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Methylated DNA Markers for Plasma Detection of Ovarian Cancer: Discovery, Validation, and Clinical Feasibility

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CRediT Author Statement

All listed authors have revised, reviewed, and accepted the submitted manuscript version and agree to be accountable for all aspects of the work. Each author's unique contributions are listed below.

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Abstract

Objective: Aberrant DNA methylation is an early event in carcinogenesis which could be leveraged to detect ovarian cancer (OC) in plasma.

Methods: DNA from frozen OC tissues, benign fallopian tube epithelium (FTE), and buffy coats from cancer-free women underwent reduced representation bisulfite sequencing (RRBS) to identify OC MDMs. Candidate MDM selection was based on receiver operating characteristic (ROC) discrimination, methylation fold change, and low background methylation among controls. Blinded biological validation was performed using methylated specific PCR on DNA extracted from independent OC and FTE FFPE tissues. MDMs were tested using Target Enrichment Long-probe Quantitative Amplified Signal (TELQAS) assays in pre-treatment plasma from women newly diagnosed with OC and population-sampled healthy women. A random forest modeling analysis was performed to generate predictive probability of disease; results were 500-fold *in silico* cross-validated.

Results: Thirty-three MDMs showed marked methylation fold changes (10 to >1000) across all OC subtypes vs FTE. Eleven MDMs (*GPRIN1, CDO1, SRC, SIM2, AGRN, FAIM2, CELF2, RIPPLY3, GYPC, CAPN2, BCAT1*) were tested on plasma from 91 women with OC (73 (80%) high-grade serous (HGS)) and 91 without OC; the cross-validated 11-MDM panel highly

discriminated OC from controls (96% (95% CI, 89–99%) specificity; 79% (69–87%) sensitivity, and AUC 0.91 (0.86 – 0.96)). Among the 5 stage I/II HGS OCs included, all were correctly identified.

Conclusions: Whole methylome sequencing, stringent filtering criteria, and biological validation yielded candidate MDMs for OC that performed with high sensitivity and specificity in plasma. Larger plasma-based OC MDM studies, including testing of pre-diagnostic specimens, are warranted.

Keywords

Ovarian neoplasm/diagnosis; carcinoma; ovarian epithelial/prevention & control; DNA methylation; liquid biopsy; cell-free nucleic acids

Introduction

Epithelial ovarian cancer (OC) constitutes the most lethal gynecologic malignancy, with a projected 21,410 new cases and 13,770 deaths recorded in the United States in 2021 (1). OC often presents at advanced stage disease, and therefore remains highly lethal despite decades of surgical and adjuvant therapy research (2). Early stage OC generally portends a favorable prognosis; however, high-grade serous OCs, the most lethal subtype, rarely presents at an early stage. Large clinical screening trials using transvaginal ultrasound and serum CA-125 have not demonstrated a reduction in OC specific mortality (3–5). Accordingly, development of sensitive early detection methods that achieve high specificity represents a critical unmet need.

Increased DNA methylation in and around gene promoter regions is an early event in carcinogenesis and has functional consequences, including altering expression of tumor suppressor genes and oncogenes (6–9). The identification of broadly informative methylation markers associated with cancer has facilitated the successful development of commercially available screening, diagnostic and prognostic assays for other solid cancers (10–16). However, despite ongoing investigations of methylated genes in OC, development of a marker panel suitable for clinical implementation is lacking (17). Data suggest that OCs may metastasize from small primary lesions, potentially arising in the fimbria of the fallopian tube (18,19), therefore requiring exquisite analytical sensitivity to achieve early detection. However, given the rarity of OC, extremely high specificity is equally important to achieve acceptable positive predictive value, especially as definitive investigation of a positive test may require an invasive procedure. Accordingly, we designed the current study to achieve three aims: 1) discovery of methylated DNA marker (MDM) candidates that discriminate OC from benign fallopian tube epithelium (FTE) and other gynecologic tissues; 2) validation of MDM candidates in tissues from an independent sample set; and, 3) assessment of performance of MDM candidates on independent plasma samples from women with and without OC.

Methods

Study Synopsis

This study was conducted in three phases (Figure 1): The first phase consisted of discovery of OC related MDMs using reduced representation bisulfite sequencing (RRBS) and technical validation using quantitative methylation specific PCR assays (qMSP) to confirm marker sequence and performance. Next, biological validation was performed by testing markers in an independent set of OC and benign tissues. Finally, MDMs, ranked by accuracy and fold-change within each subtype were tested on independent plasma samples from patients with and without OC using Target Enrichment Long-probe Quantitative Amplified Signal (TELQAS) assays. This study was approved by the Mayo Foundation Institutional Review Board.

Discovery and Technical Validation Cohort

Primary fresh frozen OC tissues from women newly diagnosed with OC who underwent primary debulking surgery were identified from within the Mayo Clinic SPORE in Ovarian Cancer biorepository. This biorepository, initiated in 1990 and supported by the Mayo Clinic SPORE in Ovarian Cancer from 2009 to the present, includes frozen tissue and blood from >3700 unique individuals with OC, ovarian borderline tumors, or benign ovarian tumors. All women presenting to Mayo Clinic with clinical findings suspicious for OC are approached to consider enrolling in the biorepository. OC histologies included high grade serous (HGS), endometrioid, clear cell, and mucinous adenocarcinomas. Benign control tissues included 1) prospectively obtained benign fallopian tube epithelium (FTE), specifically collected for this study between January 2018 and March 2018, from women who underwent opportunistic salpingectomy at the time of benign gynecologic surgery and 2) buffy coats from healthy women without cancer who were current on cervical cancer screening and mammography. All OC histologies were verified by one or more gynecologic pathologists (SEK, JKS). Tumor purity was required to be at least 70% among cases. Women who had other cancer diagnoses or who had received chemotherapy class drugs within the previous 5 years, had prior pelvic radiation, or had a prior solid organ or bone marrow transplant were excluded. Clinical variables for all subjects were abstracted from medical records.

Biological Validation Cohort

An independent cohort of women with newly diagnosed OC who underwent primary OC debulking surgery was identified for the biological validation. Formalin-fixed paraffin embedded (FFPE) primary OC tissues representing the same histologies as the discovery cohort were included. In addition, FFPE fallopian tubes from women who underwent benign gynecologic surgeries, frequency-matched based on age, were identified from clinically archived tissues from the Mayo Clinic Tissue Registry. All histologies were verified by one gynecologic pathologist (MES) who also selected the tissue macrodissection sites for DNA extraction (see below). Eligibility criteria were the same as in the discovery set. Normal, cancer-free buffy coat samples from female patients were also utilized as before.

Plasma Quality Control Cohort

Whole blood samples from women with OC and age-matched healthy women without OC that were collected between January 2018 and August 2019 into LBgard® tubes (Biomatrica, San Diego CA) were separately obtained through commercial vendors (Eastern Biologix (Bucharest, Romania) & Viomics (Phoenix, AZ)). For each subject, 6mL of plasma isolated from LBgard® tubes was utilized for cell-free DNA (cfDNA) extraction and testing. These plasma samples were used for additional preclinical marker selection, analytical performance testing, and general quality control metric assessment.

Plasma-Based Clinical Pilot Cohort

Archival EDTA-buffered plasma samples from OC cases were selected from those collected prior to primary surgical debulking from an independent cohort of women with newly diagnosed OC enrolled to the Mayo Clinic SPORE in Ovarian Cancer biorepository between 4/6/2009 and 11/5/2018. OC case inclusion criteria included postoperative histologic confirmation of epithelial OC, no prior ovarian cancer therapy, and at least 3 mL of plasma available. Cases were frequency-matched based on age to a control set of archival EDTA-buffered plasma samples from asymptomatic women without cancer in the prior 5 years, who had an intact uterus and ovaries. Participants were enrolled from a 7-county Minnesota regional population sample and enrolled between 9/13/2016 and 11/8/2019. Additional eligibility criteria were the same as in the discovery and biological validation sets. All case and control patients provided written informed consent for use of their plasma and clinical data.

Discovery—Laboratory Methods

After verification and identification of target tissue by a study pathologist, blocks of fresh frozen OC tissue, embedded in optimal cutting temperature (OCT) compound, underwent microtome cutting by the Mayo Clinic Pathology Research Core to provide ten 10-micron scrolls. Genomic DNA was purified from tissue sections using the QIAmp DNA tissue protocol, and from buffy coat samples using the QIAmp DNA blood protocol (Qiagen, Valencia, CA). The DNA samples were re-purified with AMPure XP beads (Beckman-Coulter, Brea CA) and quantified by PicoGreen (Thermo-Fisher, Waltham MA). DNA integrity was assessed using real time quantitative PCR. RRBS libraries were prepared from approximately 300ng of material following the Meissner protocol (20) with modifications. Indexed samples were combined in a 4-plex format and single end sequenced for 100 cycles by the Mayo Genomics Facility on the Illumina HiSeq 2500 instrument (Illumina, San Diego CA). Samples were randomly arranged for sequencing to reduce bias. Reads were processed by Illumina pipeline modules for image analysis and base calling. DNA from 4 OC cell lines (CAOV3, OVCAR3, SKOV3, and TOV21G) was also included to serve as guides for determining differential methylation; cell line data was not included in the formal analysis.

Technical Validation—Laboratory Methods

Quantitative methylation specific PCR assays (qMSP) were developed for differentially methylated regions (DMRs) meeting performance criteria and applied to the discovery sample cohort. This step was undertaken to confirm the validity of the sequencing data

using a targeted amplification approach. Primers were designed using MethPrimer (21) and QC checked on 20ng (~6250 genome equivalents) of positive and negative methylation controls. Multiple annealing temperatures were tested for optimal discrimination. Ten ng of sample DNA (per DMR) was bisulfite converted using the EZ-96 DNA Methylation kit (Zymo Research, Irvine CA) and amplified using SYBR Green detection on Roche 480 LightCyclers (Roche, Basel Switzerland). Samples were randomly arranged for sequencing to reduce bias. Serially diluted universally methylated DNA samples were used as positive control standards, and negative controls included bisulfite converted and unconverted leukocyte-derived genomic DNA, and converted whole genome amplified (unmethylated) DNA. The MDM results were normalized to a DNA input control (*β-actin*), analyzed using logistic regression, and filtered based on AUC, methylation signal strength, and the fold change ratio between cases and controls. MDMs which performed sub-optimally compared to the RRBS results were dropped.

Biological Validation—Laboratory methods

MDMs passing the first validation step were further tested by qMSP on DNA from independent sets of FFPE tissue. FFPE tissue blocks were macrodissected using a 1mm or 2mm core punch following gynecologic pathologist (MES) identification of the best macrodissection site. DNA was purified using the Qiagen QIAmp FFPE DNA Tissue Kit (part# 56404) and converted as described above. FFPE samples providing at least 350 ng of intact amplifiable DNA were considered adequate. The samples were blinded, randomized, and assayed as in the technical validation. Concentration-corrected copy number of each marker was ranked according to their AUC for discrimination of OC in comparison to benign FTE and buffy coat

TELQAS Design and Testing—Laboratory Methods

MDM qMSP assays were converted to the Target Enrichment Long-Probe Quantitative Amplified Signal format (TELQAS; Exact Sciences, Madison, WI). This multiplexed methodology is a modification to the FDA approved quantitative allele-specific real-time target and signal amplification assay (QuARTS) (22). It is uniquely suited to highly specific and sensitive targeted cfDNA amplification and has been validated at allele fractions < 0.01% (unpublished). The tissue validation samples were retested using the TELQAS formatted assays to ensure the performance met or bettered the qMSP results. Some MDMs required multiple oligo designs to find the most optimal hybridization sites. MDMs that failed to meet earlier performance criteria were eliminated. Additionally, several markers from the discovery phase which we were not able to optimize in the qMSP format proved workable with the TELQAS method.

Plasma Quality Control and Validation—Laboratory Methods

Further testing was undertaken to understand the performance of candidate MDMs in the plasma setting, specifically to control for background methylation in healthy circulating cfDNA, which can obscure the relatively lower analyte signature of tumor-derived material. TELQAS assay designs were tested on pooled cancer free plasma samples to define background noise for each of the candidates and those that were above 0.01 (1%) were eliminated. Additional optimization was performed using commercially sourced plasma

samples (171 cancer-free, 49 ovarian cancers, stages I-IV, various subtypes), the data from which allowed us to refine the final MDM selections for the pilot phase of the study.

Eleven MDMs were tested in independent pre-treatment plasma samples from women newly diagnosed with OC and population-sampled healthy women. Plasma samples were stored at −80C until analysis. Samples were thawed, aliquoted into identical tubes, blinded by barcode, refrozen at −80C and sent to the laboratory in randomized order. DNA was extracted and bisulfite converted as described above. The TELQAS assay was configured to run 5 triplex and 2 biplex reactions, targeting the 11 cancer-specific MDMs and methylated *B3GALT6,* a normalizing marker for total human DNA input in each sample. Detailed steps of the assay protocol have been published previously (23). Briefly, 12 cycles of multiplex PCR amplification of the MDMs were performed on the bisulfite converted DNA and then diluted 10-fold with a 10 mmol/L Tris-HCl and 0.1 mmol/L EDTA solution. Ten μL of the diluted amplicons were used in LQAS assays, performed on ABI 7500DX Equipment (Applied Biosystems).

Statistical Analysis—Discovery

Streamlined Analysis and Annotation Pipeline for RRBS (SAAP:RRBS), an in-house analysis software package, was used for quality scoring, sequence alignment, and annotation to a UCSC reference genome (24,25). First, candidate CpGs were filtered by *a priori* read-depth (≥10), significance of differential %-methylation between OC histologies and benign controls, coverage of CpG across samples, and target to background ratio in the benign control group. CpG islands are typically biochemically defined by an observed to expected CpG ratio >0.6 (26). However, for this model, tiled units of CpG analysis or differentially methylated regions (DMRs) were created based on regions where %-methylation was observed below a set background level in the benign controls (FTE, buffy coat) and a set distance between CpG site locations (>100bp) for each chromosome. Regions with five or fewer CpGs were excluded. To account for varying read depths across individual subjects, an over-dispersed logistic regression model was used, where dispersion parameter was estimated using the Pearson Chi-square statistic of the residuals from the fitted model. Statistical significance was determined by over-dispersed logistic regression of the average methylation percentage per candidate DMR. Candidate DMRs were filtered according to their significance level, AUC, and fold-change difference between OC cases and benign controls. This approach has been validated in the establishment of methylation profiles for colon and pancreatic cancer (11,25). Sample size considerations were based on the desired statistical power to detect a 10% difference in the %-methylation between any two groups, recommending a sample size of 18 for each group.

Statistical Analysis—Biological Validation and Plasma-Based Clinical Pilot

Distributions of individual markers were examined using boxplots and marker intensity maps. Areas under the receiver operating characteristics curve (AUC) were generated for each marker to assess accuracy. Random forest (rForest) models were used to classify samples using predictive probability of being an OC case. This included bootstrap sampling to generate 500 training sets to derive a prediction algorithm of OC. For each of the 500 training sets, a single recursive partition tree was derived, and the overall prediction of OC

was the average number of trees classifying a sample as OC. Since training of the model is based on bootstrap random sampling, an individual sample was not used in training in approximately 1/3 of the trees within the rForest. Cross-validation was achieved by using all trees within the rForest model, independent of an individual sample (i.e., sample was not used in training a set of trees within the rForest model), to obtain the predicted probability of OC for that sample. Sample size considerations for biological, tissue-based validation were based on minimizing the width of a 95% confidence interval (95% CI) for sensitivity and specificity. With an assumed specificity of 95% a control set of 29 would provide a 95% CI no wider than ± 10%. To achieve a 95% CI that was no wider than ± 7% for a target sensitivity of 90%, a minimum of 84 samples was required. Sample size estimates for the plasma based clinical pilot were based on being able to detect an AUC of 0.70 from the null AUC of 0.50. With 91 OC cases and 91 healthy women there was greater than 80% power to detect this difference using a one-sided test at a 5% significance level. The effect of covariates on the rForest model was evaluated by comparing stratified AUCs.

Results

Ovarian Cancer MDM Discovery and Technical Validation

RRBS was conducted on 57 primary OCs, including 18 HGS, 18 endometrioid, 15 clear cell, and 6 mucinous OCs, in addition to 14 benign FTE and 19 buffy coat samples from cancer-free women. The stage distribution included 25 FIGO stage I (44%), 8 stage II (14%), 19 stage III (33%), and 5 stage IV (9%). Clinicopathologic characteristics of the discovery cohort are detailed in Supplemental Table 1.

There were 3.4 million CpG sites captured in the samples with at least 10 reads and 335,000 CpG sites were selected for further analysis after meeting group coverage and variance criteria with 526 DMRs identified as statistically significant based on variance inflated logistic regression models. A quasi-binomial likelihood was used to estimate the over dispersion parameters due to biological variation beyond what would be predicted for binomial data. Comparisons included 1) pooled OC cases vs benign FTE and buffy coat sample controls and 2) individual OC histology subtypes vs controls. MSP primers were designed for 54 candidate DMRs to proceed through technical validation. Selection was based on performance metrics, marker complementarity, and individual CpG methylation patterns. In general, all candidates had AUCs >0.85, fold-change levels >5-fold over controls and were at least 20% methylated at every CpG. Amplicon lengths were 45–120bp and addressed 5–8 CpGs per MDM assay. qMSP was performed on DNA from the same 90 cases and controls that had underwent RRBS. Forty-four of the 54 candidate genes identified in the discovery set had an AUC >0.90, with a signal-to-noise ratio of over 10-fold and a control group methylation of <5%.

Biological Validation of Candidate Ovarian Cancer MDMs

Independent biological validation was performed on 33 MDMs based on their performance in the qMSP technical validation: *AGRN, BANK1, BCAT1, BCL2L11, C2CD4D, CAPN2, CDO1, CELF2, DNMT3A, GATA2, GDF6, GPRIN1, GYPC, IFFO1, KCNA3, MAML3, MAX.chr1:1477, MAX.chr6:1038, MAX.chr11:1492, MAX.chr14:1055, MT1A (region 1),*

MT1A (region 2), NCOR2, NR2F6, PALLD, PARP15, PDRM14, RIPPLY3, SIM2, SKI, SLC12A8, TACC2, ZMIZ1. qMSP was conducted on DNA from an independent, blinded set of 105 OCs, including 34 HGS, 2 low-grade serous (LGS), 28 endometrioid, 25 clear cell, and 16 mucinous in addition to 31 benign FTE. Clinicopathologic characteristics of this cohort are described in Supplemental Table 2. All 33 MDMs showed marked methylation fold changes (10 to >1000) across all OC histologies in comparison with benign FTE (Figure 2). In addition, 26 MDMs demonstrated high cancer discrimination (AUC >0.90) in 1 or more OC histologic subtype vs either FTE or buffy coat controls (or both). Nineteen MDMs were 100% discriminate between OC and benign controls. All of these MDMs were retested in the TELQAS format to confirm performance, including a small number of DMRs we identified in the discovery which we had wanted to validate initially but were not able to be transitioned to a qMSP format. However, we were successful in designing functional and optimized TELQAS assays for them, including *FAIM2*, *JAM3*, *LRRC41*, *SRC*, and *TSHZ3*. Of these, two in particular – *FAIM2* and *SRC* – had sensitivities of 71.9% and 66.7%, respectively, at 95% specificity.

Testing Candidate Ovarian Cancer MDMs in Plasma of Women with and Without Ovarian Cancer

After testing and optimization of TELQAS designs in the commercially sourced plasma samples, we chose 11 MDMs for the Mayo collected and phenotyped plasma sample pilot. The decision tree around the selection was a combination of those loci with a minimum of methylation noise in normal plasma cfDNA (<1%, generally), having a variety of OC subtype specificity to cover all prospective comers (even though we were highly weighted toward the high-grade serous subtype), and the initial performance of individual MDMs and panels of complementary MDMs in both the tissue cancer samples (N=105) and the test plasma cancer samples (N=49). The 11 final MDMs (*GPRIN1, CDO1, SRC, SIM2, AGRN, FAIM2, CELF2, RIPPLY3, GYPC, CAPN2,* and *BCAT1*) were then tested on plasma from 91 women with OC and 91 healthy population-sampled control women without OC. OC cases included 73 HGS, 4 LGS, 8 endometrioid, 4 clear cell, and 2 mucinous. Clinicopathologic characteristics of both the OC case and healthy control groups are detailed in Table 1. When comparing pooled OC cases to controls, the 11 MDMs individually had marked methylation fold changes compared to benign controls (Figure 3A) and the best individual MDM AUC (0.82 (95% CI 0.76, 0.82)) was observed in *SIM2*. Table 2 lists the AUCs for each of the 11 OC MDMs tested in plasma.

The cross-validated combined 11-MDM panel discriminated OC from healthy controls with 96% (95%CI 89–99%) specificity, 79% (69–87%) sensitivity, and an AUC 0.91 (0.86 – 0.96)) (Figure 3B). When dichotomizing clinical covariates of age, BMI (both based on median values in pooled OC cases and benign controls), smoking (ever smoker v. others), and menopausal status (postmenopausal v. others), there was a trend toward a higher AUC (0.95 compared to 0.85) associated with the lower BMI category (p=0.053). There was not a statistically significant difference in the 11-MDM panel performance when age, smoking, and menopausal status were considered (Table 3).

Overall, at a 95% specificity, the 11-MDM panel correctly identified 63 (86%) of the 73 HGS OCs, including all 5 of the stage I/II HGS OCs. Additionally, the 11-MDM panel correctly identified 1 (25%) of the 4 LGS OCs, 3 (75%) of the 4 clear cell OCs, 4 (50%) of the 8 endometrioid OCs, and 1 (50%) of the 2 mucinous OCs.

Biological Roles of OC MDMs

To assess the potential functional significance of the identified OC MDMs, we sampled a random selection of the 526 initial DMRs, used genomic coordinates to map to highly annotated genes (RefSeq), and then queried Uniprot for molecular and biological roles. DMRs most commonly mapped to either 5-prime regulatory sequences or intronic gene body locations. Gene-protein function for all DMRs included operative pathways known to be important for driving tumorigenesis; these included transcriptional regulation, cell cycle, growth, signaling, and apoptosis. For the final 11 OC MDMs, we confirmed pathway associations relevant to cancer and identified previously published evidence of cancer-related actions for each these genes (Supplementary Table 3) (27–45).

Discussion

Using whole methylome sequencing with stringent filtering criteria and biological validation, we identified 11 candidate MDMs that have high sensitivity and specificity in discriminating women with OC from women without OC based on plasma testing. Importantly, the panel correctly identified 100% of stage I/II HGS OCs. As such, these OC MDMs hold promise in the development of a blood-based detection method that may also allow for earlier detection of OC.

The detection of circulating tumor DNA, including methylated tumor DNA, for early diagnosis and monitoring of cancer is a rapidly expanding area of research. Recent advancements in assay technology have facilitated increased analytical sensitivity, thereby increasing the potential for early detection of cancers in plasma when circulating levels of tumor DNA are low (46,47). In particular, the TELQAS assay chemistry represents one such advancement imparting an analytical sensitivity threshold of 2–4 DNA strands/mL of plasma. This method has been previously demonstrated by our group to detect methylated DNA from esophageal cancer, gastric cancer, hepatocellular carcinoma, and colorectal cancer in plasma samples with a high sensitivity and specificity (23,48–50). Here we demonstrate for the first time, discrimination of both early and advanced stage OC patients from healthy control women using TELQAS assays with plasma samples. These findings support further evaluation of the 11-MDM OC panel in larger case-control studies with aims that include testing the panel for complementarity or superiority to CA-125 in women presenting with an adnexal mass and reducing the panel to the smallest number of required MDM candidates. Cohort designs are anticipated to ultimately test the OC MDM panel in high and average risk asymptomatic women.

While methylation-based diagnostic and/or early detection test development has been successfully translated to the clinic in colorectal cancer (11), the heterogeneity of OC histologies poses a challenge in the development of a broadly representative yet highly sensitive and specific biomarker panel for OC. Not only is there variability in stage

distribution and clinical behavior among the histologies, but others have shown through gene expression profiling and methylation analyses that distinct molecular differences exist among the histologic subtypes (51,52). To account for these phenomena, our approach to discovery and validation of candidate OC MDMs included the spectrum of most common OC histologies.

An additional challenge is that the most common OC histology, HGS, appears to arise from small fallopian tube serous tubal intraepithelial carcinomas (STICs) (18,19) that exfoliate onto the ovary or into the peritoneal cavity where clinical growth of symptomatic disease at an advanced stage usually prompts the evaluation and diagnosis. While 86% of HGS OCs in our study were correctly identified via the 11-MDM OC panel, the majority were advanced stage. A promising finding, however, is that among those HGS OCs that were early stage, the 11-MDM panel also identified 100% of them. And recently, Pisanic and colleagues demonstrated that STICs are indeed epigenetically similar to HGS OCs and dissimilar to benign FTE (53). However, it remains unknown whether the presence of STICs could be identified via a blood-based assay.

Strengths of this study include the intentional representation of the most common OC histologic subtypes in both discovery and validation cohorts and the confirmation of each histologic diagnosis by gynecologic pathologists. To complement that, robust clinical exclusion criteria were utilized, including the exclusion of patients who had another cancer diagnosis within 5 years prior to or 3 years after their OC diagnosis. This was especially important in the setting of mucinous OC histologies given the potential for them to represent metastatic disease from gastrointestinal tract primary malignancies (54). An additional strength of our study includes the use of control samples from both benign FTE and buffy coat. By controlling for methylation patterns in benign FTE, the precursor tissue to most HGS OC, and white blood cells, the highest contributors of circulating DNA in plasma, we were able to establish and validate the specificity of our selected MDM panel (46,55). Additionally, many of the methylation markers we identified, including all 11 in the final panel were corroborated in their functional roles in carcinogenesis by a search of the PubMed database (27–45). While the cancers already known to be associated with these genes are not ovarian malignancies, their relevance can be extrapolated based on the roles the genes play in tumorigenesis overall. Further, these functionally significant roles provide a measure of external validity and biological relevance to our marker selection methods.

This study also has limitations. While the 11-MDM panel performed promisingly well, larger studies with increased representation of non-HGS histologies, larger numbers of early stage OCs, as well as studies including women with benign ovarian masses are needed. The availability of ample banked plasma volume among the less common histologies for the translational pilot contributed to this limitation and opportunities may exist to streamline volume needed. Additionally, in this study we were not able to meaningfully incorporate CA-125 into the plasma-based panel given the high proportion of advanced stage and HGS histologies resulting in a substantially higher than upper limit of normal median CA-125 among the OC cases. However, future studies in women with isolated adnexal masses and/or early stage OC should include CA-125 within the biomarker panel. We also excluded patients who received chemotherapy within the five years prior to OC diagnosis, had prior

pelvic therapeutic radiation, or had received a transplant secondary to the potential for these factors to interfere with methylation levels. Further research is needed to assess the applicability of the identified OC MDMs in these populations. All women included in this study were from a single institution and were predominantly White. A larger, more diverse population-based study set with representative sampling will allow estimation of OC MDM positive and negative predictive values. In addition, the identification of higher risk groups based on age and genetic risk factors is essential in optimizing positive predictive value.

In summary, we utilized robust methodologies and quality control in the identification of a panel of OC MDMs in tissue and demonstrated the feasibility to detect these OC MDM in plasma, highly discriminating between the presence and absence of OC. Sensitivity and specificity for the HGS histologic subtype in plasma were promisingly high and larger plasma-based studies with expanded populations of non-HGS histologic subtypes, earlier OC stages, benign neoplasms, and more diverse populations of women are warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Whole methylome sequencing identified novel ovarian cancer methylated DNA markers.
- An 11-MDM ovarian cancer panel discriminated between ovarian cancer and no cancer in plasma.
- In plasma, the 11-MDM panel identified all 5 early-stage high grade serous ovarian cancers.

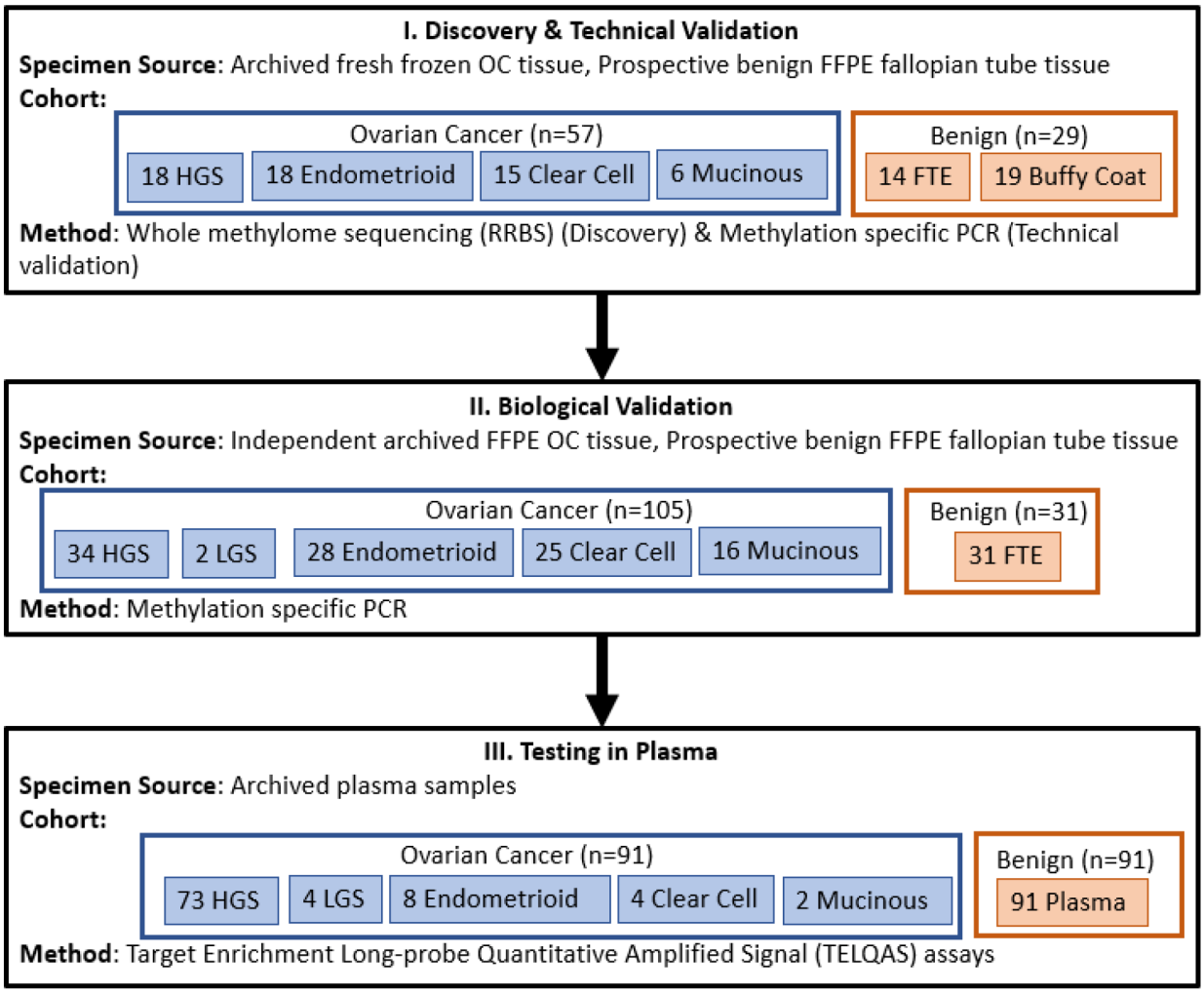

Figure 1.
Overall study flow diagram.

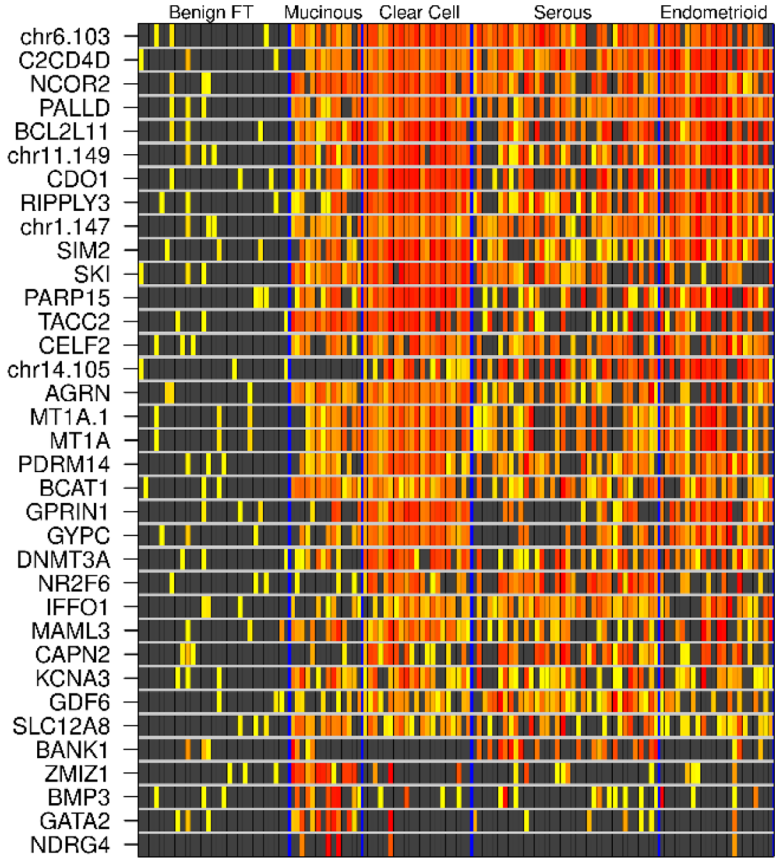

Figure 2.

Heatmatrix for tissue-based biological validation. Increasing deciles of methylation intensity are depicted on a yellow-red color spectrum. Each row is a candidate MDM, each column is a patient tissue sample.

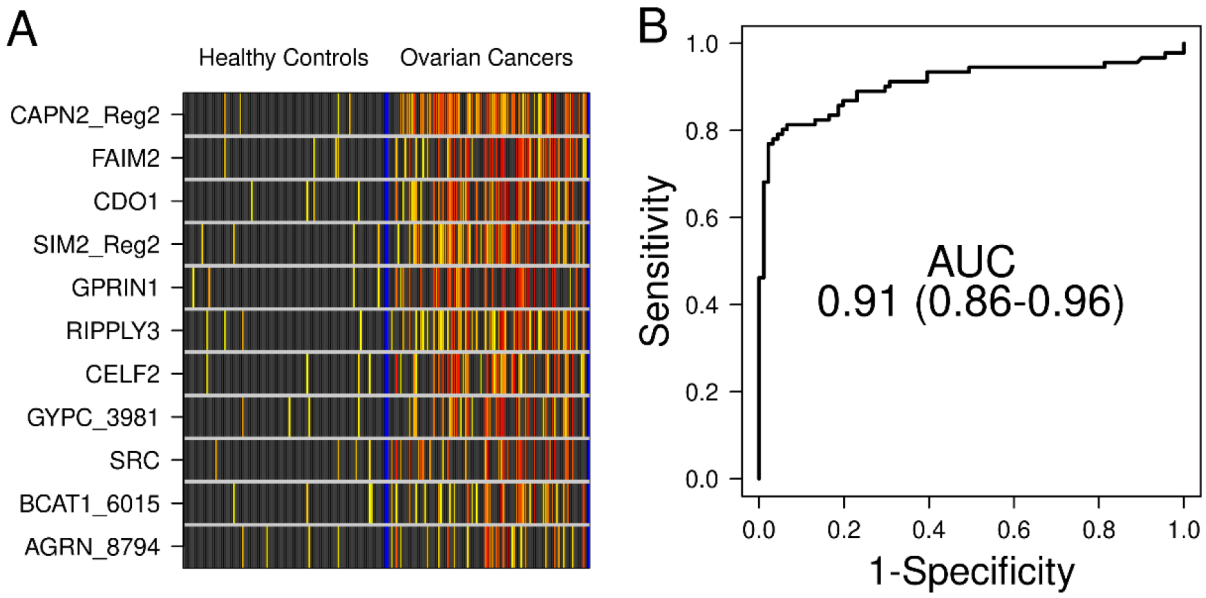

Figure 3.

A. Methylation intensity of top 11 OC MDMs in plasma within the plasma-based clinical pilot. Black boxes indicate values of *B3GALT6*-normalized MDM qMSP product below the control samples' 95th percentile. For products above that threshold, increasing deciles of intensity are depicted on a yellow-red color spectrum. Each row is a candidate MDM, each column is a patient sample. **B.** In plasma, the 11-MDM panel discriminated between OC cases and healthy controls with an AUC of 0.91.

Table 1.

Clinicopathologic characteristics of plasma-based clinical pilot OC cases and healthy population-sampled control women.

Characteristic	Ovarian cancer (n=91)	Healthy controls (n=91)
Age, years (median [IQR])	61 [57–68]	61 [58–66]
BMI, kg/m^2 (median [IQR])	27.25 [24.66–30.5]	27.64 [22.95–31.45]
Pregnancies (median [IQR])	2 [1–3]	2 [1–4]
Live births (median [IQR])	2 [1–3]	2 [1–3]
Race		
White	75 (83%)	89 (98%)
Non-White	13 (14%)	1 (1%)
Unknown	3 (3%)	1 (1%)
Tobacco Use		
Current	9 (10%)	13 (14%)
Previous	23 (25%)	26 (29%)
Never	59 (65%)	52 (57%)
Menopausal Status		
Premenopausal	7 (8%)	9 (10%)
Perimenopausal	5 (5%)	5 (5%)
Postmenopausal	74 (81%)	77 (85%)
Unknown	5 (5%)	0 (0%)
Histology		
High grade serous	73 (80%)	-
FIGO grade 1 or 2 endometrioid	8 (9%)	-
Clear cell	4 (4%)	-
Low grade serous	4 (4%)	
Mucinous	2 (2%)	-
Stage		
I	10 (11%)	-
II	5 (5%)	-
III	64 (70%)	-
IV	12 (13%)	-
CA-125 U/mL (Median [IQR])	358.4 [119.2 – 1044.8]	8.6 [5.8–12.5]

BMI, body mass index; IQR, interquartile range

Table 2.

Individual performance of each of the 11 OC MDMs in plasma-based clinical pilot.

MDM	AUC (95% CI)
SIM2	0.82 (0.76, 0.82)
SRC	0.78 (0.72, 0.78)
RIPPLY3	0.77 (0.7, 0.77)
AGRN	0.77 (0.7, 0.77)
CDO1	0.75 (0.68, 0.75)
BCAT1	0.75 (0.68, 0.75)
GYPC	0.73 (0.66, 0.73)
CELF2	0.73 (0.66, 0.73)
CAPN2	0.72 (0.64, 0.72)
FAIM2	0.64 (0.55, 0.64)
GPRIN1	0.56 (0.47, 0.56)

MDM, methylated DNA marker; AUC, area under the receiver operator curve; CI, confidence interval

Table 3.

rForest fit AUC (95% CI) in plasma stratified by clinical covariates.

Characteristic	Yes	No	p value
Age ≥ 61	0.92 (0.86–0.99)	0.90 (0.82–0.97)	0.583
BMI ≥ 27.5	0.85 (0.76–0.94)	0.95 (0.91–1)	0.053
Ever smoker	0.89 (0.79–0.98)	0.91 (0.86–0.97)	0.656
Postmenopausal	0.92 (0.87–0.97)	0.87 (0.70–1)	0.5694

AUC, area under the receiver operator curve; CI, confidence interval; BMI, body mass index