Featured Article

Detection of Breast Cancer in Nipple Aspirate Fluid by CpG Island Hypermethylation

Rachel Krassenstein,[1] Edward Sauter,[3] Essel Dulaimi,[1] Cristina Battagli,[1] Hormoz Ehya,[2] Andres Klein-Szanto,[2] and Paul Cairns[1,2]

Departments of [1]Surgical Oncology and [2]Pathology, Fox Chase Cancer Center, Philadelphia, Pennsylvania, and [3]Department of Surgery, Ellis Fischel Cancer Center/University of Missouri, Columbia, Missouri

Abstract

Purpose: **New approaches to the early detection of breast cancer are urgently needed as there is more benefit to be realized from screening. Nipple aspiration is a noninvasive technique that yields fluid known to contain breast epithelial cells. Silencing of tumor suppressor genes such as $p16^{INk4a}$, BRCA1, and hMLH1 have established hypermethylation as a common mechanism for tumor suppressor inactivation in human cancer and as a promising target for molecular detection.**

Experimental Design: **Using sensitive methylation-specific PCR, we searched for aberrant promoter hypermethylation in a panel of six normally unmethylated genes: *glutathione S-transferase π 1* (GSTP1); *retinoic acid receptor-β2* (RARβ2); $p16^{INK4a}$; $p14^{ARF}$; *RAS association domain family protein 1A* (RASSF1A); and *death-associated protein kinase* (DAP-kinase) in 22 matched specimens of tumor, normal tissue, and nipple aspirate fluid collected from breast cancer patients.**

Results: **Hypermethylation of one or more genes was found in all 22 tumor DNAs (100% diagnostic coverage) and identical gene hypermethylation detected in 18 of 22 (82%) matched aspirate fluid DNAs. In contrast, hypermethylation was absent in benign and normal breast tissue and nipple aspirate DNA from healthy women.**

Conclusions: **Promoter hypermethylation of important cancer genes is common in breast cancer and could be detected in matched aspirate DNAs from patients with ductal carcinoma *in situ* or stage I cancer. Promoter hypermethylation represents a promising marker, and larger studies may lead to its useful application in breast cancer diagnosis and management.**

Introduction

Breast cancer is the most common and second most lethal cancer in women in the Western world. Mammographic screening aids physical examination to detect smaller, earlier stage breast cancers, resulting in improved survival (1). Although breast cancer mortality has begun to decline because of improved screening techniques, >40,000 patients in the United States will die in 2002 of breast cancer (2). Novel approaches to detection of this disease are therefore extremely important. Nipple aspiration is a noninvasive technique that provides a fluid sample containing both cells shed from the breast duct lining and proteins secreted and concentrated into the duct lumen (3). Because epithelial cells are known to be present in nipple aspirate fluid, it is a suitable clinical sample for DNA-based detection of breast cancer. Molecular tests have been used for the successful detection of several types of cancer in bodily fluids that drain from the organ of interest, notably bladder cancer in urine, using the genetic alterations of loss of heterozygosity and point mutation as targets (4).

Another genetic alteration found in neoplastic cells, aberrant methylation, can also be used as a target for the molecular detection of neoplasia. In cancer cells, several tumor suppressor genes such as $p16^{INK4a}$, VHL, hMLH1, and BRCA1 have been found to have hypermethylation of normally unmethylated CpG islands within the promoter regions. The hypermethylation is associated with transcriptional silencing of the gene (5, 6). Hypermethylation can be analyzed by the sensitive methylation specific-PCR (MSP) technique, which can identify up to 1 methylated allele in 1000 unmethylated alleles, appropriate for the detection of neoplastic cells in a background of normal cells (7). MSP has been used in recent studies for the successful detection of cancer cell DNA in bodily fluids such as liver (8), lung (9) and head and neck cancer in serum (10), lung cancer in both sputum (11) and bronchial lavage (12), and prostate cancer in urine (13).

In a feasibility study, we examined the methylation status of a small panel of six tumor suppressor and other cancer genes, GSTP1, RARβ2, $p16^{INK4a}$, $p14^{ARF}$, RASSF1A, and DAP-kinase (14–19) known to be hypermethylated in breast neoplasia but unmethylated in normal breast cells, in paired breast tumor and aspirate fluid DNA samples. Promoter hypermethylation identified in the tumor DNAs was used as a molecular target for cancer detection in the corresponding nipple aspirate fluid DNA. We found each of the 22 tumors to have hypermethylation of at least one gene from the panel, thereby providing a target that we successfully detected in 18 (82%) of the matched aspirate fluids with 100% specificity.

Materials and Methods

Subjects and Aspiration Technique. After approval from the Institutional Review Board, we obtained matched breast tumor and normal breast tissue through the Fox Chase

Table 1   Clinicopathological and hypermethylation detection data in tumors and aspirate fluids of breast cancer patients

TNM, tumor-node-metastasis staging classification, all tumors were M_0; stage, Stage Grouping, American Joint Committee on Cancer; mf, multifocal; grade, histologic grade; cytology, cytology of nipple aspirate fluid; Meth.Gene, hypermethylated gene(s) in tumor used as target in paired aspirate fluid DNA; Detected, hypermethylation of identical gene(s) detected in aspirate fluid DNA, Y, yes or N, no.

Patient	Age (yrs)	TNM	Stage	Size (cm)	Grade	Cytology	Meth.Gene	Detected
1	44	$T_{is}N_0$	0	0.25	2	Malignant	GSTP1,p16	Y,Y
2	42	$T_{is}N_0$	0	0.6	2	Normal	RARβ,RASSF1A	N,N
3	50	$T_{is}N_0$	0	0.9	2	Normal	RARβ	Y
4	46	$T_{is}N_0$	0	1.1	2	Benign	RASSF1A	Y
5	51	$T_{is}N_0$	0	mf	2	Normal	RASSF1A	Y
6	60	$T_{is}N_0$	0	mf	2	Normal	p14	Y
7	47	$T_{is}N_0$	0	mf	3	Malignant	GSTP1,p14	Y,Y
8	75	T_1N_0	I	0.2	1	Normal	RARβ	Y
9	43	T_1N_0	I	0.9	2	Malignant	GSTP1	Y
10	51	T_1N_0	I	1	3	Normal	RASSF1A	N
11	56	T_1N_0	I	1.7	2	Normal	RARβ	Y
12	60	T_1N_0	I	2.0	2	Normal	RARβ	Y
13	46	T_2N_0	IIA	3	3	Malignant	GSTP1	N
14	46	T_2N_1	IIB	0.8	3	Malignant	p14	Y
15	47	T_2N_1	IIB	1.2	3	Malignant	GSTP1,p16	Y,Y
16	65	T_2N_1	IIB	2.4	3	Malignant	RARβ,p16	N,N
17	53	T_2N_1	IIB	3.5	3	Malignant	p16	Y
18	42	T_2N_1	IIB	4	3	Malignant	GSTP1,RASSF1A	Y,Y
19	35	T_2N_1	IIB	4.5	3	Malignant	RARβ,p14	Y,Y
20	34	T_2N_1	IIB	4.5	3	Malignant	DAP-K	Y
21	43	T_2N_1	IIB	mf	3	Malignant	p16	Y
22	31	T_3N_1	IIIA	7	2	Malignant	GSTP1,p14	Y,Y

Cancer Center Tumor Bank Facility and nipple aspiration fluid from 22 women with breast cancer ages 31–75 years. Subjects included women with recently diagnosed, biopsy-proven ductal carcinoma *in situ* (DCIS) or invasive carcinoma (Table 1). In all cases, aspirate fluid was collected from the breast with active disease. Nipple fluid was aspirated by a trained physician or nurse clinician using a modified breast pump (3). Fluid in the form of droplets was collected in capillary tubes. The quantity of fluid varied from 1–200 μl in different patients, but in the majority of cases amounted to 20–50 μl. Nipple aspirate fluid was available from 5 healthy women and fibrocystic tissue from 5 separate cases of benign breast disease.

Specimen Preparation, Cytological Analysis, and DNA Extraction.   Breast tumor tissue was microdissected with the assistance of a pathologist. The aspirate fluid specimens were rinsed into containers holding 3% polyethylene glycol in denatured alcohol (Shandon Lipshaw, Pittsburgh, PA). Part of the fluid specimen was cytocentrifuged onto glass slides for staining and cytological examination (3). Genomic DNA was extracted from the remaining fluid in capillary tubes or, when the quantity of fluid was limiting, from scraping the cytocentrifuged slides. Cytological review confirmed whether the specimens contained atypical and/or malignant breast epithelial cells (Table 1). DNA was extracted from both fluid and tissue using standard phenol/chloroform techniques (13). One-tenth volume of 10 M ammonium acetate and 2 μl of glycogen (Roche Diagnostics Corporation, Indianapolis, IN) were used with 2.5 volumes of 100% ethanol, incubation at -20°C and centrifugation at top speed (16,000 relative centrifugal force) for isolation of small amounts of DNA from the fluid, whereas DNA was simply spooled out from the tissue specimens after ethanol precipitation.

MSP.   Specimen DNA (0.1–1 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 in the breast cancer detection panel were *GSTP1* (14), *RARβ2* (15), *p16INK4a* (7), *p14ARF* (20), *RASSF1A* (18), and *DAP-kinase* (19). The primer sequences used have all been reported previously and can be found in the report referenced after each gene. The primers for *RASSF1A* include CpG site positions 7–9 on the forward primer and 13–15 on the reverse primer as described in Dammann *et al.* (18). The primer sequences for *RARβ2* are as previously described (15) but with the addition of 2 bp to the 5′ end of both primers for the unmethylated product to aid in discrimination between the unmethylated and methylated *RARβ2* products. PCR was performed for 31–35 cycles at 95°C denaturing, 58–62°C annealing, and 72°C extension with a final extension step of 5 min. 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. Cycle number also depended upon the starting amount of template DNA. Typically, aspirate DNA was subjected to 1 or 2 extra cycles compared with the primary tumor or normal tissue DNA. For each set of modifications and PCR, a cell line or tumor with known hypermethylation as a positive control, normal lymphocyte DNA as a negative control, and water with no DNA template as a control for contamination were included. In the absence of a tumor cell line with known hypermethylation of a particular gene, normal human lymphocyte DNA *in vitro* methylated with *Sss*I 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 analyzed. PCR was repeated for each positive sample.

Results and Discussion

We examined hypermethylation in a small panel of six normally unmethylated genes comprising the detoxifying gene *GSTP1* (reported to be hypermethylated in 31% of breast tumors): the tumor suppressor gene *p16^INK4a* (17%); the potential metastasis inhibitor gene *DAP-kinase* (7%); the putative suppressor genes *RARβ2* (38%) and *RASSF1A* (62%; Refs. 17, 21, 22); and the tumor suppressor gene *p14^ARF* (hypermethylated in 5 of 22 or 23% of tumors in this study) in 22 breast tumor and matched nipple aspirate DNAs. It was necessary to use a panel of genes because no one gene was known to be hypermethylated in more than a proportion of breast tumors (17). To detect promoter hypermethylation, we used the sensitive MSP assay, which can detect 0.1% cancer cell DNA from a heterogeneous cell population (7). We first screened the tumor DNAs only to conserve the limited amount of DNA isolated from the aspirate fluids (Fig. 1*A*). We did not screen each of the 22 tumors with all six genes in the panel because it was necessary only to identify hypermethylation of one gene in each tumor as a target to be examined in the aspirate fluid DNA. *A priori*, because each gene in the panel is normally unmethylated and could be PCR amplified in a robust manner, hypermethylation of any one gene is an equally as valid target as hypermethylation of any other gene in the panel. Each of the 22 tumor DNAs showed hypermethylation of at least one gene from the panel (Table 1). The diagnostic coverage (whether a hypermethylated gene was available as a target in each case) of our panel was therefore 100%. Hypermethylation was found in patients of all ages, which suggested that methylation of the genes in the panel was not an age-related event (Table 1).

Once an individual tumor was known to be positive for hypermethylation of a gene(s) in the panel, the corresponding aspirate fluid DNA was analyzed for hypermethylation of that particular gene(s). We detected hypermethylation of the identical gene(s) in 18 of 22 (82%) nipple aspirate fluid DNAs (Fig. 1*B*). In those tumors positive for more than one gene, the promoter hypermethylation pattern was identical in the matched aspirate fluid DNA (Table 1). The positive nipple aspirates included 6 of 7 cases of DCIS and 4 of 5 stage I breast cancer (Table 1). Hypermethylation was detected in breast cancer patient aspirates with normal or benign cytology, as well as in aspirates with malignant cytology (Fig. 2). Hypermethylation was not detected in 4 (18%) aspirate fluid DNAs. Cytological analysis had revealed normal cytology in 2 of these cases and malignant cytology, but with low numbers of epithelial cells, in the other 2 cases (Table 1). As is routine in PCR methodology, we chose to limit PCR to a maximum number of 35 cycles because it is known that specificity can decrease in MSP, as in other PCR protocols, with increased cycle number (23). It is possible that a higher number of cycles would have resulted in the 4 negative aspirate fluid DNAs being positive. A two-stage (nested) MSP approach has been reported to allow detection of 1 hypermethylated allele in >50,000 unmethylated alleles and resulted in successful MSP detection of lung cancer in sputum
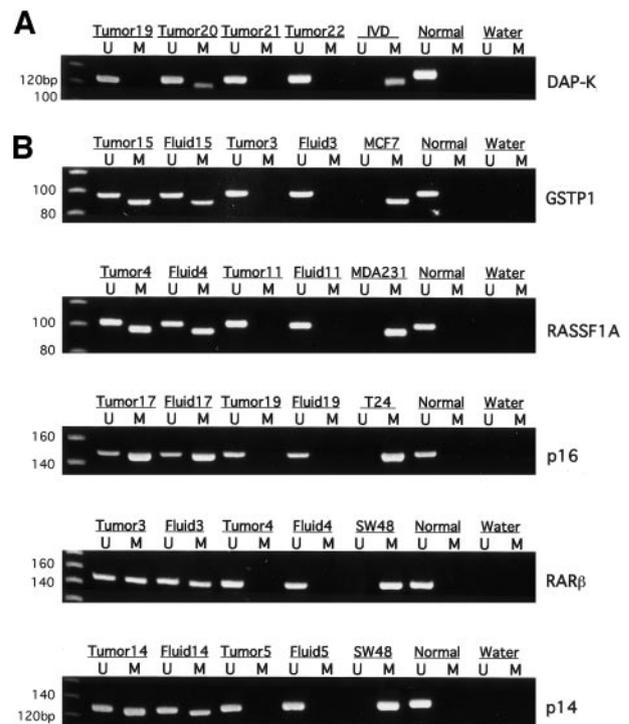

Fig. 1 Methylation-specific PCR (MSP) of cancer genes in breast tumor and nipple aspirate fluid DNAs. *A*, MSP of *death-associated protein kinase* (*DAP-Kinase*) in breast tumor DNAs. The presence of a visible PCR product in the methylated lane (*M*) of the tumor DNA from patient 20 indicates the presence of methylated alleles of *DAP-Kinase*. The absence of a PCR product in the methylated lane (*M*) of tumors 19, 21, and 22 indicates that these tumors have unmethylated alleles only. The PCR product in the unmethylated lane (*U*) of the tumor DNAs most likely arises from normal cell contamination of the tumor specimen. *In vitro*-methylated DNA as a positive control for *DAP-Kinase* methylation, normal lymphocyte DNA as a negative control, a water control for contamination in the PCR reaction (right), and a 20-bp molecular ruler as a molecular weight marker (*far left*) are also shown. *B*, MSP of *glutathione S-transferase π 1* (*GSTP1*), *RAS association domain family protein 1A* (*RASSF1A*), *p16^INK4a*, *retinoic acid receptor*-β2 (*RARβ2*), and *p14^ARF* in breast tumor and nipple aspirate fluid DNAs. Viewed from left to right, 2 patients are shown in each gel. In each case, the first patient's tumor DNA and aspirate DNA show hypermethylation. The second patient's tumor DNA is not methylated, and the corresponding aspirate fluid DNA also shows no hypermethylation (*M*), although a product can be clearly seen in the unmethylated lane (*U*). All gel panels follow the example of *GSTP1* where the presence of a visible PCR product in the methylated lane (*M*) of the tumor DNA from patient 15 indicates the presence of methylated alleles of *GSTP1*. A PCR product is also present in the methylated lane (*M*) of the aspirate DNA from patient 15, indicating the presence of neoplastic DNA. Tumor 3 is unmethylated for *GSTP1*, and the paired aspirate fluid DNA is also unmethylated. Controls are as in *A*, but tumor cell line MCF-7 DNA was used for *GSTP1*, MDA231 DNA for *RASSF1A*, T24 DNA for *p16^INK4a* and SW48 DNA for *RARβ2*, and *p14^ARF* as a positive control.

where a conventional (nonnested) MSP approach had previously been unsuccessful (24). Advances in fluid collection techniques, neoplastic cell or DNA enrichment, and PCR technology could enhance sensitivity.

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. We only included in the hypermethyla-

Fig. 2 Cytological analysis of nipple aspirate fluid. *A*, abundant cancer cells, patient 9. *B*, scant cancer cells, patient 21. *C*, foam cells only, normal control 1.
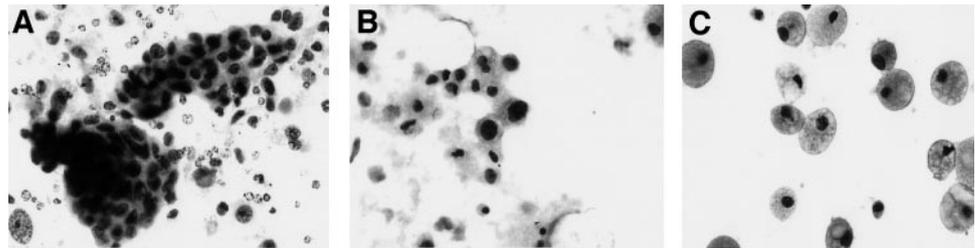
tion panel genes reported to be unmethylated in normal cells (14–19). As a control for specificity, we examined the aspirate fluid DNA for the methylation status of a gene known to be unmethylated in the tumor. A particular gene that is unmethylated in tumor DNA should always be unmethylated in the corresponding aspirate DNA (9, 10, 13). For example, tumor 3 in Fig. 1*B* was negative for *GSTP1* hypermethylation, and the matched aspirate fluid DNA was also negative. Additional examples are shown in the gel panels of Fig. 1*B*. There was no case where a aspirate fluid DNA gave a positive methylation result in the absence of methylation in the corresponding tumor (potential false positive). Moreover, we did not observe gene hypermethylation in nipple aspirate fluid DNA from 5 women with no clinical evidence of breast cancer or in 5 fibrocystic breast tissue DNAs (Fig. 3). Although we also observed no hypermethylation in paired normal breast tissue from the breast cancer patients (Fig. 3), it should be noted that the systemic nature of breast cancer might result in histologically normal tissue DNA from a neoplastic breast showing hypermethylation due to the presence of a preneoplastic clone or occult neoplastic cells. Thus, we observed absolute specificity, albeit in a limited study. Future studies could use sufficient controls to address larger issues beyond our exploratory study.

Promoter hypermethylation of a gene is not necessarily specific to only one type of cancer. Hypermethylation as with other mechanisms of inactivation of suppressor genes, deletion and point mutation, can be found in different types of cancer. *GSTP1* is frequently hypermethylated in breast cancer (14), prostate cancer (25), and liver cancer (26) but infrequently methylated in other cancer types (14, 17) and is therefore a suitable marker for breast cancer in women. It is likely that genes hypermethylated exclusively or more frequently in breast cancer than in cancers of other organ sites will be identified in the future (27, 28). For example, *BRCA1* hypermethylation has been reported in breast and ovarian cancer only (17, 29), and inclusion of this gene in a panel would provide additional specificity for breast cancer. Algorithms could be developed to score a gene hypermethylation panel for the detection of breast cancer. As more genes are found to be hypermethylated in breast cancer, future studies of the gene hypermethylation profile in large, representative series of breast cancers will determine both the number of genes and which genes to be screened to obtain optimal diagnostic coverage.

A recent study reported a diagnostic coverage of 87.5% using a panel of three genes, including *RARβ2*, in breast cancer and positive detection of hypermethylation in ductal lavage specimens from 2 of 7 DCIS and 17 of 20 invasive breast

carcinoma patients. Hypermethylation was also observed in ductal lavage from 5 of 45 women with healthy mammograms, 2 of whom were subsequently diagnosed with breast cancer (30). Detection of $p16^{INK4a}$ hypermethylation in 5 of 8 plasma DNAs from breast cancer patients using a methylation sensitive restriction enzyme approach has also been reported previously (31). The detection of hypermethylation in tumors and matched serum, ductal lavage, and nipple aspirates from patients with DCIS and stage I breast cancer suggest that hypermethylation is both frequent and a relatively early event in breast tumorigenesis and is therefore a suitable target for early detection of breast cancer.

Our hypermethylation panel of 6 genes provided 100% diagnostic coverage and is certainly manageable in terms of time and economy particularly in view of recent chip, array, and high-throughput technology. If the timing of hypermethylation of certain genes was found to be associated with a defined pathological stage of breast cancer, the panel could be easily extended in the future to simultaneously provide molecular staging and prognostic information in addition to detection. In support of a recent study of detection of hypermethylation in ductal lavage (29), we have demonstrated the feasibility of
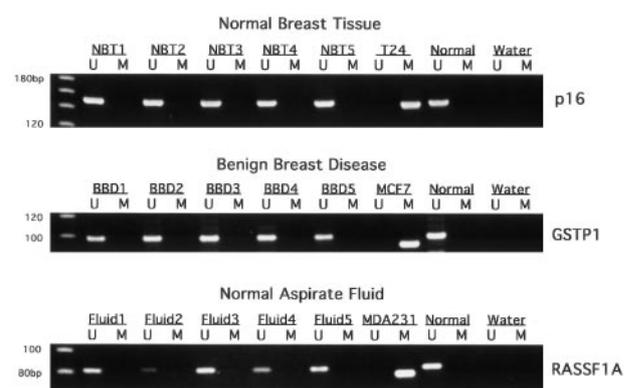
Fig. 3 Methylation-specific PCR of cancer genes in normal and inflammatory control DNAs. The absence of a PCR product in the methylated lane (*M*) of $p16^{INK4a}$ in normal breast tissues 1–5, *glutathione S-transferase π 1* (*GSTP1*) in benign breast tissues 1–5, and *RAS association domain family protein 1A* (*RASSF1A*) in nipple aspirate fluid DNA from healthy women 1–5 indicates that these specimens have unmethylated alleles (*U*) only. Tumor cell line T24, MCF7, and MDA231 DNAs as a positive control for methylation of $p16^{INK4a}$, *GSTP1*, and *RASSF1A*, respectively, normal lymphocyte DNA as a negative control, a water control for contamination in the PCR reaction (*right*), and a 20-bp molecular ruler as a molecular weight marker (*far left*) are also shown.

simple, 82% sensitive, and 100% specific detection of breast cancer, including DCIS and stage I cancer, in nipple aspirate fluid. If these results are confirmed in larger studies, promoter hypermethylation may have useful clinical application in breast cancer diagnosis and management. Our findings, reported here will hopefully provide a stimulus for such additional studies.

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