Tumor Cell-Specific BRCA1 and RASSF1A Hypermethylation in Serum, Plasma, and Peritoneal Fluid from Ovarian Cancer Patients

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ABSTRACT

Because existing surgical and management methods can consistently cure only early-stage ovarian cancer, novel strategies for early detection are required. Silencing of tumor suppressor genes such as p16INK4a, VHL, and hMLH1 have established promoter hypermethylation as a common mechanism for tumor suppressor inactivation in human cancer and as a promising target for molecular detection in bodily fluids. Using sensitive methylation-specific PCR, we screened matched tumor, preoperative serum or plasma, and peritoneal fluid (washes or ascites) DNA obtained from 50 patients with ovarian or primary peritoneal tumors for hypermethylation status of the normally unmethylated BRCA1 and RAS association domain family protein 1A tumor suppressor genes. Hypermethylation of one or both genes was found in 34 tumor DNA (68%). Additional examination of one or more of the adenomatous polyposis coli, p14ARF, p16INK4a, or death associated protein-kinase tumor suppressor genes revealed hypermethylation in each of the remaining 16 tumor DNA, which extended diagnostic coverage to 100%. Hypermethylation was observed in all histologic cell types, grades, and stages of ovarian tumor examined. An identical pattern of gene hypermethylation was found in the matched serum DNA from 41 of 50 patients (82% sensitivity), including 13 of 17 cases of stage I disease. Hypermethylation was detected in 28 of 30 peritoneal fluid DNA from stage IC-IV patients, including 3 cases with negative or atypical cytology. In contrast, no hypermethylation was observed in nonneoplastic tissue, peritoneal fluid, or serum from 40 control women (100% specificity). We conclude that promoter hypermethylation is a common and relatively early event in ovarian tumorigenesis that can be detected in the serum DNA from patients with ovary-confined (stage IA or B) tumors and in cytologically negative peritoneal fluid. Analysis of tumor-specific hypermethylation in serum DNA may enhance early detection of ovarian cancer.

INTRODUCTION

There will be an estimated 25,580 new cases and 16,090 deaths from ovarian cancer in the United States this year (1). The highly lethal nature of ovarian cancer is related to the absence of symptoms in the majority of women with early stages of the disease. Seventy percent of patients have advanced disease (stage III or IV) upon presentation with a 5-year survival at best of 15 to 20% despite aggressive treatment. Yet, when the cancer is detected early, women with stage I disease have a 5-year survival of 77 to 87%, and with stage I tumors that are well differentiated, the 5-year survival is 94% (2). Current techniques to screen for ovarian cancer, e.g., physical exam, computed tomography scan, ultrasound, and the CA-125 serum marker, have shown limited success (3–5). The BRCA1 and BRCA2, site-specific ovarian cancer and hereditary nonpolyposis colorectal cancer autosomal dominant familial syndromes, account for an estimated 10% of ovarian cancer (6) and represent a high risk group for screening. Thus, new approaches to early detection of ovarian cancer are urgently needed because existing surgical and management methods can consistently cure only early-stage cancer.

There is broad agreement that the genetic and epigenetic alterations, which initiate and drive cancer, can be potentially useful in the diagnosis and management of cancer (7). Silencing of tumor suppressor genes such as p16INK4a, VHL, and the mismatch repair gene hMLH1 have established promoter hypermethylation as a common mechanism for tumor suppressor inactivation in human cancer and a promising new target for molecular detection (8, 9). Several cancer genes of clear biological significance, including p16INK4a and BRCA1, have been found to have hypermethylation of normally unmethylated CpG islands within the promoter region in ovarian cancer cells (10–12). Hypermethylation can be analyzed by the sensitive methylation-specific PCR (MSP) technique, which can identify 1 methylated allele in 1000 unmethylated alleles (13), appropriate for the detection of few neoplastic cells in a background of normal cells. MSP also allows rapid analysis of multiple gene loci, does not require prior knowledge of epigenetic alteration, and can potentially provide a “yes or no” answer for the detection of cancer (13, 14).

Bodily fluids that surround or drain the organ of interest from patients with various solid malignancies have been successfully used for MSP-based detection. These include detection of lung cancer in serum (15), sputum (16) and bronchial lavage (17), head and neck cancer in serum (18), breast cancer in ductal lavage (19), and prostate (20) or renal cancer (21) in urine. However, ovarian cancer has not yet been tested. Peritoneal washings or ascites (peritoneal fluid), known to contain cancer cells in ovarian cancer patients, are one potential choice of specimen in which to screen for hypermethylated DNA. We hypothesized that the more readily accessible serum or plasma from patients with ovarian cancer might also contain hypermethylated DNA from tumor cells amenable to MSP analysis. As a feasibility study, we therefore screened a series of matched ovarian tumor, serum, and peritoneal fluid DNA, as well as normal and benign disease control DNA for aberrant promoter hypermethylation of BRCA1, RAS association domain family protein 1A (RASSF1A), and other tumor suppressor genes.

MATERIALS AND METHODS

Specimen Collection and Preparation. After approval from the Institutional Review Board, we obtained tumor or cyst tissue specimens via the Fox Chase Cancer Center Tumor Bank Facility and matched preoperative serum or plasma via the Fox Chase Cancer Center Biospecimen Repository from 60 patients, ages 18 to 87 years, diagnosed with an ovarian or primary peritoneal lesion who underwent laparotomy or laparoscopy. Thirty-five patients had histologically verified ovarian tumors comprising 21 papillary serous, 3 mucinous, 4 clear cell, 5 endometroid, 1 transitional cell and 1 undifferentiated. Ten patients had borderline neoplasms of low malignant potential; 5 papillary serous, 4 mucinous, and 1 mixed. Five patients had papillary serous tumors of primary peritoneal origin. Tumors were graded and staged according to Amer-
ican Joint Committee on Cancer guidelines (22). An additional 10 patients had benign ovarian cysts. Approximately 20 to 50 mL of ascites or peritoneal washing were aliquoted from routine collection for cytological analysis in 42 of the 50 cases. An additional 21 archival stage I tumor specimens without matched fluid or serum were also obtained. Twenty serum specimens from normal healthy age-matched women were obtained via the Fox Chase Cancer Center Biospecimen Repository. Ten specimens of histologically normal (non-neoplastic) ovarian tissue were collected from the unaffected ovary in 2 cases of unilateral disease and from 8 female bladder cancer patients who underwent cystectomy.

Tumor tissue was obtained immediately after surgical resection and subsequently microdissected with the assistance of a pathologist. DNA was extracted from tissue, ~50 mL of peritoneal fluid, or 1.5 mL of serum using a standard technique of digestion with proteinase K in the presence of SDS at 37°C overnight followed by phenol/chloroform extraction (23). Tumor specimen DNA was spooled out after precipitation with 100% ethanol. Serum or peritoneal fluid DNA was precipitated with one tenth volume of 10 mM ammonium acetate, 2 μL of glycogen (Roche Diagnostics Corporation, Indianapolis, IN), and 2.5 volumes of 100% ethanol, followed by incubation at ~20°C and centrifugation at top speed (16,000 relative centrifugal force). Approximately 50 ng of DNA were obtained from 1 mL of serum. For paraffin-embedded tissue, 10 7-μm sections were cut with a microtome and put on glass slides. A tumor cell-rich area or cyst, indicated by the pathologist (H. Ehyai), was removed with a razor blade or needle depending on size, using an inverted microscope. The dissected tissue was placed directly into a microcentrifuge tube, washed with xylene, and DNA isolated as above.

MSP. Specimen DNA (0.05 to 1 μg) was modified with sodium bisulfite, converting all unmethylated but not methylated cytosines to uracil followed by amplification with primers specific for methylated versus unmethylated DNA. The genes used for ovarian tumor cell DNA detection were BRCA1 (11), RASSF1A (24), adenomatous polyposis coli (APC; ref. 25), p14ARF (26), p16INK4A (13), and death associated protein-kinase (DAP-kinase; ref. 27). The primer sequences used have all been previously reported and can be found in the report referenced after each gene. The primers for RASSF1A include CpG site positions 7 to 9 on the forward primer and 13 to 15 on the reverse primer as described previously (24). PCR amplification of tumor DNA was performed for 31 to 37 cycles at 95°C denaturing, 58 to 66°C annealing, and 72°C extension with a final extension step of 5 minutes. Cycle number and annealing temperature depended upon the primer set to be used, each of which had been previously optimized for the PCR technology in our laboratory. In each set of DNA modified and PCR amplified, a cell line or tumor with known hypermethylation as a positive control, normal lymphocyte, or normal ovarian tissue DNA as a negative control and water as no DNA template as a control for contamination were included. If no tumor cell line with known hypermethylation of a particular gene was available, normal human lymphocyte DNA in vitro methylated with SsII methylase according to the manufacturers instructions (New England Biolabs, Beverly, MA) was used as a positive control. After PCR, samples were run on a 6% nondenaturing acrylamide gel with appropriate size markers and the presence or absence of a PCR product analyzed.

Statistical Analysis. The sensitivity of MSP-based detection of hypermethylation in peritoneal fluid or serum was calculated as number of positive tests/number of cancer cases. The specificity was calculated as number of negative tests/number of cases without hypermethylation in serum or peritoneal fluid as assessed using Fisher’s exact test. Results were considered statistically significant if the two-sided P was ≤0.05.

RESULTS

The hypermethylation status of the normally unmethylated BRCA1 and RASSF1A tumor suppressor genes was examined in 50 ovarian or primary peritoneal tumor and matched serum and peritoneal fluid DNA by the sensitive MSP assay, which can detect 0.1% cancer cell DNA from a heterogeneous cell population (13). The frequency of promoter hypermethylation of BRCA1 was 12 of 50 (24%) and RASSF1A 25 of 50 (50%) tumors. Thirty-four of the 50 (68%) tumor DNA showed hypermethylation of one or both genes (Table 1). To increase the diagnostic coverage (whether a hypermethylated gene was available as a target in each case), we screened the 16 tumors with unmethylated alleles of BRCA1 and RASSF1A for hypermethylation of the APC, p14ARF, p16INK4A, and DAP-kinase tumor suppressor genes. We found all 16 tumors to have hypermethylated alleles of one or more of these genes (Table 1). Potential diagnostic coverage was further assessed in an additional 21 archive stage I tumor DNA without matched serum or fluid. Twenty of 21, and therefore overall 70 (37 of 38 stage I, 33 of 33 stage III to IV) of 71 (99%) tumor DNA, showed hypermethylation of at least one of the six genes in the panel. Hypermethylation was observed in all histologic cell types (papillary serous, mucinous, endometroid, and clear cell), in all pathological stages and stages of ovarian cancer examined, including well-differentiated stage IA or B tumors, and in borderline neoplasms of low malignant potential. Thus, promoter hypermethylation of the tumor suppressor genes in the panel can be a relatively early event in ovarian tumorigenesis. Hypermethylation was found in patients of all ages (Table 1).

We then determined the hypermethylation status of the same genes in the matched serum and peritoneal fluid DNA and compared the pattern of gene hypermethylation found to that of the corresponding tumor DNA. We detected an identical pattern of gene hypermethylation in 41 of 50 (82%) matched serum or plasma DNA (Fig. 1A and Table 1). The serum-positive cases included 13 of 17 cases of stage I and 28 of 33 stage III to IV tumors. No hypermethylation was detected in serum DNA from nine (18%) patients. There was no statistical association between the tumor stage and positive detection in serum (13 of 17 stage I versus 28 of 33 stage III to IV, P = 0.47, Fisher’s exact test). Twenty-eight of the 30 stage IC-IV patient peritoneal fluid DNA were MSP positive, whereas 26 of 30 were cytologically positive (P = 0.67, Fisher’s exact test). One of the 12 peritoneal fluid DNA from the stage IA or B patients showed methylation (patient 12; Table 1).

In contrast, we did not observe hypermethylation of the gene panel in cyst tissue, serum, or peritoneal fluid DNA from 10 patients with benign ovarian disease or in serum DNA from 20 normal, healthy age-matched women. Hypermethylation was also absent in 10 normal (nonneoplastic) ovarian tissue DNA (Fig. 1B and Table 2). Furthermore, a gene negative for hypermethylation in the tumor DNA was always negative in the matched serum or peritoneal fluid DNA, e.g., patient 27 in the RASSF1A gel panel shown in Fig. 1A. The specificity of the hypermethylated gene panel was therefore 100%.

DISCUSSION

Successful detection of tumor specific aberrant hypermethylation in bodily fluids that surround or drain the organ of interest has been demonstrated in several tumor types (15–20); however, ovarian cancer has yet to be tested. In many patients with ovarian cancer, tumor cells are present in peritoneal fluid by cytological examination. By definition, peritoneal fluid from stage IA and B cancer patients does not contain tumor cells by cytological examination (22), although it is not known whether free neoplastic DNA can be present. Molecular diagnosis in peritoneal fluid may be useful for early detection in high-risk populations and also may complement traditional cytology for molecular staging. For the general population at risk of sporadic ovarian cancer, serum is a preferable choice of bodily fluid for molecular detection because it is readily accessible in all individuals from a peripheral blood sample, is currently used for CA-125 testing, and is enriched for tumor DNA in cancer patients (28). Several recent studies have shown that it is possible to detect tumor-specific genetic or
epigenetic alterations in serum DNA from head and neck, lung, and colon cancer patients (15, 18, 29). Importantly, tumor cell-specific DNA alterations in serum were not limited to patients with metastatic cancer but were also present in serum from patients with early or organ-confined tumors (15, 18, 29). Neoplastic DNA in the serum most likely arises from cells that have left the site of the primary lesion and have invaded the circulatory system but lack the capacity of metastasis to new organs or may be released from the primary tumor as free DNA from nonviable (apoptotic) neoplastic cells (7).

The use of DNA-based methods for the early detection of ovarian cancer has several potential advantages. Because some genetic and epigenetic events will occur early in the disease process, molecular diagnosis may allow detection before symptomatic or overt radiographic manifestations. The epigenetic alteration of aberrant promoter hypermethylation can be detected at sensitive levels by PCR (1 in 1000) and importantly, because the alteration is a qualitative change, can provide a “yes or no” answer and is thus potentially very specific (13, 14).

More than 80% of ovarian cancer is of epithelial origin consisting of papillary serous, mucinous, endometroid, and clear cell histologic cell types. There is also primary papillary serous carcinoma of the peritoneum, which is histologically identical to primary serous carcinoma of the ovary but is suspected to have a multifocal origin from the epithelial lining of the peritoneal cavity. A clinically distinct, intermediate form of epithelial ovarian cancer also exists: the ovarian tumor of low malignant potential (6). The heterogeneity of gene alterations within and between distinct histologic types mandates the use of a panel of genes. Indeed, no single gene is known to be hypermethylated in more than a proportion of ovarian tumors (10, 30, 31). It will likely be necessary to use a panel of genes to maximize detection of any type of adult sporadic cancer, analogous to the need for analysis of several genes for the diagnosis of familial breast cancer or hereditary nonpolyposis colorectal cancer.

Until recently, the few genes identified as hypermethylated in ovarian cancer included GPC3 on the X chromosome (32), NOEY2 in an imprinted region of 1p (33), and myoD1 hypermethylated in
ovarian tumors (34) but also reported to be methylated in normal tissue (35). Only genes hypermethylated in a cancer-specific manner can be used in molecular detection strategies based on conventional MSP analysis. In addition to infrequent **p16 INK4a** hypermethylation (10%), we found slightly more frequent hypermethylation (15%) of two more genes, **p14 ARF** and **DAP-kinase**, in ovarian tumors. Furthermore, hypermethylation of **BRCA1** has been reported in 15 to 20% of sporadic ovarian tumors (11, 12) and a recent profile of hypermethylation reported **RASSF1A** to be hypermethylated in 41%, and **APC** in 18%, of ovarian cancer (31). Thus, it was timely to examine hypermethylation as a target for detection of ovarian cancer in bodily fluids.

Using the **BRCA1**, **RASSF1A**, **APC**, **p14ARF**, **p16INK4a**, and **DAP-Kinase** tumor suppressor genes, we have demonstrated that promoter hypermethylation is common in ovarian cancer, including stage I disease, and can be readily detected in a specific manner in serum and peritoneal fluid DNA. In this initial feasibility study, we observed a sensitivity of 82% in serum. Of interest was that methylation was detected in the serum DNA of four of six patients with CA-125 values of >35 (Table 1). Also, one nonneoplastic control patient with a fibroma had a CA-125 value of 63, but no methylation was detected in the paired serum DNA (data not shown). Overall, hypermethylation was not detected in nine (18%) serum DNA from cancer patients. In these samples, neoplastic DNA may have been present in an amount lower than can currently be detected by conventional MSP. As is routine in PCR methodology, we chose to limit PCR to a maximum number of cycles (n = 37) because it is known that specificity can decrease in MSP, as in other PCR protocols, with increased cycle number (36). It is possible that a higher number of cycles or a two-stage (nested) MSP approach (16) would have resulted in the

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<th>Table 2</th>
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NOTE: Peritoneal fluid (PF) was available from only 8 of 10 patients with cystic disease.

Abbreviation: PF, peritoneal fluid.
positive detection of hypermethylation in the negative serum DNA. We observed no significant difference in detection frequency between stage I disease and more advanced stage III and IV disease, which suggested that tumor stage was not the main determinant of positive detection in serum. Hypermethylation was detected in 28 and cytology was positive in 26 of the 30 peritoneal fluids from stage IC-IV patients. Three peritoneal fluids with negative or atypical cytology were positive for hypermethylation (patients 22, 33, and 36); however, one cytology positive fluid was negative for methylation (patient 28). Hypermethylation in peritoneal fluid may be useful to accurately identify women that have a higher risk of developing recurrence and may be candidates for adjuvant therapy. Methylation was observed in only 1 peritoneal fluid from 15 stage I A or B patients, but 11 of the 15 paired sera were positive for methylation. This suggests that free neoplastic DNA from ovary-confined disease accesses the bloodstream more readily than the peritoneum. We believe the sensitivity of methylation-based detection can likely be improved by advances in collection techniques, enrichment of neoplastic cells or DNA from the fluid or serum by antibody or oligo-based magnetic bead technology, and improvements in PCR technology.

For a feasibility study of detection, it is important that the target genetic alteration is cancer specific and not present in normal or benign cells. Although we only included in the hypermethylation panel genes reported to be unmethylated in normal cells, we still performed several controls to determine specificity. First, we tested and did not observe gene hypermethylation in cyst tissue, serum, and peritoneal fluid DNA from 10 patients with nonneoplastic ovarian disease or in serum from 20 normal, healthy controls (Fig. 1B). Second, we examined the serum and peritoneal fluid DNA for the methylation status of a gene known to be unmethylated in the tumor DNA. This approach has been validated in previous MSP-based detection studies (15, 18, 20). There was no case where a serum or peritoneal fluid DNA gave a positive methylation result in the absence of methylation in the corresponding tumor (potential false positive; Table 1). For example, tumor 27 in Fig. 1A did not have RASSF1A hypermethylation, and the matched serum and peritoneal fluid DNA were also negative. Third, we examined 10 nonneoplastic ovarian tissue DNA and observed no hypermethylation at our routine PCR amplification sensitivity (Fig. 1B). Our findings in the 40 control women indicate that serum or peritoneal fluid hypermethylation is highly specific for cancer (Table 2). In addition, a recent study on the hypermethylation profile of ovarian cancer found no hypermethylation of BRCA1 or APC in 16 nonmalignant ovarian tissue specimens, although 2 specimens showed hypermethylation of RASSF1A (31).

We used primer sequences to different RASSF1A promoter CpG sites in our MSP analysis. A recent study reported DAP-kinase hypermethylation in normal human lymphocytes by quantitative real-time MSP analysis (37). However, at our routine number of amplification cycles for conventional MSP, we did not observe DAP-kinase methylation in nonneoplastic DNA (Table 2). The inclusion of several classical tumor suppressor genes, invariably inactivated in tumor cells only, as opposed to less well-defined cancer genes in our detection panel is likely one reason for the high specificity we observed. Future studies could employ sufficient controls to address larger issues beyond this pilot study.

It is probable that genes hypermethylated exclusively or more frequently in ovarian cancer will be identified in the near future (10, 38, 39). Inclusion of such genes in an ovarian cancer detection panel would provide greater specificity for ovarian cancer. Algorithms could be developed to score the specificity of a particular gene hypermethylation panel for the detection of ovarian cancer compared with other cancer types. At present, BRCA1 hypermethylation provides some specificity because this gene is methylated in breast and ovarian cancer only (11, 12). Furthermore, whether particular genes were methylated or not might aid in the prediction of the behavior of individual tumors within a particular pathological stage. The heterogeneity of genetic alterations between tumors, for example, which tumor suppressor gene pathways are abrogated in an individual tumor, is likely one underlying cause of differences in tumor behavior and response to therapy. The panel used here contained genes of clear biological significance such as the p16INK4a, p14ARF, and APC genes involved in the p16/Rb and p53/p14 tumor suppressor gene pathways (40) and the Wnt signaling pathway (41), respectively. A recent study linking methylation of a Fanconi’s anemia gene to cisplatin sensitivity of ovarian cancer (42) indicates the potential of tumor profiling.

Molecular detection of loss of heterozygosity or new alleles by microsatellite analysis has been reported in 17 of 20 (85%) serum and 12 of 19 (63%) peritoneal fluid DNA from ovarian cancer patients (43) and by digital single nucleotide polymorphism analysis in 19 of 20 (95%) ascitic fluids from ovarian carcinoma patients (44). Successful detection of p53 point mutation in matched peritoneal fluid from three patients has also been demonstrated (45); however, p53 is not mutated in the majority of ovarian tumors (6). MSP-based detection has several advantages over microsatellite or point mutation-based detection of ovarian cancer. MSP has greater sensitivity, which will be important for detection of early, small, or precursor lesions. Also MSP, unlike point mutation, does not require prior knowledge of the gene status. At the protein level, telomerase-based detection was found to compare favorably with cytological examination of peritoneal fluid (46) and the potential of proteomic-based strategies for early detection has also been demonstrated (47). Although the sensitivity of our MSP-based detection was lower than that reported in this proteomics study (47), our study detected alterations of well-characterized tumor suppressor genes known to be present in tumor cells. In the future, different screening modalities and marker combinations, optimized for sensitivity and specificity, will likely be examined in concert for diagnosis of ovarian cancer.

The hypermethylation panel of six genes tested here provided ~100% diagnostic coverage of 71 ovarian or primary peritoneal cancers, including all major histologic cell types and pathological stages, and is certainly manageable in terms of time and economy in view of current array and high-throughput technology. The potential of microarray technology for simultaneous screening for cancers of several different organ types may also partly address the issue that the relatively low incidence of ovarian cancer in the general population has been cited as one obstacle to screening for this disease (4, 5). In the near term, MSP-based detection could be used alongside an established marker, CA-125, to improve sensitivity and specificity. A typical 10-mL peripheral blood sample taken for CA-125 analysis would also provide enough serum for MSP analysis. In this study, we have demonstrated for the first time the feasibility of hypermethylation-based, sensitive (82%) and 100% specific (no false positives) detection of ovarian cancer DNA in serum from patients with well-differentiated, organ-confined stage I tumors, as well as advanced disease. If these results are confirmed in larger studies, promoter hypermethylation may have useful clinical application in ovarian cancer diagnosis and management.

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