Differential Methylation Profile of Ovarian Cancer in Tissues and Plasma

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An accurate biomarker for detection of ovarian cancer may reduce cancer-related mortality. Using a previously developed microarray-based technique, we evaluated differences in DNA methylation profiles in a panel of 56 genes using sections of serous papillary adenocarcinomas and uninvolved ovaries ($n = 30$) from women in a high-risk group. Methylation profiles were also generated for circulating DNA from blood of patients ($n = 33$) and healthy controls ($n = 33$). Using the most differentially methylated genes for naïve Bayesian analysis, we identified ten of these profiles as potentially informative in tissues. Various combinations of these genes produced 69% sensitivity and 70% specificity for cancer detection as estimated under a stratified, fivefold cross-validation protocol. In plasma, five genes were identified as informative; their combination had 85% sensitivity and 61% specificity for cancer detection. These results suggest that differential methylation profiling in heterogeneous samples has the potential to identify components of a composite biomarker that may detect ovarian cancer in blood with significant accuracy. (*J Mol Diagn* 2009, 11:60–65; DOI: 10.2353/jmoldx.2009.080072)

Despite its relatively low prevalence,¹ ovarian cancer is the most frequent cause of death from gynecological malignancies. At early stages, women are mostly asymptomatic or present with vague and non-specific symptoms, so early ovarian cancer is difficult to diagnose. Considering that patients diagnosed with stage I ovarian cancer have a 5-year survival rate over 90%, early detection of ovarian cancer may reduce cancer-related mortality.

It has been suggested that a screening test for ovarian cancer should have a positive predictive value of 10% or more; such that 10 women would undergo exploratory surgery to diagnose one cancer.² The low prevalence of ovarian cancer requires that a screening test has a sensitivity of at least 75% and a specificity of at least 99.6%.² The screening test should also be simple, inexpensive,

and produce only minimal discomfort for the patient. Such a test has yet to emerge.

The most widely used procedure for ovarian cancer detection and monitoring is a blood-based test for cancer antigen 125 (CA125).^{3,4} Its specificity for early-stage disease is high (96% to 100%), but the sensitivity is low,⁵ so the test must be combined with other diagnostic techniques. A two-line screening procedure has been suggested in which candidates with high CA125 undergo follow-up transvaginal ultrasonography.⁶ Unfortunately, this combination still has only a limited sensitivity because of the low sensitivity of the initial CA125 test.⁷ Even when high-risk women are screened, the test does not provide considerable advantages⁸; it does not detect tumors early enough to influence outcomes.⁹ As a result, low sensitivity and a high rate of false-negative results of the CA125 test reduce access to transvaginal ultrasonography; on the other hand, the low sensitivity of transvaginal ultrasonography for early cancer suggests that the effect on prognosis would be negligible.⁹

To improve detection, combinations of CA125 with other antigens have been suggested.¹⁰ The paradigm involves combinations of blood-based markers as the first line followed by confirmatory transvaginal ultrasonography.⁴ Among other analytes, DNA has certain advantages—it is a relatively stable molecule that can be amplified in polymerase chain reaction providing high analytical sensitivity; it can be recovered from the blood (eg,¹¹) and can be used as a biomarker either directly¹¹ or as a substrate for genetic testing. It can also be used to test for abnormal DNA methylation, which has been found in ovarian tumors.¹²

We hypothesized that methylation of a single gene would not provide sufficient accuracy, but that a combination of several informative genes (a composite biomarker) would. To test this hypothesis, we investigated methylation in 56 genes in each clinical sample using DNA from ovarian

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tumors and unaffected ovaries, and cell-free plasma DNA from ovarian cancer patients and healthy controls.

Materials and Methods

Clinical Specimens

This project was approved by the Institutional Review Board at Northwestern University. Formalin-fixed paraffin-embedded tissues were provided by the Pathology Core Facility of the Robert H. Lurie Comprehensive Cancer Center, Feinberg School of Medicine, Northwestern University. Serous papillary adenocarcinoma (stage 3 in over 80% of samples) with mostly endometrioid components was selected; the tumor description from the Surgical Pathology final report was confirmed by a single pathologist. The control group included ovarian tissues from subjects of the high-risk group defined as women with family history of ovarian cancer, personal history of breast cancer or women with a mutation in BRCA1 gene; in most cases follicular and luteal cysts were present. Plasma from women with serous papillary adenocarcinoma was provided by the Fox Chase Cancer Center Biosample Repository. Blood specimens were collected from ovarian cancer patients before tumor removal or initiation of chemotherapy. Stages of disease and tumor grades were extracted from the Surgical Pathology final report. Plasma from healthy female volunteers of similar age and race was deposited in the same Repository. A brief description of the samples, including stage of the disease, grade of the tumor, and age of donors is presented in Table 1.

DNA Isolation

One 10- μ m section from a paraffin block was used for DNA isolation. After deparaffination and ethanol precipitation, the pellet was processed using a DNAeasy Tissue kit (Qiagen, Valencia, CA). Purified DNA was dissolved in 10 mmol/L Tris pH7.8, 0.5 mmol/L EDTA. DNA from plasma (0.2 ml) was purified using DNAzol reagent (Molecular Research Center, Cincinnati, OH).

Microarray Mediated Methylation Assay: Overall Approach

The assay for methylation analysis (MethDet test) was initially developed using gel-based detection (methylation-sensitive restriction enzyme digestion with PCR).¹³ To increase throughput, microarray-based detection was adopted (microarray mediated methylation assay or M³-assay).¹⁴ In the MethDet test, one portion of each genomic DNA sample was digested with a methylation-sensitive restriction enzyme while another portion of the same sample served as a control. Selected regions of the genomic DNA from each of the digested and undigested DNA samples were amplified by PCR using gene-specific primers that flank designated restriction sites. In the digested portion, only fragments with methylated sites were resistant to digestion and were capable to serve as templates, whereas in the undigested (control) portion, all fragments were amplified. The two sets of PCR products was compared by competitive hybridization to custom-designed microarrays (M³-assay).¹⁴ Fluorescent signals of hybridized fragments in the M³-assay were separately scored, and the ratio between the signals from control and digested DNAs was calculated. This ratio was used to assign methylated or unmethylated "calls." The data were statistically assessed to select groups of informative fragments, which were then analyzed together as a composite biomarker. Detailed description of the method is presented in Melnikov et al.¹⁴

Statistical Analysis

Methylation calls were made independently for each spot, and final gene-specific calls were made according to the majority call from the triplicate spots for that gene. If there was no majority, the final call was not assigned. As with expression microarray analysis,¹⁵ non-specific filtering removed uninformative spots (detectable calls in less than 2/3 of the samples or less than 10% differential methylation across the entire sample set). Informative genes with $P < 0.10$ were selected by Fisher's Exact Test for differential methylation in gene-specific analyses

Table 1. Clinical Specimens Used in the Study

	Age				Histology	Stage Range	Grade
	Mean	Median	SD	Range			
Tissue specimens							
Disease	59	58.5	14	29–80	Serous papillary adenocarcinoma	1c–4	1–3
Control	47.4	44	8.5	32–61	NA	NA	NA
Plasma specimens							
Disease	65	63	8.4	50–80	Serous papillary adenocarcinoma	3a–4	1– 3c
Control	65	63	8.5	50–81	NA	NA	NA

Tissue specimens in the Disease group were from women with the diagnosis of serous papillary adenocarcinoma in the final pathology report. Tissue specimens in the Control group were from women of the high risk group (see Materials and Methods for description) undergoing preventive oophorectomy with follicular cysts being the most frequent pathology. Plasma specimens in the Disease group were from women with serous papillary adenocarcinoma collected before surgery and chemotherapy. Plasma from healthy donors (no pathology, NA) was used for the Control group.

comparing methylation status for cancer and normal samples. The moderate P value of 0.10 was chosen to include informative genes with occasionally inflated P values due to random subsetting of the data in cross-validation. The previously reported independence of methylation sites,¹⁶ also evident in our samples, suggested use of the naïve Bayes classifier.¹⁷ Naïve Bayes classifiers were constructed using the e1071 R (R Development Core Team, 2005) package,¹⁸ using an uninformative prior with probabilities of 0.5 for normal or cancer classification. The predictive ability of the naïve Bayes classifier was estimated using 25 rounds of fivefold cross-validation. For each round of cross-validation, the data were partitioned into five sets with an equal distribution of diseased and control specimens. Each set then served as a test set based on training of the naïve Bayes classifier with the other four sets. Sensitivity and specificity were estimated and averaged over all five runs and over 25 random partitionings of the data into five groups. Gene selection and classifier parameter estimation were performed anew with each round of cross-validation.

A more detailed description and validation of the technique was presented earlier.¹⁴

Results

MethDet-Test

Development and validation of the assay for methylation analysis (MethDet-test) was described¹³ and its application to clinical specimens (M^3 -assay) was demonstrated.¹⁴ Microarray-based detection by the M^3 -assay was used in this project.

Clinical Specimens

The age of the subjects and their corresponding tumor descriptions are presented in Table 1. Most (26 of 30 or 86.7%) of ovarian cancer cases ($n = 30$) had advanced disease (stage 3b and higher), and only four cases had lower stages. Most of the tumors were either moderately or poorly differentiated (90% of samples were grade 2 or higher) and only three tumors were either grade 1 or borderline. The histology of the tumors was predominantly serous papillary adenocarcinoma (70%) with additional endometrioid components present in 30% of the cases. As ovarian tissues from healthy women were not available, the control group ($n = 30$) contained tissues from women at high risk for ovarian cancer undergoing preventive bilateral salpingo-oophorectomy. This high risk group included women with family history of ovarian cancer or personal history of breast cancer, and six women had confirmed mutations of *BRCA1*. According to the pathology reports, no neoplastic changes were detected in specimens from this group although a possibility of occult neoplasia could not be excluded. Most of the samples (83.3%) contained multiple cysts. Five specimens contained benign tumors (cystadenoma, adenofibroma, teratoma), and surface epithelial hyperplasia was noted for two samples. Cancer cases were on average

older than controls, with mean age 59 vs. 47.4 ($P < 0.001$ using a two sample Student's t -test).

Plasma samples were obtained from a different cohort of healthy women ($n = 33$) and women with serous papillary adenocarcinoma (confirmed after surgery; $n = 33$). All samples were collected before surgery and/or chemotherapy. Cases and controls were age-matched (average age 65 in both groups). All cancer cases had disease at stage 3A or higher; of the 22 cases where the tumor grade was established only three were well-differentiated (grade 1), while all of the rest were poorly differentiated (grade 3 and higher).

Statistical analysis was done as described in Materials and Methods. Results are presented as 2×2 contingency tables (Table 2) with summary statistics shown in Table 3. Sensitivity was determined as the number of positive tests among the cancer cases divided by the total number of cancer cases. Specificity was determined as the number of negative tests among the controls divided by the total number of controls. Accuracy was determined as the number of correct tests divided by the total number of tests. Positive predictive value was determined as the proportion of correctly diagnosed cancer samples, while negative predictive value was determined as the proportion of correctly diagnosed controls.

Genes of the Composite Biomarker

Ten genes were consistently predictive for ovarian cancer detection in multiple rounds of cross-validation when tissue samples were used (Table 4A), while five were important for cancer detection using plasma samples (Table 4B). Hypomethylation in tumor samples compared to controls was always important for the classification, which was consistent with the design of the MethDet-test.^{13,14} Only the unmethylated signal in a heterogeneous tumor sample could be unequivocally assigned to tumor cells; on the other hand, methylated signals could originate from any part of the specimen, including stroma, so their informative value for tumor characterization was very low.

Five genes were consistently selected for detection using circulating DNA (Table 4A), and three of them (*BRCA1*, *PGR*, and *THBS1*) were parts of the tissue-based composite biomarker. For the remaining two genes, methylation of *HIC1* was identified in ovarian tumors,¹⁹ but *PAX5* involvement was not reported previously. Our results correlated well with data from the Cairns' group, who described increased methylation of *BRCA1* and *RASSF1A* in serum of ovarian cancer patients²⁰; while *RASSF1A* was among the genes tested, it was not selected as an informative gene in our analysis. The same was true for hypermethylation of *MLH1*, which was identified as a predictor of poor survival for ovarian cancer patients after carboplatin/taxol chemotherapy.²¹

Discussion

Current knowledge of ovarian cancer is insufficient for the development of mechanistic biomarkers, but correlative

Table 2. Cancer Detection in Tissue and Plasma Specimens

Tissue Specimens (30 per group)				Plasma Specimens (33 per group)			
		True				True	
		Cancer	Normal			Cancer	Normal
Predicted	pCancer	TP = 0.694 (20.82)	FP = 0.298 (8.94)	Predicted	pCancer	TP = 0.851 (28.08)	FP = 0.389 (12.84)
	pNormal	FN = 0.306 (9.18)	TN = 0.702 (21.06)		pNormal	FN = 0.149 (4.92)	TN = 0.611 (20.16)

Values predicted by the MethDet test (Predicted) are compared with true values (True) in 2×2 contingency tables, and true positive (TP), false positive (FP), false negative (FN), and true negative (TN) were determined. Raw numbers (average for 25 rounds of fivefold cross-validation) are in parenthesis.

biomarkers may have a positive impact on cancer treatment. Correlative biomarkers based on abnormal DNA methylation have significant appeal, because multiple individual markers (differentially methylated CpG sites) are present in each sample and can be analyzed as a group, while the use of PCR ensures high analytical sensitivity of the technique. In addition, abnormally methylated DNA has been consistently detected in the bloodstream of patients with different cancers including ovarian cancer; this provides the opportunity to develop a minimally invasive test that can be used for screening of asymptomatic women. The test has to accommodate the inherent heterogeneity of DNA extracted from tumor or blood to be clinically applicable. In this project, we have explored the feasibility of a sensitive and specific methylation biomarker for ovarian cancer based on DNA from ovarian tumors or from patients' blood. While the developed test cannot be immediately used for ovarian cancer detection, the results suggest that the approach has merits and that methylation-based cancer detection may be practical.

The MethDet test includes two stages: detection of methylation by digestion with a methylation-sensitive restriction enzyme and detection of the signal for each fragment either by gel electrophoresis (methylation-sensitive restriction enzyme PCR)¹³ or by the microarray-mediated methylation assay (M³-assay).¹⁴ This procedure has been previously validated with alternative methods of methylation detection¹³; it has also been used for methylation detection in clinical samples of breast cancer¹⁴; extensive discussion of the MethDet has been presented.¹⁴

By design and similar to methylation-specific PCR, MethDet evaluates methylation only in a few CpG sites in each promoter, so no rigorous correlation between gene expression and MethDet results is expected.¹⁴

We have used the MethDet test to compare DNA methylation in ovarian tumors and in control ovaries. It is important to emphasize that most tissue specimens in the control group have been removed from women of the high-risk group (family or personal history of breast cancer, family history of ovarian cancer, and mutations in

BRCA1 gene), so the possibility of an occult neoplasm has to be considered.

Initially, we asked whether MethDet could detect any methylation changes in ovarian tumors when compared to normal ovarian tissue. Indeed, differences in methylation of ten out of 56 genes were sufficiently pronounced to warrant their selection as components of the potential composite biomarker (Table 4A). This result confirms that differential methylation can be detected in heterogeneous samples of ovarian tumors and normal ovaries, indicating that a diagnostic biomarker based on DNA methylation in tissues is feasible.

Analysis of components of the biomarker shows that tumors have higher frequencies of methylation in all of the contributing genes compared to normal ovarian tissues. Complete or partial inactivation of several of these genes is well-established for ovarian cancer: *BRCA1* is either mutated²² or its promoter is methylated²³; loss heterozygosity (LOH) is frequent at the 22q13 locus that contains *EP300*;²⁴ a combination of LOH and methylation is found for *DNAJC15* (*MCJ*)²⁵ and *MLH1*;²¹ frequent methylation is observed in promoters of *TP73*¹⁹ and *PYCARD* (*TMS1*).²⁶ For other genes (*CDKN1C*-p57, *PGR*, and

Table 4. Informative (Unmethylated) Genes of the Composite Biomarkers
A

	Control	Cancer
Tissue		
<i>BRCA1</i>	20 (66.7%)	8 (26.7%)
<i>EP300</i>	17 (56.7%)	9 (30%)
<i>NR3C1</i> (<i>GR</i>)	19 (63.3%)	5 (16.7%)
<i>MLH1</i>	22 (73.3%)	7 (23.3%)
<i>DNAJC15</i> (<i>MCJ</i>)	21 (70%)	11 (36.7%)
<i>CDKN1C</i> (<i>p57kip2</i>)	19 (63.3%)	3 (10%)
<i>TP73</i>	25 (83.3%)	8 (26.7%)
<i>PGR</i> (<i>prox</i>)	16 (53.3%)	1 (3.3%)
<i>THBS1</i>	27 (90%)	12 (40%)
<i>PYCARD</i> (<i>TMS1</i>)*	20 (76.9%)	9 (34.6%)

N = 30 for each group.

*TMS was detected in 26 samples.

B

	Control	Cancer
Plasma		
<i>BRCA1</i>	16 (48.5%)	2 (6.1%)
<i>HIC1</i>	16 (48.5%)	7 (21.2%)
<i>PAX5</i>	14 (42.4%)	7 (21.2%)
<i>PGR</i> (<i>prox</i>)	18 (54.5%)	6 (18.2%)
<i>THBS1</i>	16 (48.5%)	3 (9.1%)

N = 33 for each group.

Table 3. Summary Statistics of the Test

	Sensitivity	Specificity	Accuracy	PPV*	NPV*
Tissue	69.40%	70.20%	69.80%	69.96%	69.64%
Plasma	85.10%	61.10%	73.10%	68.60%	85.70%

*PPV and NPV depend on prevalence, which in this case is 0.5.

Sensitivity [TP / (TP + FN)], specificity [TN / (FP + TN)], accuracy [(TP+TN)/(P+N)], positive [PPV = TP / (TP+FP)] and negative [NPV = TN / (TN+FN)] predictive values are presented.

THBS1), there is a good correlation between increased methylation in tumors (this study) and reduced expression in ovarian cancer.²⁷

The accuracy of the biomarker for cancer detection has been established by stratified cross-validation as described in the Materials and Methods. Both sensitivity and specificity are moderate (Table 3); this may result in part from the presence of tissues with occult neoplasia in the control group and/or on the suboptimal selection of genes for MethDet test. Nevertheless, cancer detection in tissue samples is important as a demonstration that multiplexed analysis of DNA methylation in heterogeneous samples can produce meaningful results and can be useful for tumor detection. Clinical utility of the tissue-based detection is limited to analysis of surgically removed or biopsy samples and may not translate to a diagnostic assay.

Analysis of the methylation of circulating DNA from blood holds a greater promise for cancer detection. Such DNA is present in blood of ovarian cancer patients,¹¹ so we have analyzed cell-free circulating DNA from ovarian cancer patients and healthy gender- and age-matched controls with the MethDet test. To exclude treatment-related changes in methylation, plasma samples have been obtained either before or during surgery and before initiation of therapy. The sensitivity of plasma-based detection has been much better than for tissues (85%), but the specificity has remained low (Table 3) likely reflecting suboptimal selection of genes for the assay. It is encouraging that similar genes have been identified for both the tissue-based and blood-based potential biomarkers (Table 4), suggesting that selected correlative biomarkers do detect certain mechanistic features of ovarian cancer.

Significant sensitivity of blood-based detection was achieved, suggesting that the chosen approach can be optimized. One of the obvious directions is improvement of target selection for MethDet: if a considerable sensitivity can be achieved within the existing analytical space of 56 promoters, one can expect that a rational choice of targets will improve the accuracy to the clinically relevant level. While additional optimization is essential, our results support the feasibility of a composite biomarker for ovarian cancer based on methylation detection in circulating DNA.

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