Tumor Suppressor Gene Promoter Hypermethylation in Serum of Breast Cancer Patients

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ABSTRACT

Purpose: Breast cancer is the most common malignancy in American women and the second leading cause of death from cancer. The genetic and epigenetic alterations that initiate and drive cancer can be used as targets for detection of neoplasia in bodily fluids. Tumor cell-specific aberrant promoter hypermethylation can be detected in nipple aspirate and ducal lavage from breast cancer patients. In this study, we examine serum, a more readily accessible bodily fluid known to contain neoplastic DNA from individuals with cancer, for methylation-based detection of breast neoplasia.

Experimental Design: We examined the promoter methylation status of three normally unmethylated biologically significant cancer genes, RAS association domain family protein 1A (RASSF1A), adenomatous polyposis coli (APC), and death-associated protein kinase (DAP-kinase), by sensitive methylation-specific PCR in 34 breast tumor and paired preoperative serum DNA. The 34 patients comprised 7 ductal carcinoma in situ (CIS), 3 lobular CIS, 5 stage I and 15 stage II to IV invasive ductal carcinomas, and 4 invasive lobular carcinomas. Normal and benign tissue and serum control DNA were also examined to determine the specificity of hypermethylation.

Results: Hypermethylation of one or more genes was found in 32 of 34 (94%) breast tumor DNA. APC was hypermethylated in 15 of 34 (47%), RASSF1A in 22 of 34 (65%), and DAP-kinase in 17 of 34 (50%) tumors. Twenty-six (76%) of the corresponding serum DNA were positive for promoter hypermethylation, including ductal CIS, lobular CIS, stage I disease, and lobular carcinoma patients.

Conclusions: Tumor cell specific promoter hypermethylation of APC, RASSF1A, and DAP-kinase is present in ductal CIS, lobular CIS, and all grades and stages of invasive breast cancer. Hypermethylation can be detected by methylation-specific PCR analysis in serum DNA from patients with preinvasive and early-stage breast cancer amenable to cure. If confirmed in additional studies, hypermethylation-based screening of serum, a readily accessible bodily fluid, may enhance early detection of breast cancer.

INTRODUCTION

Detection of breast cancer at an early stage is the key to successful treatment and outcome. In tumors confined to the breast at diagnosis, stage is the most important prognostic factor. Precursor lesions include at least two histologically defined types. Ductal carcinoma in situ (CIS) is a preinvasive lesion detected more often because the availability of mammography. Patients with localized ductal CIS are candidates for breast conserving surgery. A second entity, lobular CIS, is believed to represent a marker of increased risk for the development of invasive cancer. Within the category of invasive breast cancers, stage I (<2 cm) tumors have a better disease-free survival rate than higher stage tumors. However, ~30% of women diagnosed with localized breast cancer develop metastatic disease. In the roughly 15% of invasive breast tumors not detected by mammography, lobular carcinomas are overrepresented. Despite the availability of mammography and prevalence of self-examination, there is still additional benefit to be gained from additional screening methodologies (1, 2). Novel approaches to early detection of breast neoplasia therefore merit examination.

The genetic and epigenetic alterations that initiate and drive tumorigenesis are promising targets for early detection because they may precede clinically obvious cancer, can be detected at sensitive levels, may be specific to tumor cells, and can potentially provide information about the prognosis and treatment of the disease (3, 4). CpG islands located in promoter regions of genes are normally unmethylated. In cancer cells, aberrant hypermethylation of these promoter regions is associated with transcriptional silencing. Hypermethylation is therefore an alternative mechanism for inactivation of tumor suppressor genes (5, 6). Because gene hypermethylation has been found to be a common and early alteration in many tumor types (7–9), including breast (10, 11), it has emerged as a promising target for detection strategies in clinical specimens (3, 4). Several tumor suppressor and other cancer genes have been found to be hypermethylated in breast cancer but to be unmethylated in...
normal cells, e.g., RAS association domain family protein 1A (RASSF1A), adenomatous polyposis colii (APC), and death-associated protein kinase (DAP-kinase) genes (9, 10, 12, 13).

Detection of tumor suppressor gene hypermethylation in nipple aspirate fluid has been reported by ourselves (14) and in ductal lavage by others (15). Serum or plasma is a more readily accessible bodily fluid, and provision of a specimen does not require the presence of a specialist. DNA is known to be released into serum/plasma, which is enriched for tumor DNA in cancer patients (16). 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 (17–19). 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 (17–19). 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 (3, 4).

To determine the feasibility of detection of hypermethylated tumor DNA, we examined the methylation status of the APC, RASSF1A, and DAP-kinase genes in paired tumor and serum DNA from 34 patients with breast neoplasia, including ductal CIS, lobular CIS, stage I ductal and invasive lobular carcinoma, as well as in normal and benign disease tissue and serum control DNA.

MATERIALS AND METHODS

Specimen Collection and DNA Extraction. After approval from the Institutional Review Board, we obtained breast tumor 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 34 patients, ages 34 to 90 years, with histologically verified breast neoplasia. There were 7 ductal CIS, 3 lobular CIS, 20 invasive ductal, and 4 cases of invasive lobular cancer. Tumors were graded and staged according to American Joint Committee on Cancer guidelines (20). Serum specimens from 20 normal, healthy age-matched women and 8 women with benign breast disease were also obtained via the Fox Chase Cancer Center Biospecimen Repository. DNA previously extracted from five specimens of fibrocystic breast disease and five specimens of histologically normal (nonneoplastic) breast tissue were available for analysis (14). Tumor tissue was obtained immediately after surgical resection and subsequently microdissected with the assistance of a pathologist. DNA was extracted from tissue or from ∼1.5 mL of serum or plasma using a standard technique of digestion with proteinase K in the presence of SDS at 37°C overnight followed by phenol/chloroform extraction (21). Tumor specimen DNA was spooled out after precipitation with 100% ethanol. Serum DNA was precipitated with one tenth volume of 10 mol/L 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. The area of lobular CIS or ductal CIS, indicated by the pathologist, 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.

Methylation-Specific PCR (MSP). Specimen DNA (0.05 to 1 μg) was modified with sodium bisulfite, converting all unmethylated, but not methylated, cytosine to uracil followed by amplification with primers specific for methylated versus unmethylated DNA. The genes used in the breast cancer detection panel were APC (8), RASSF1A (22), and DAP-kinase (23). 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 (22). PCR amplification of template DNA was performed for 31 to 36 cycles at 95°C denaturing, 58°C to 66°C annealing and 72°C extension with a final extension step of 5 minutes. Cycle number and annealing temperature depended on the primer set to be used, each of which had been previously optimized for the PCR technology in our laboratory. For each set of DNA modification and PCR, a cell line or tumor with known hypermethylation as a positive control, normal lymphocyte, or normal tissue DNA as a negative control and water with no DNA template as a control for contamination were included. If no tumor or cell line with known hypermethylation of particular gene was available, normal human lymphocyte DNA in vitro methylated with SssI methylase according to the manufacturers instructions (New England Biolab, Beverly, MA) was used as a positive control. After PCR, samples were run on a 6% nondenaturing acrylamide gel with a 20-bp ladder and analyzed.

Statistical Analysis. The sensitivity of MSP-based detection of hypermethylation in serum was calculated as number of positive tests divided by number of cancer cases. The specificity was calculated as number of negative tests divided by number of cases without cancer and in a second, distinct approach as number of negative tests divided by number of cases without hypermethylation in serum. The association of tumor stage with positive detection in serum was compared using Fisher’s exact test. Results were considered statistically significant if the two-sided P was ≤0.05.

RESULTS AND DISCUSSION

We examined the methylation status of the normally unmethylated APC and RASSF1A tumor suppressor genes and the candidate metastasis inhibitor gene DAP-kinase, previously reported to be frequently hypermethylated in breast cancer (8–10, 12–14), in 34 breast tumor and matched serum or plasma DNA. The tumor set we studied included preinvasive and early-stage lesions (three lobular CIS, seven ductal CIS, and five stage I invasive ductal carcinomas) that have a better chance of cure with existing treatment options (2). To detect promoter hypermethylation, we used the sensitive MSP assay, which can detect 0.1% cancer cell DNA from a heterogeneous cell population (24). We found APC to be hypermethylated in 15 of 34 (47%), RASSF1A in 22 of 34 (65%), and DAP-kinase in 17 of 34 (50%) tumor DNA. Because no one gene was known to be hyper-
methylated in more than a proportion of breast tumors, it was necessary to use a panel of genes to provide a target for detection in the paired sera. Overall, 32 of 34 tumor DNA showed hypermethylation of at least one of the three genes from the panel, which provided a diagnostic coverage (whether a hypermethylated gene was available as a target in each case) of 94%. Hypermethylation was found in ductal CIS, lobular CIS, and invasive ductal and lobular tumors of all pathological grades and stages, which indicated that hypermethylation can be a relatively early event in breast tumorigenesis. Frequent hypermethylation of \textit{RASSF1A} in ductal CIS has been reported previously (10). In regard to whether lobular CIS is a high-risk marker or a precursor lesion, it will be interesting to additionally examine tumor suppressor gene hypermethylation in lobular CIS. Hypermethylation was found in patients of all ages (Table 1). Three of 3 lobular CIS, 5 of 7 ductal CIS, 3 of 5 stage I, and 15 of 19 stage II to IV, and in total 26 of 34 (76%), patient serum DNA were positive for hypermethylation of a gene known to be methylated in the corresponding tumor DNA (Table 1 and Fig. 1). Positive detection of hypermethylation in serum was not associated with tumor stage (11 of 15 ductal CIS, lobular CIS, and stage I invasive ductal carcinoma versus 15 of 19 stage II to IV invasive ductal carcinoma and invasive lobular carcinoma; \( P = 1.0 \) Fisher’s exact test). In four cases (patients 2, 10, 13, and 29), where more than one gene was hypermethylated in the tumor DNA, not all of the methylated genes were detected in the paired serum DNA. This is likely attributable to differences in PCR amplification efficiencies between primer sets but will require further study.

We wished to investigate detection of hypermethylation in serum because it is a more readily accessible bodily fluid compared with nipple aspirate or ductal lavage fluid. Obtaining a peripheral blood sample does not require the presence of a specialist and is relatively painless. Several studies have detected tumor-derived genetic and epigenetic alterations in serum or plasma DNA from cancer patients (17–19). Hypermethylation was detected at only a slightly lower sensitivity (76%) in serum compared with 82% in our previous study of nipple aspirate fluid (14). A pioneering report of detection of hypermethylation in ductal lavage had a diagnostic coverage of 87.5% and found fluid DNA from 2 of 7 ductal CIS and 17 of 20 invasive breast carcinoma patients to contain hypermethylated DNA (15). Another previous study detected methylation of the \textit{p16\textsuperscript{INK4a}} tumor suppressor gene by a methylation-sensitive re-

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NOTE. All patients were female. Cell type: LCIS, lobular CIS; DCIS, ductal CIS. Stage, stage grouping, American Joint Committee on Cancer; TNM, Tumor-Node-Metastasis staging classification; grade, histologic grade, LCIS are not graded; M/M, tumor DNA methylated/serum DNA methylated; U/U, tumor DNA unmethylated/serum DNA unmethylated; M/U, tumor DNA methylated/serum DNA unmethylated; no cases of U/M means tumor DNA unmethylated/serum DNA methylated were identified.
Detection of Promoter Hypermethylation in Breast Cancer

example, tumor 14 in Fig. 1 did not have

dated in previous MSP-based detection studies (17, 18, 26). For
the matched serum DNA (Table 1). This approach has been vali-
unmethylated in the tumor DNA was found to be unmethylated in
our study with several different controls. In all cases, a gene

provements in PCR technology.

by antibody or oligo-based magnetic bead technology, and im-

that the sensitivity level of hypermethylation-based detection can
remain manageable given emerging array technology. We believe

geneity of breast cancer. Significantly larger panels of genes will

a larger panel of genes reflects the genetic and epigenetic hetero-
genomes hypermethylated in breast cancer to the panel. The need for
coverage can likely be increased to 100% by addition of other
genes hypermethylated in breast cancer to the panel. The need for

of sensitivity observed in our study. Most immediately, diagnostic
product in the unmethylated lane (U) from all tumor DNA arises from normal cell contam-
ination of the tumor specimen or from an unmethylated allele. In vitro
methylated DNA (APC and DAP-K) or tumor cell line MDA231
(RASSF1A) as a positive control, 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.

There are several potential approaches to improving the level of sensitivity observed in our study. Most immediately, diagnostic
can likely be increased to 100% by addition of other genes hypermethylated in breast cancer to the panel. The need for
a larger panel of genes reflects the genetic and epigenetic hetero-
genity of breast cancer. Significantly larger panels of genes will
remain manageable given emerging array technology. We believe
that the sensitivity level of hypermethylation-based detection can
also be improved upon by the study of optimal serum collection
techniques, enrichment of neoplastic cells or DNA from the serum
by antibody or oligo-based magnetic bead technology, and
improvements in PCR technology.

We examined the tumor cell specificity of the three genes in
our study with several different controls. In all cases, a gene
unmethylated in the tumor DNA was found to be unmethylated in
the matched serum DNA (Table 1). This approach has been vali-
dated in previous MSP-based detection studies (17, 18, 26). For
example, tumor 14 in Fig. 1 did not have APC hypermethylation,
and the matched serum DNA was also negative. Moreover, we did
not observe APC, RASSF1A, or DAP-kinase hypermethylation in

restriction enzyme site-based assay in 6 of 10 matched plasma
DNA from invasive breast cancer patients (25).

Of particular interest is the detection of hypermethylation in normal serum. It has been reported that hypermethylation of
several genes can be detected in the serum of patients with cancer,
and this has led to the development of serum-based diagnostic tests
for various types of cancer. However, the sensitivity and specificity
of these tests are still being evaluated.

We examined the tumor cell specificity of the three genes in
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example, tumor 14 in Fig. 1 did not have APC hypermethylation,
and the matched serum DNA was also negative. Moreover, we did
not observe APC, RASSF1A, or DAP-kinase hypermethylation in

serum DNA from 20 normal, healthy age-matched controls and 8
patients with the benign breast disease (Fig. 2 and Table 2). We had
previously examined six cases of fibrocystic disease and histolog-
ically normal breast tissue containing nonneoplastic cells from six
breast cancer patients and observed no RASSF1A or DAP-kinase
hypermethylation in these nonneoplastic cells (14). We also found
these 12 nonneoplastic DNA to have unmethylated alleles of APC.
Methylation of APC has been reported to be absent in previous
studies of normal breast cells (13). These findings indicate that
aberrant promoter hypermethylation of tumor suppressor genes in
serum can be highly specific for cancer. A recent study reported
DAP-kinase hypermethylation in normal human lymphocytes by
quantitative MSP analysis (27). However, at our routine cycle
numbers for conventional MSP, we did not observe DAP-kinase
methylation in normal tissue or serum DNA. Future studies could
use sufficient controls to address larger issues beyond our pilot
study.

Table 2  Hypermethylation detection data in normal and
benign controls

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NOTE. The methylation status of RASSF1A and DAP-kinase in
normal and inflammatory breast disease tissue was reported previously
(14).
Methylation-based detection might also provide differential diagnosis of breast cancer. The E-cadherin and DAP-kinase genes have been reported to show different frequencies of methylation in ductal versus lobular carcinomas (28, 29). The methylation status of other genes such as the estrogen receptor gene, may aid in prognostic subgrouping (30). A recent study reported that DNA methylation in serum was an independent prognostic marker in breast cancer patients (31). It is also possible to screen for more than one tumor type, for example, breast and ovarian neoplasia, simultaneously in serum from high-risk individuals with inherited BRCA1 or BRCA2 mutation. Methylation profiling of breast and ovarian cancer will likely identify genes methylated exclusively in one but not the other of these cancer types (32, 33).

In this study, we have demonstrated sensitive and specific detection of hypermethylation in serum from breast cancer patients. The sensitivity observed in preinvasive or stage I lesions was 73% only slightly lower than the 79% in stage II to IV cancer. All three cases of lobular CIS examined demonstrated hypermethylation in the paired serum. One lobular CIS patient had a bilateral mastectomy and the other two patients had a lumpectomy. All three lobular CIS patients were free of disease 6 to 22 months after surgery. Our study also provides the first demonstration of methylation-based detection of ductal CIS and stage I ductal carcinoma in serum. Interestingly, we detected hypermethylation in the serum DNA from all four invasive lobular carcinoma patients. It is known that invasive lobular carcinoma is more often missed by mammography. Additional studies of the use of hypermethylation-based screening of serum should address improvements in sensitivity, the validation of specificity in larger numbers and examination of high-risk populations. If our results are confirmed in such studies, promoter hypermethylation may have useful clinical application in the detection of breast neoplasia.

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Tumor Suppressor Gene Promoter Hypermethylation in Serum of Breast Cancer Patients

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