. We identified an ovarian CCC methylation profile that was distinct from the other histologic subtypes of ovarian cancer. Through analysis of genes showing strong associations between DNA methylation and levels of transcription, we identified important biological mechanisms contributing to characteristic features of ovarian CCC⁴ that appear to be deregulated through coordinate epigenetic modifications in this disease.

Material and Methods

Detailed methods are provided in Supporting Information.

Cell lines and clinical samples

The immortalized noncancerous cell lines were maintained as described.²²⁻²⁴ Ovarian cancer cell lines, including 14 CCC and 32 non-CCC cell lines (Supporting Information Table 1), were cultured in RPMI1640/FBS/pen-strep medium (Sigma-Aldrich, St. Louis, MO) with 10% heat-inactivated fetal bovine serum (v/v; Invitrogen, Carlsbad, CA) in an atmosphere of 5% CO₂ at 37°C. The short tandem repeat (STR) genotypes of all ovarian cancer cell lines were analyzed to authenticate the cell lines using either the PowerPlex® 1.2 System (Promega, Madison, WI) at The Fragment Analysis Facility of Johns Hopkins University or the AmpFℓSTR® Identifier® Plus PCR Amplification Kit (Applied Biosystems, Carlsbad, CA) at the University of Colorado Cancer Center, DNA Sequencing and Analysis Core. The STR genotypes of ovarian cancer cell lines that are available from ATCC,

RIKEN BioResource Center Cell Bank or the Japanese Collection of Research Bioresources (JCRB) Cell Bank were identical to the source genotypes as reported within their respective STR databases and all other noncommercially available cell lines were shown to be derived from females with unique genotypes.

Tissue specimens were derived from 85 patients with ovarian cancer treated at Duke University Medical Center, (13 CCC, 53 SAC, 11 EAC and eight MAC) and from eight patients without malignant disease (four ovarian surface epithelium samples and four fallopian tube epithelium samples). All patients provided written informed consent and specimens were used under a protocol approved by the Duke University Institutional Review Board. For all tumor specimens, representative sections were mounted on slides and stained with hematoxylin and eosin to confirm that at least 60% of the cellular content comprised cancer cells with less than 20% necrosis. Tumors were histologically classified according to World Health Organization (WHO) criteria.

Extraction of genomic DNA and bisulfite treatment

Genomic DNA was extracted from cells cultured in 10-cm dishes to 70-90% confluence using the QIAamp® DNA Mini Kit (Qiagen, Valencia, CA) or from 5 to 20 mg of clinical tissues using the AllPrep® DNA/RNA/Protein Mini Kit (Qiagen) for methylation beadchip analysis. Puregene Reagents (Qiagen) were used for DNA extraction after Decitabine treatment (see below). Five hundred nanograms of genomic DNA was bisulfite modified using the EZ DNA Methylation™ Kit (Zymo Research, Irvine, CA) according to the manufacturer's protocol for the Infinium methylation assay, and 800 ng of genomic DNA was bisulfite modified using the same kit but according to standard protocol for methylation analysis of individual genes.

Illumina Infinium HumanMethylation BeadChip Assays

Bisulfite-converted genomic DNA was analyzed using the Illumina Infinium HumanMethylation27 BeadChip for cell line samples (GEO Accession Number GSE51688) and HumanMethylation450 Beadchip for clinical tissue specimens (GEO Accession Number GSE51820) (Illumina, San Diego, CA). The HumanMethylation450 BeadChip includes 90% of the content contained on the HumanMethylation27 BeadChip ([http:// dl.dropbox.com/u/6904769/50CellLines_b-value_submission.txt.zip](http://dl.dropbox.com/u/6904769/50CellLines_b-value_submission.txt.zip)). Chip processing was performed according

to the manufacturer's protocol by Expression Analysis (Durham, NC), an Illumina Certified Service Provider. Data were extracted using Illumina GenomeStudio v2010.3 software (Illumina). Methylation values for each CpG locus are represented as β -values, a quantitative measure of DNA methylation, with levels ranging from 0 (completely unmethylated) to 1 (completely methylated). Bisulfite conversion efficiency (*i.e.*, the conversion of non-CpG cytosines to uracils) was assessed for all samples by Pyrosequencing (see below) before data generation using the Illumina BeadChips.

Methylation-specific polymerase chain reaction

DNA methylation of estrogen receptor alpha (ERalpha) network genes (*ESR1*, *BMP4*, *DKK1*, *SOX11*, *SNCG* and *MOSCI1*) was validated using methylation-specific polymerase chain reaction (MS-PCR). Primer sequences and PCR conditions are provided in Supporting Information Table 2a.

Pyrosequencing

All primers for pyrosequencing assays were designed using PSQ assay design software version 1.0.6 (Biotage, Uppsala, Sweden). The pyrosequencing assays were designed such that we analyzed the same CpG sites as those present on the Illumina BeadChip for 11 genes (*ESR1*, *HNFI1A*, *HNFI1B*, *C14orf105*, *KIF12*, *MIA2*, *PAX8*, *SERPINA6*, *SGK2*, *SRC* and *TM4SF4*) and for adjacent CpG sites for the *F2* gene. Primer sequences and PCR conditions are shown in Supporting Information Table 2b. Methylation values for each CpG site were calculated using Pyro Q-CpG software 1.0.9 (Biotage).

Real-time reverse transcription-polymerase chain reaction

Two micrograms of total RNA prepared from 36 ovarian cancer cell lines (13 CCC and 23 non-CCC; using RNA Stat60, Teltest, Friendswood, TX) or 49 frozen tissues (13 CCC and 36 non-CCC; using the AllPrep® DNA/RNA/Protein Mini Kit; Qiagen) were used to generate cDNA in a 40 μ l volume with the Superscript II kit (Invitrogen). Two microliters of the cDNA was used as template for PCR using TaqMan assays in a 20 μ l volume (Applied Biosystems) for *HNFI1B* (Hs01001602), *SGK2* (Hs00367639), *C14orf105* (Hs00216847), *F2* (Hs01011988), *ESR1* (Hs00174860), *CRIP1* (Hs00832816), *SOX11* (Hs00846583), *IGFBP4* (Hs01057900) and *BMP4* (Hs00370078). *B2M* (Hs 00187842) was used as an internal control. Relative expression was calculated using the delta delta Ct method.

5-Aza-2'-deoxycytidine (Decitabine) treatment

RMG-2, RMG-5 and KOC-7C cell lines were seeded into six-well plates and the following day they were treated in triplicate by adding RPMI1640/FBS/pen-strep media (mock) or the same media containing 5 μ M 5-aza-2'-deoxycytidine (Decitabine; Sigma-Aldrich). Media was replaced daily (with/without Decitabine as relevant) for 3 days after which the cells had reached 70–90% confluence and were harvested for DNA extraction as described above or RNA extraction using RNA Stat60 (Teltest).

Real-time reverse transcription (RT)-PCR and pyrosequencing analyses were performed as described above.

Bioinformatics

Identification of genes showing differential methylation. Identification of genes differentially methylated between groups was done by comparing β -values using unpaired *t*-tests with thresholds of $p < 0.01$ and difference of average β -values > 0.2 (20% methylation) for the comparison between ovarian cancer cell lines and normal cells or between CCC and non-CCC. Genes annotated by gene name or gene ID (Illumina annotation file) were used for the analyses.

Unsupervised consensus clustering. ConsensusCluster software²⁵ was used to evaluate the similarity between genes or specimens.

Identification of genes regulated by DNA methylation in ovarian cancer. Two cell line microarray datasets [Gene Expression Omnibus (GEO) Accession Number: GSE25428 and GSE29175^{13,26}] were used for the identification of candidate genes whose mRNA expression levels are regulated by DNA methylation in ovarian cancer. The ComBat normalizing algorithm was used before performing any analysis to reduce the likelihood of batch effects.²⁷ Candidate genes functionally regulated by methylation were identified based on the following criteria: (i) the *p*-value of unpaired Student *t*-tests is less than 0.01; (ii) the average β -value difference is more than 0.2 (20% methylation) between 14 CCC cell lines and 32 non-CCC cell lines and (iii) a significant inverse correlation ($p < 0.05$) between expression microarray values and β -values from the Infinium assay for the 42 ovarian cancer cell lines with gene expression data.

Categorical analyses. The biological characteristics of the CCC-specific methylation signature were evaluated using the enrichment of Molecular Signatures Database (MSigDB) gene sets (v2.5 updated April 7 2008)²⁸ with the R package allez 1.0.²⁹ Briefly, for each gene set, the proportion of the annotated genes in the CCC hypermethylated or hypomethylated gene sets was compared to that for all probeset genes. A gene set was considered significantly enriched if the *z*-score was more than 4.0.³⁰

Pathway analyses. The relationship of hypermethylated or hypomethylated genes to particular pathways was evaluated using MetaCore™ software (GeneGo; <http://www.genego.com/>), an integrated knowledge database and software suite for evaluation of the association between a particular gene list and known pathways.

Heatmap of β -values and expression. Absolute β -values are represented by color gradient intensity, from white; β -value = 0, to red; β -value = 1, using Java TreeView (<http://jtreeview.sourceforge.net/>). Average-linkage hierarchical clustering was performed in published expression microarray datasets representing clinical ovarian cancer samples (GSE6008 and

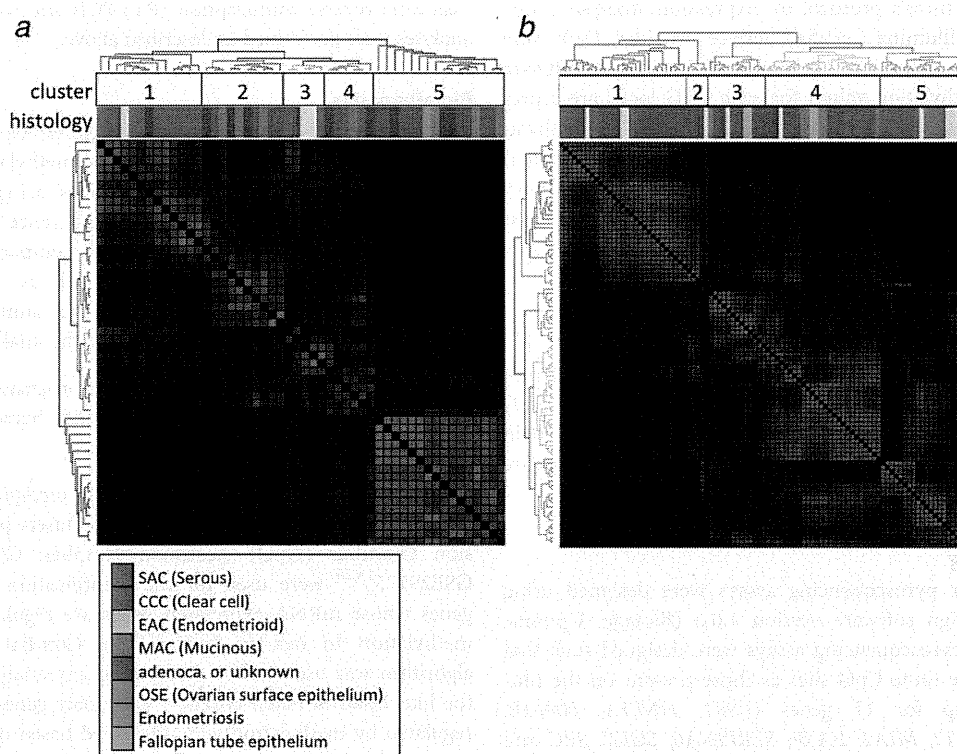


Figure 1. Consensus clustering of DNA methylation profiles for 46 ovarian cancer and four noncancerous cell lines (a), and 85 clinical ovarian cancer specimens and eight normal counterparts (b). Red–black coloration represents the similarity of methylation profiles between samples from similar to divergent methylation patterns, respectively. The same specimens are ordered identically in the individual rows and columns. The colored dendrogram indicates the five different clusters and the color bar indicates the histological subtype derivation of each specimen.

GSE2109^{31,32}) using genes transcriptionally regulated by DNA methylation with Cluster version 3.0 (<http://rana.lbl.gov/eisen/>).

Statistical analyses

The Student's *t*-test was used to compare continuous variables between groups. Fisher's exact test was used for analysis of categorical variables. Correlations between expression microarray values and methylation β -values from the Infinium assay or between β -values and % methylation values from pyrosequencing were evaluated using Pearson's correlation. *p*-values less than 0.05 were considered statistically significant.

Results

Divergent methylation profiles between ovarian cancer cells and noncancerous cells

By comparing methylation β -values, 2,003 genes (2,561 CpG sites) of a total possible 14,495 genes (27,578 CpG sites) (13.8%) in ovarian cancer cell lines showed methylation levels that differed relative to noncancerous specimens (Supporting Information Table 3). Of these differentially methylated genes, 1,870 genes (2,404 CpG sites; 93.4%) were hyperme-

thylated (HM), whereas only 139 genes (157 CpG sites; 7.0%) were hypomethylated (UM) in ovarian cancer cell lines.

In the clinical samples analyzed with the larger probeset, 3,432 genes (6,919 CpG sites) of 21,231 genes (365,860 CpG sites) (16.2%) showed methylation levels that differed compared to normal specimens. Of the differentially methylated genes, 2,383 genes (4,830 CpG sites; 65.6%) were HM, whereas 1,253 genes (2,089 CpG sites; 34.5%) were UM in ovarian cancer tissues (Supporting Information Table 3).

CCCs possess a unique methylation profile

Unsupervised consensus clustering generated a distinct CCC-specific cluster in the cell line dataset (Fig. 1a). The noncancerous cell lines clustered together with nine of 16 SAC (56.3%) cell lines. This data indicates that CCCs possess a distinct epigenomic pattern when compared to the other histologic types of ovarian cancer and noncancerous specimens. Similar to the clustering results in the cell lines, a distinctive CCC-specific cluster was again generated from analysis of clinical specimens. Like the cell lines, 25 of 51 (49.0%) SAC clinical specimens clustered together with normal specimens (Fig. 1b). Among the tissue specimens, six of eight MAC and five of 11 EAC samples were grouped within the cluster

containing the majority of the CCC samples. However, both the MAC and EAC subclusters are distinct from the CCC subcluster, indicating that the methylation profiles of MAC and EAC share higher similarity to CCC when compared to the other tissue specimens.

To evaluate the similarity between cell lines and clinical samples, the number of differentially methylated probes that overlap between these datasets was determined among the 25,978 CpG probes in common between the HumanMethylation27 and HumanMethylation450 BeadChips. Of these evaluable CpG probes, 1,042 and 77 CpGs showed hypermethylation in CCC compared to non-CCC cell lines and patient specimens, respectively, with a statistically significant overlap of 25 probes ($p < 0.0001$). We also found that the cell lines and clinical samples had 54 and 53 CpG probes showing hypomethylation, respectively, in CCC relative to non-CCC, with six CpG probes in common, a finding that is also statistically significant ($p < 0.0001$). These 25 hypermethylated and six hypomethylated probes are shown in Supporting Information Tables 4a and 4b. These results support that there are specific methylation profiles in cell lines that are also detectable in patient samples.

Identification of genes transcriptionally regulated by DNA methylation in CCC

Next, we examined the biological significance of the CCC-specific DNA methylation. For this purpose, the genes most likely to be transcriptionally regulated by DNA methylation in CCC were identified using cell line data because of the availability of both methylation and expression microarray data and the similarity of methylation status between the cell lines and clinical specimens. First, 856 genes (1,042 CpG sites) showed increased methylation, and 44 genes (54 CpG sites) showed decreased methylation in CCC when compared to non-CCC (Supporting Information Fig. 1a). Next, potential functional relationships between expression and methylation of the 856 HM and 44 UM CCC genes were assessed by determining if there were correlations between these values. Of 625 evaluable HM genes, 276 genes showed a significant inverse correlation ($r < -0.3051$, $p < 0.05$) between methylation and expression. Of the 33 evaluable UM genes, 22 showed a significant inverse correlation ($r < -0.3217$, $p < 0.05$) between methylation and expression (Supporting Information Fig. 1b). We considered these 276 HM and 22 UM genes as candidate CCC-specific epigenetically regulated genes (Supporting Information Tables 5a and 5b, respectively).

Hypomethylation of the HNF1 pathway and hypermethylation of estrogen receptor pathway genes in CCC

Biological functions of the 22 CCC-specific UM genes were characterized by analyzing the targeted pathways and “biological process” Gene Ontology (GO) terms. Pathway analysis using MetaCore™ indicated that nine of these 22

UM genes (41%) are members of the HNF1 pathway (Fig. 2a). Furthermore, the nine CCC-specific UM HNF1 pathway genes are not randomly deregulated but rather synchronously altered by methylation in CCC (Fig. 2c), suggesting that hypomethylation in CCC targets HNF1 pathway genes. Analyses using allez showed significant enrichment of all HNF1-binding motifs among the CCC UM genes including categorical terms V\$HNF1_01, RGTAMWNATT_V\$HNF1_01, V\$HNF1_Q6 and V\$HNF1_C ($p < 0.00001$; Supporting Information Table 6). Allez showed that the 22 CCC UM genes were enriched for stress response-related GO terms including “response to oxidative stress” (z -score > 4.0 ; Table 1, top).

Among the 276 CCC-specific HM genes, pathway analysis showed that 64 genes were involved in the estrogen receptor pathway (Fig. 2b). Similar to the synchronous decreased methylation of HNF1 pathway genes in CCC, these 64 genes exhibit coordinate hypermethylation in CCC relative to non-CCC (Fig. 2d). These results indicate that the estrogen receptor pathway is a target of hypermethylation in CCC. A categorical analysis using allez to identify GO terms associated with the HM genes showed that the 276 genes HM in CCC included many with tumor-suppressive functions having GO annotations of “cell cycle arrest” and “negative regulation of cell cycle,” and development- and organogenesis-related genes with annotations of “negative regulation of cytoskeleton organization and biogenesis” (Table 1, bottom).

Validation of hypomethylation and increased expression of HNF1 pathway genes in CCC

Eleven hypomethylated genes in the HNF1 pathway (*HNF1A*, *HNF1B*, *C14orf105*, *KIF12*, *MIA2*, *PAX8*, *SERPINA6*, *SGK2*, *SRC*, *TM4SF4* and *F2*) identified through analysis of CCC versus noncancerous specimens and from comparisons between CCC versus non-CCC cell lines were analyzed by bisulfite pyrosequencing. The ten genes for which the same CpG sites were analyzed showed very strong positive correlations between the Infinium assay β -values and the percent methylation as measured by pyrosequencing ($r > 0.9605$, $p < 0.000001$ for all genes; Supporting Information Fig. 2). Adjacent CpG sites for these same ten genes also showed significant positive correlations between the β -values of the Infinium assay CpG sites and the percent methylation by pyrosequencing, as did the adjacent CpG sites measured for the *F2* gene ($0.7494 < r < 0.9906$, $p < 0.000001$ for all genes; Supporting Information Fig. 3). Confirming the results of the methylation beadchip analyses, the pyrosequencing results also showed significantly lower methylation levels in CCC relative to those in non-CCC cell lines for these 11 genes ($p < 0.01$ for all genes; Fig. 3a). In 85 clinical ovarian cancer specimens, ten of the 11 HNF1 network genes showed overall hypomethylation in ovarian CCC specimens and six of these 11 genes showed significantly decreased methylation in CCC compared to non-CCC (*HNF1A*, $p = 0.0036$; *HNF1B*, $p = 0.0041$; *C14orf105*, $p = 0.0194$; *SERPINA6*, $p = 0.0291$; *SRC*,

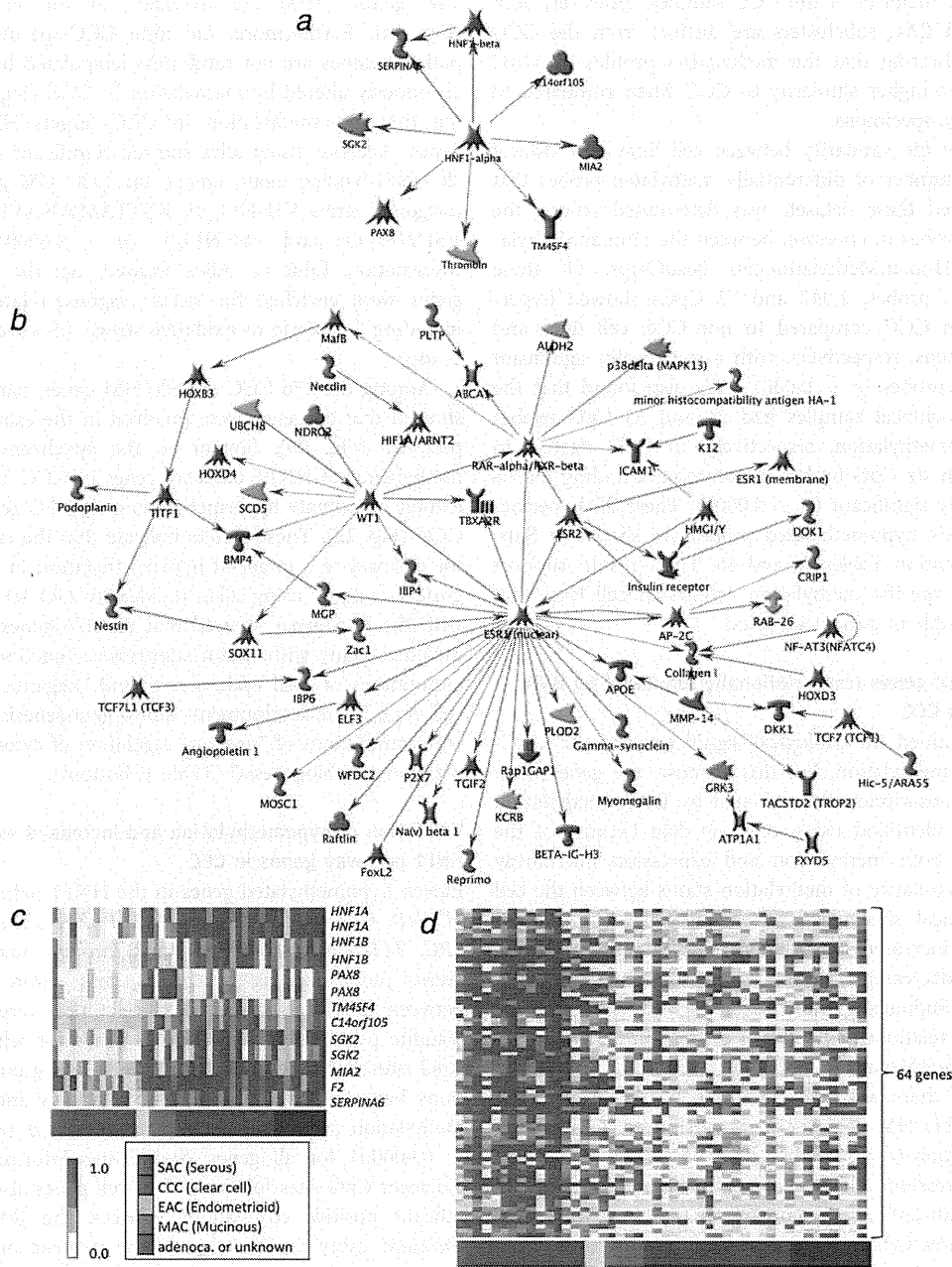


Figure 2. MetaCore™ pathway analyses of the 22 CCC-specific UM genes (a) and the 276 CCC-specific HM genes (b), and heatmaps showing methylation profiles of nine HNF1 pathway genes (c) and 64 ERalpha network genes (d) in 46 ovarian cancer cell lines. Of the 22 CCC-specific UM genes for which expression and methylation are inversely correlated, nine were identified as members of the HNF1 transcriptional network (a). Of 276 CCC-specific HM genes for which expression and methylation are inversely correlated, 65 were members of the ERalpha (encoded by *ESR1*) network (b). In the heatmaps, red to white coloration represents the Infinium β -value from completely methylated to completely unmethylated, respectively. Genes within the HNF1 pathway (c) and ERalpha network (d) show synchronous hypomethylation and hypermethylation, respectively, in CCC versus non-CCC cell lines.

p = 0.0004 and *F2*, *p* = 0.0003; Fig. 3b). When CCC was compared with SAC, eight genes were significantly hypomethylated in CCC (*HNF1A*, *p* = 0.0007; *HNF1B*, *p* = 0.0002;

C14orf105, *p* = 0.0073; *MIA2*, *p* = 0.0480; *SERPINA6*, *p* = 0.0071; *SRC*, *p* < 0.0001; *TM4SF4*, *p* = 0.0452 and *F2*, *p* < 0.0001; Fig. 3b and data not shown).

Table 1. Categorical analysis of “biological process” gene ontology (GO) terms for the 22 UM genes (top) and 276 HM genes (bottom) in CCC

MSigID.c5.bp	n-Probes	z-Score	p-Value
22 CCC-specific UM genes			
POSITIVE REGULATION OF TRANSCRIPTION DNA DEPENDENT ¹	202	8.78	<0.000001
POSITIVE REGULATION OF RNA METABOLIC PROCESS ²	205	8.70	<0.000001
POSITIVE REGULATION OF TRANSCRIPTION ¹	242	7.90	<0.000001
POSITIVE REGULATION OF NUCLEOBASE NUCLEOSIDE NUCLEOTIDE AND NUCLEIC ACID METABOLIC PROCESS ²	260	7.58	<0.000001
POSITIVE REGULATION OF CELLULAR METABOLIC PROCESS ²	396	5.83	<0.000001
POSITIVE REGULATION OF METABOLIC PROCESS ²	407	5.73	<0.000001
PROTEIN IMPORT INTO NUCLEUS TRANSLOCATION ¹	21	4.66	0.000002
RESPONSE TO OXIDATIVE STRESS ²	78	4.65	0.000002
TYROSINE PHOSPHORYLATION OF STAT PROTEIN	26	4.14	0.000017
GLUTAMINE FAMILY AMINO ACID METABOLIC PROCESS	27	4.06	0.000025
276 CCC-specific HM genes			
NEGATIVE REGULATION OF CYTOSKELETON ORGANIZATION AND BIOGENESIS ³	18	7.19	<0.000001
MICROTUBULE POLYMERIZATION OR DEPOLYMERIZATION ³	21	6.58	<0.000001
PYRIMIDINE NUCLEOTIDE METABOLIC PROCESS	15	5.86	<0.000001
CELL CYCLE ARREST GO 0007050 ⁴	115	4.78	0.000001
SENSORY ORGAN DEVELOPMENT ³	23	4.53	0.000003
AEROBIC RESPIRATION	24	4.40	0.000005
NEGATIVE REGULATION OF CELL CYCLE ⁴	157	4.33	0.000008
NEGATIVE REGULATION OF CELLULAR COMPONENT ORGANIZATION AND BIOGENESIS ³	45	4.05	0.000026

¹GO terms related to transcriptional activation.

²GO terms related to stress response.

³GO terms related to development and organogenesis.

⁴GO terms related to tumor suppression.

We selected four HNF1B network genes for expression validation studies using both cell lines and clinical specimens. Messenger RNA levels of *HNF1B*, *F2*, *C14orf105* and *SGK2* were significantly increased in 13 CCC cell lines compared to 23 non-CCC cell lines ($p = 0.0018$, $p = 0.0057$, $p = 0.0046$ and $p = 0.0158$, respectively; Fig. 4a). Clinical samples also showed higher expression for all four genes comparing CCC ($N = 13$) to non-CCC ($N = 36$) specimens (*HNF1B*, $p = 0.0002$; *F2*, $p < 0.0001$; *C14orf105*, $p = 0.0002$ and *SGK2*, $p = 0.0019$; Fig. 4b).

We used publicly available ovarian cancer expression microarray datasets representing clinical ovarian cancer samples (GSE6008 and GSE2109) to assess the expression levels of the genes we had identified as functionally regulated by methylation. Supervised hierarchical clustering was performed using only the genes regulated by DNA methylation listed in Supporting Information Tables 5a and 5b. The CCC specimens were clearly divided from non-CCC samples based on the expression levels of these genes. The CCC-specific UM genes from our analysis were enriched among a distinct cluster of genes that are highly expressed in the CCC clinical specimens when compared to the presence of CCC-specific

HM genes in this cluster (16 of 30 *versus* 0 of 378 in GSE6008, and 18 of 30 *versus* 1 of 378 in GSE2109; $p < 0.0001$ for both; Supporting Information Figs. 4a and 4b).

Validation of hypermethylation and decreased expression of estrogen receptor pathway genes in CCC

Representative ER pathway genes including *ESR1*, *BMP4*, *DKK1*, *SOX11*, *SNCG* and *MOSCI* were investigated for their methylation status in cell lines using MS-PCR. Twelve of 14 (86%) CCC cell lines exhibited methylation of the *ESR1* promoter, whereas 17 of 32 (53%) non-CCC cell lines showed methylation of this gene ($p = 0.0487$). Similarly, more of the CCC *versus* non-CCC cell lines showed methylation at the *BMP4* and *DKK1* promoter regions (*BMP4*: 12 of 14 CCCs *versus* 8 of 32 non-CCCs, $p = 0.0002$; *DKK1*: 10 of 14 CCCs *versus* 9 of 32 non-CCCs, $p = 0.0094$; Table 2, top and Supporting Information Figs. 5a–5f). For *SOX11* and *SNCG*, all CCC cell lines exhibited methylation while there were several non-CCC unmethylated cell lines (*SOX11*: 13 of 13 CCCs *versus* 18 of 23 non-CCCs, $p = 0.1363$; *SNCG*: 13 of 13 CCCs *versus* 21 of 24 non-CCCs, $p = 0.5382$). The *MOSCI* gene showed evidence of methylation in all cell lines

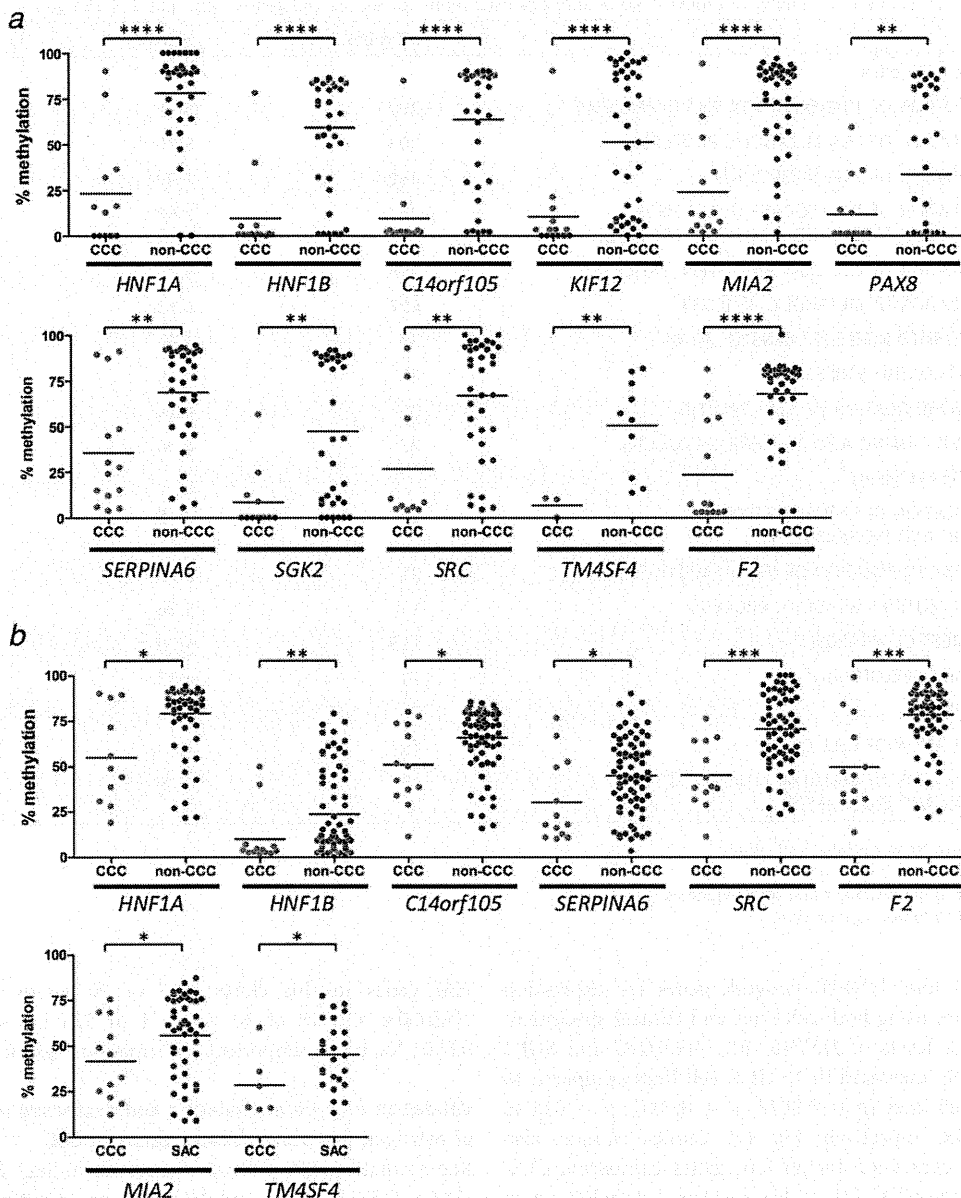


Figure 3. Quantitative methylation analyses of 11 HNF1 network genes (*HNF1A*, *HNF1B*, *C14orf105*, *KIF12*, *MIA2*, *PAX8*, *SERPINA6*, *SGK2*, *SRC*, *TM4SF4* and *F2*) in cell lines by bisulfite pyrosequencing show lower levels of methylation in CCC versus non-CCC, supporting the results from the Infinium BeadChip (a). For clinical specimens, six of 11 HNF1 network genes show significantly decreased methylation in CCC compared to non-CCC. When CCC is compared with SAC in clinical samples, eight genes (*HNF1A*, $p = 0.0007$; *HNF1B*, $p = 0.0002$; *C14orf105*, $p = 0.0073$; *SERPINA6*, $p = 0.0071$; *SRC*, $p < 0.0001$ and *F2*, $p < 0.0001$, included in data shown in Fig. 3b, top; *MIA2*, $p = 0.0480$ and *TM4SF4*, $p = 0.0452$ are shown below) are significantly hypomethylated in CCC (b). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

analyzed. We also investigated these six genes in clinical specimens, and three of the six (*SOX11*, *BMP4* and *MOCS1*) were methylated more often in CCC than in non-CCC, with *SOX11* showing a significant difference ($p = 0.0116$) and *BMP4* and *MOCS1* approaching significance ($p = 0.0645$ and 0.1784 , respectively; Table 2, bottom and Supporting Information Figs. 6a–6f).

Transcript levels for five ER pathway genes (*ESR1*, *CRIP1*, *SOX11*, *IGFBP4* and *BMP4*) were decreased in CCC cell lines when compared to non-CCC cell lines, all of which were significant except for *SOX11* ($p = 0.0112$, $p = 0.0046$, $p = 0.2108$, $p = 0.0038$ and $p = 0.0472$, respectively; Fig. 4a). Among the clinical specimens, four genes showed lower average expression levels in CCC compared to non-CCC, with a

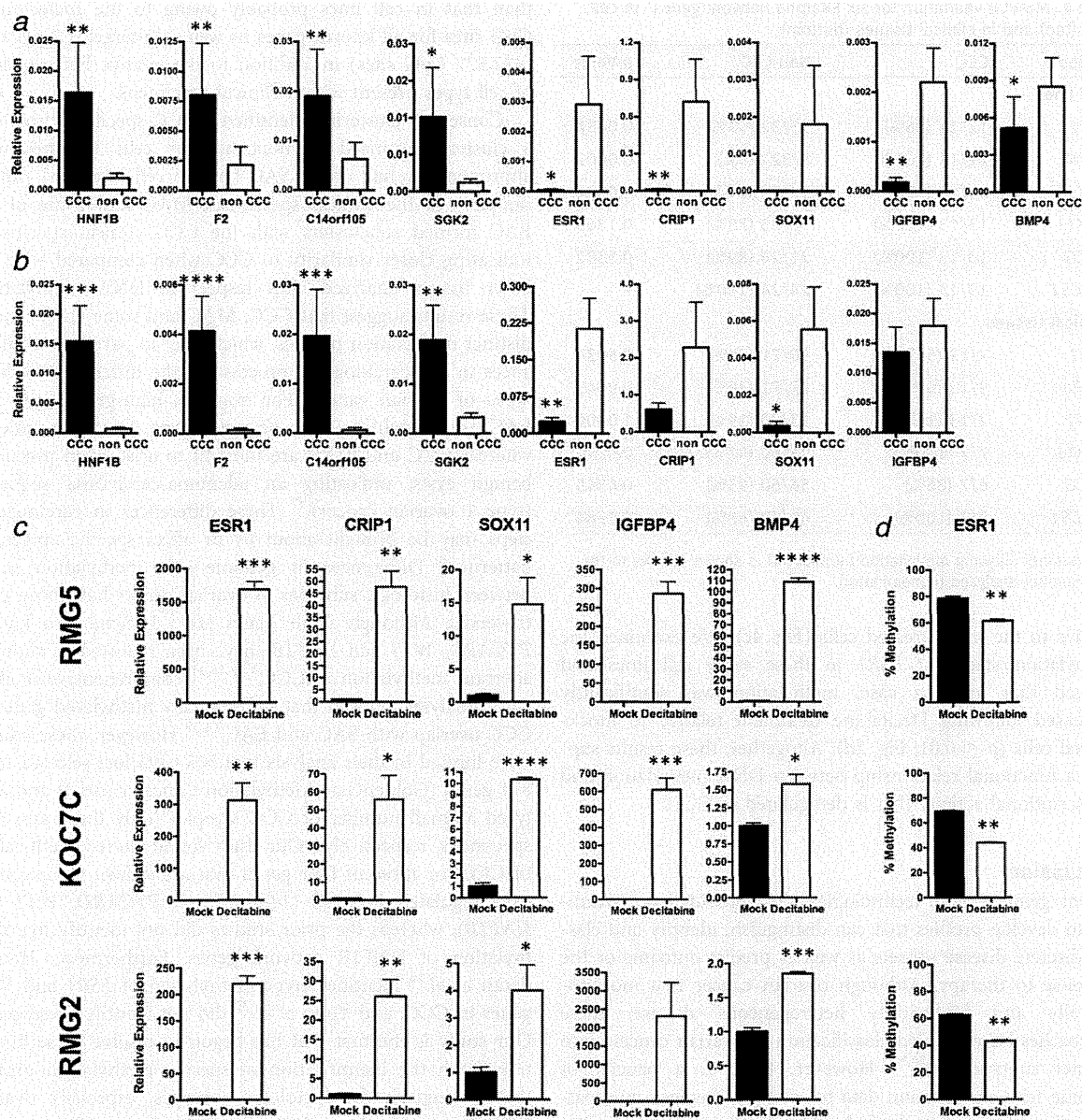


Figure 4. Validation of altered expression of HNF1 network and ERalpha pathway genes in CCC and reactivation and demethylation of ERalpha pathway genes with 5-aza-2'-deoxycytidine (Decitabine) treatment. Quantitative RT-PCR of HNF1 network genes (*HNF1A*, *F2*, *C14orf105* and *SGK2*) and ERalpha pathway genes (*ESR1*, *CRIP1*, *SOX11*, *IGFBP4* and *BMP4*) using cell lines (a) and clinical samples (b). The expression of ERalpha pathway genes (*ESR1*, *CRIP1*, *SOX11*, *IGFBP4* and *BMP4*) is markedly induced by demethylating agent Decitabine in three CCC cell lines (RMG-5, KOC-7C and RMG-2) (c). Methylation at the *ESR1* promoter is decreased in response to Decitabine treatment (d). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

significant difference for two (*ESR1*, $p = 0.0014$; *CRIP1*, $p = 0.1853$; *SOX11*, $p = 0.0476$ and *IGFBP4*, $p = 0.991$; Fig. 4b). When compared to SAC, *IGFBP4* mRNA levels did not differ in CCC ($p = 0.9521$), but expression of *ESR1*, *CRIP1* and *SOX11* was significantly decreased in CCC ($p = 0.0022$, $p = 0.0476$ and $p = 0.0017$, respectively).

The potential to reactivate expression of five genes, *ESR1*, *CRIP1*, *SOX11*, *IGFBP4* and *BMP4*, was evaluated in three CCC cell lines (RMG-2, RMG-5 and KOC-7C) using the DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine (Decitabine). There was a marked and significant increase in mRNA levels for all five genes in cells treated with Decitabine

Table 2. MS-PCR validation for six ERalpha network genes¹ in cell lines (top) and in clinical tissues (bottom)

Genes	CCC	Non-CCC	p-Value
Cell lines			
<i>ESR1</i>	12/14 (86%)	17/32 (53%)	0.0487
<i>BMP4</i>	12/14 (86%)	8/32 (25%)	0.0002
<i>DKK1</i>	10/14 (71%)	9/32 (28%)	0.0094
<i>SOX11</i>	13/13 (100%)	18/23 (78%)	0.1363
<i>SNCG</i>	13/13 (100%)	21/24 (89%)	0.5382
<i>MOCS1</i>	13/13 (100%)	24/24 (100%)	–
Clinical tissues			
<i>ESR1</i>	7/12 (58%)	50/71 (70%)	0.5034
<i>BMP4</i>	9/13 (69%)	27/71 (38%)	0.0645
<i>DKK1</i>	2/13 (15%)	13/70 (19%)	1.0000
<i>SOX11</i>	7/7 (100%)	28/60 (47%)	0.0116
<i>SNCG</i>	6/7 (86%)	56/60 (93%)	0.4345
<i>MOCS1</i>	7/7 (100%)	41/60 (68%)	0.1784

¹The number showing methylation (numerator) is shown relative to the total number analyzed (denominator).

relative to the mock-treated cells (Fig. 4c). We examined the methylation status of *ESR1* in these same cell lines and showed that in each case, methylation was significantly decreased following Decitabine treatment relative to mock-treated cells ($p \leq 0.01$; Fig. 4d). Altogether, these results support a functional relationship between DNA methylation and transcriptional activity that is deregulated in CCC.

Discussion

Recent genome-wide technologies have provided opportunities to develop profiles that can distinguish, identify and classify discrete disease subsets as well as predict outcome or the response to therapy. Although ovarian cancer is a morphologically and biologically heterogeneous disease, these approaches have enabled classification of ovarian cancers into distinct subtypes.^{2,31,33,34} However, there is a paucity of genome-wide methylation data to examine if epigenomic patterns discriminate histological subtypes of cancers, including ovarian cancer.

The comparison of methylation profiles between ovarian cancer and noncancerous cells indicated that of the 2,003 genes that showed differential levels of methylation, as many as 93.4% showed hypermethylation in the cancer cell lines. Based on prior focused studies of promoter CpG island methylation and next-generation sequencing, acquisition of methylation occurs more frequently than loss of methylation in almost all types of primary tumors when compared to their normal counterparts.^{35,36} Because 26,956 (97.7%) of the 27,578 CpG sites are located at promoter regions on the Illumina Infinium HumanMethylation27 BeadChip, our results are comparable with those of published reports. The ratio of HM genes (65.5%) in primary cancer tissues was smaller

than that in cell lines probably owing to the inclusion of CpG sites for all known genes as well as intergenic CpG sites (485,577 CpG sites) in addition to the relative heterogeneity of cell types present within clinical specimens.

Consensus clustering identified a CCC-specific cluster and a cluster comprised of noncancerous cells together with approximately half of the SAC both in cell lines and clinical samples. In the clinical specimens, MAC and some of the EAC formed subclusters with the CCC clinical specimens, indicating closer similarity to CCC when compared with the other tissues analyzed with respect to DNA methylation. These results suggest that CCC, MAC and some EAC acquire distinct methylation profiles, which may in part reflect differences in the carcinogenic process for the different histologic types of ovarian cancer. For example, high-grade SAC and EAC are thought to arise *de novo* (type 2 ovarian cancer), whereas CCC and MAC are thought to arise from precursor benign cysts, indicating an adenoma-carcinoma sequence (type 1 ovarian cancer).³⁷ These differences in carcinogenic steps may be brought about by or affect specific epigenetic patterns.³⁸ Differences in genome-wide methylation status between histologic subtypes of ovarian cancer have been controversial. Although single genes (*14-3-3 sigma* also known *PYCARD*, *WT1* and *HNF1B*) have been reported as showing aberrant methylation in CCC,^{1,13,16,21} comprehensive methylation analyses showed that differentially methylated genes in CCC overlap with SAC and EAC.^{14,39} However, these studies were limited in their analysis to 1,505 CpG loci selected from 871 genes (GoldenGate Methylation Cancer Panel I) and analyzed a small number of CCC samples (only three and four specimens, respectively). Our study found altered methylation in CCC for three of four genes that are known as epigenetically regulated in CCC (*14-3-3 sigma*, *PYCARD*, *WT1* and *HNF1B*), whereas the prior studies did not identify hypomethylation of *HNF1B* network genes. Furthermore, Houshdaran *et al.*¹⁴ identified hypermethylation of *ESR1* and *WT1* genes in CCC, and Yoon *et al.*³⁹ did not identify these genes. Our study is the first that has begun to resolve these limitations with the identification of specific methylation profiles that distinguish the histologic subtypes, especially ovarian CCC, suggesting that epigenetic regulation contributes to the ontogeny of epithelial ovarian cancer.

Although many single genes have been reported as regulated by DNA methylation in ovarian cancer, the biological role of genome-wide methylation in ovarian carcinogenesis has not been elucidated, with questions remaining as to whether or not changes in methylation reflect cause or consequence. We considered the genes showing an inverse correlation between expression and methylation as functionally important genes that are regulated at least in part through epigenetic mechanisms. For our purposes, the data from cell lines were used to evaluate expression-methylation correlations and proved more powerful at revealing these relationships than the clinical samples, likely because of the heterogeneity in cell type composition of the clinical tissue

specimens and because the DNA and RNA were extracted from different parts of the same piece of tissue. Therefore, to analyze how DNA methylation influences biological features of CCC, we identified genes for which transcription and methylation are coordinately related and that are CCC-specific. Interestingly, our pathway and categorical analyses showed that genes with HNF1 transcription factor-binding sites as well as *HNF1B* were synchronously hypomethylated in CCC. Furthermore, genes that function in the ERalpha network in CCC were among the 276 HM genes, including *ESR1* and *WT1*. These findings suggest that hypomethylation and hypermethylation in CCC appear to target genes belonging to specific and highly relevant pathways in this particular histologic subtype of ovarian cancer.

Previously, we reported that HNF1 transcription is activated in CCC relative to the other ovarian cancer histologic subtypes, and this activation is related to loss of DNA methylation at *HNF1B*.^{13,21,40} Our finding that activation occurs of multiple components of the HNF1 pathway by synchronous hypomethylation is a significant step forward in understanding this process and supports previous studies. HNF1B is involved in glucose homeostasis and may be responsible for the prominence of glycogen in CCC cells^{41,42} causing the cytoplasm to be clear in appearance, a defining feature of CCC. Our findings suggest that this morphologic feature of CCC is at least in part epigenetically regulated. Ovarian CCC is characterized by unique biology, including slow growth and resistance to chemotherapy and oxidative stress. Ovarian CCC also exhibits the molecular phenotypes of HNF1 pathway activation, PI3K pathway activation and MAPK activation, traits that have been collectively referred to as “ovarian CCC-likeness.”⁴ Using GO term analysis, CCC-specific UM genes were enriched with genes related to oxidative stress, consistent with “ovarian CCC-likeness.” These results indicate that loss of DNA methylation appears to target specific pathways and biological functions, with hypomethylation influencing pathways that help sculpt the characteristic biology of the disease.

ESR1, which is a key molecule in the ERalpha network that we found is hypermethylated in ovarian CCC, is a known target of methylation, and the absence of ERalpha expression in CCC has been reported.^{43–45} *WT1*, one of the genes regulated by ERalpha signaling, has previously been shown to be inactivated by promoter methylation in ovarian CCC.⁴⁶ These results support our findings and underscore the importance of epigenetic regulation of this pathway in CCC. Endometriotic cysts of the ovary are precursors of ovarian cancer, especially of the CCC and EAC histologic subtypes.⁴ Akahane *et al.* showed that reductions in ERalpha expression occurred with progression from endometriosis to atypical endometriosis in CCC specimens, whereas ERalpha expression increased with malignant transformation in EAC.⁴⁷ Estrogen signaling through estrogen receptors has divergent outcomes in that it can induce activation of cell proliferation but also inhibit cell cycle progression.^{48,49} Our results indicate that the ERalpha signaling-independent

growth of CCC is largely attributable to aberrant epigenetic alterations in CCC. Although changes in CCC phenotype cannot be specifically assessed using demethylating agents, these findings support our hypothetical model for the malignant transformation of endometriosis, in which EAC is induced by estrogen and CCC is influenced by the unique microenvironment within endometriotic cysts, including persistent exposure to oxidative stress and chronic inflammation.⁴ Epigenetic changes can be the earliest initiation factor and complement single driver mutations in a human cancer;⁵⁰ our results suggest that coordinate deregulation of DNA methylation leads not only to tumor development but also defines the biological features of the malignancy.

We previously reported suppression of TGFbeta pathway activity by DNA methylation in ovarian cancers, primarily of serous histology,²⁶ while our study demonstrates that there are distinct pathways influenced by hypomethylation and hypermethylation in ovarian CCC. Genetic and epigenetic disruption plays a fundamental role in cancer development. However, mutations in *ARID1A* and *PIK3CA* are the only genetic alterations that have been identified in ~50% and 40% of CCC cases, respectively. Thus, gene mutations alone cannot distinguish histologic subtypes of ovarian cancer. Our results indicate that coordinate changes in DNA methylation occur that affect expression of genes belonging to specific and relevant pathways in ovarian CCC, which contrasts with the more isolated pattern of genetic mutations observed in this disease. In high-grade SAC, *TP53* mutations initiate carcinogenesis and also contribute to the rapid growth that characterizes this histologic subtype, a “genetically based disposition.” On the other hand, *ARID1A* mutations lead to malignant transformation from endometriosis to CCC and the specific biological characteristics of CCC and “ovarian CCC-likeness” are developed through alteration of DNA methylation, an “epigenetically based disposition.” Furthermore, although changes in methylation levels induced by environmental stress such as oxidative stress and inflammation were not analyzed in this study, our current and prior results collectively suggest that CCC tumor development is brought about by microenvironment-mediated effects resulting from oxidative stress that lead to these CCC-specific methylation profiles.¹³ Inflammatory diseases such as ulcerative colitis and chronic gastritis resulting from *Helicobacter pylori* infection induce increased DNA methylation and are associated with increased cancer risk.⁵¹ Together, these findings suggest that an “epigenetically based disposition” may be involved in inflammation-induced carcinogenesis.

In summary, our genome-wide methylation analyses have revealed that ovarian CCC has a distinct methylation profile relative to the other histological subtypes of epithelial ovarian cancer. The CCC-specific methylation profile includes synchronous gain of promoter methylation for multiple genes in the ERalpha pathway and loss of promoter methylation for multiple genes in the HNF1 pathway. Further work will be required to more precisely delineate the functions of these

two pathways in this disease, to develop these changes as epigenetic biomarkers and to explore new modalities of treatment for CCC, including epigenetic therapies.

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Hepatocyte Nuclear Factor-1 β (HNF-1 β) Promotes Glucose Uptake and Glycolytic Activity in Ovarian Clear Cell Carcinoma

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Ovarian clear cell carcinoma (OCCC) is a morphologically and biologically distinct subtype of ovarian carcinomas that often arises in ovarian endometriosis. We previously reported that a unique carcinogenic environment, especially iron-induced oxidative stress in endometriotic cysts may promote development of OCCC. We also identified a gene expression profile characteristic of OCCC (the "OCCC signature"). This 320-gene OCCC signature is enriched in genes associated with stress response and sugar metabolism. However, the biological implication of this profile is unclear. In this study, we have focused on the biological role of the *HNF-1 β* gene within the OCCC signature, which was previously shown to be overexpressed in OCCC. Suppression of HNF-1 β in the HNF-1 β -overexpressing human ovarian cancer cell line RMG2 using short hairpin RNA resulted in a significant increase in proliferation. It also facilitated glucose uptake, glycolytic activity, and lactate secretion along with increased expression of the glucose transporter-1 (*GLUT-1*) gene and several key enzymes in the glycolytic process. Conversely, forced expression of HNF-1 β in the serous ovarian cancer cell line, Hey, resulted in slowed cellular growth and repressed glycolytic activity. These data suggest that HNF-1 β represses cell growth, and at the same time, it promotes aerobic glycolysis which is known as the "Warburg effect." As the Warburg effect is regarded as a characteristic metabolic process in cancer which may contribute to cell survival under hypoxic conditions or in a stressful environment, overexpression of HNF-1 β may play an inevitable role in the occurrence of OCCC in stressful environment.

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Key words: ovarian clear cell carcinoma; HNF1 β ; glucose metabolism

INTRODUCTION

Ovarian cancer is the leading cause of death among gynecological malignancies. Epithelial ovarian cancer consists of four histologically distinct subtypes, namely, serous, mucinous, endometrioid, and clear cell carcinoma. Ovarian clear cell carcinoma (OCCC) has distinct clinical features as compared to other subtypes: it is generally chemo-resistant, is often accompanied by a thromboembolic complications, and shows slow growth but with unfavorable outcomes. The most notable feature of OCCC is its relationship with ovarian endometriosis. According to epidemiological surveys, approximately 1% of ovarian endometriosis eventually transforms into carcinoma, primarily of the clear cell and endometrioid subtypes. This strongly suggests that there is a specific carcinogenic trajectory that directs the malignant transformation of endometriosis [1–6].

We previously reported that the unique composition of endometriotic cyst fluid, especially the high free iron concentration, promotes carcinogenesis through persistent oxidative stress [7]. We also identified a microarray-based gene signature that is

specific to OCCC, which we designated as the "OCCC signature." Analyses of the OCCC signature revealed that OCCC-specific gene expression is characterized by the involvement of many stress-related genes, which further supports the association between OCCC and stress. Pathway analysis of the OCCC signature demonstrated that an intracellular signaling pathway consisting of a large number of genes, especially stress-related genes, is activated in OCCC. A second prominent gene category overrepresented in

Abbreviations: OCCC, ovarian clear cell carcinoma; *HNF-1 β* , hepatic nuclear factor-1 β gene; shRNA, short hairpin RNAs; qRT-PCR, quantitative real-time polymerase chain reaction; GSEA, gene set enrichment analysis

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