Detection of Gene Promoter Hypermethylation in Fine Needle Washings from Breast Lesions


ABSTRACT

Purpose: Fine needle aspiration (FNA) is used widely in diagnostic assessment of breast lesions. However, cytomorphological evaluation depends heavily on the proficiency of cytopathologists. Because epigenetic alterations are frequent and specific enough to potentially augment the accuracy of malignant disease detection, we tested whether hypermethylation analysis of a panel of genes would distinguish benign from malignant breast FNA washings.

Experimental Design: FNA washings were collected from 123 female patients harboring suspicious mammary lesions. Sodium bisulfite-modified DNA was amplified by methyl-specific PCR (MSP) for CDH1, GSTP1, BRCA1, and RARβ to detect gene promoter CpG island methylation. Paired samples of 27 breast cancer tissue and 7 fibroadenomas and 12 samples of normal breast tissue, collected post-operatively, were also analyzed. MSP results were compared with conventional cytomorphological diagnosis.

Results: FNAS were cytomorphologically diagnosed as benign (25 cases), malignant (76 cases), suspicious for malignancy (6 cases), and unsatisfactory (16 cases). Percentages of methylated CDH1, GSTP1, BRCA1, and RARβ in FNA washings were 60, 52, 32, and 16%, and 65.8, 57.9, 39.5, and 34.2% for benign and malignant lesions, respectively. These differences did not reach statistical significance. In all of the paired benign lesions tested, there was absolute concordance. Sixty-seven percent (18 of 27) of FNA washings displayed hypermethylation patterns identical to malignant paired tissue. No methylation was found in the normal breast samples for any of the genes.

Conclusions: Detection of gene hypermethylation in FNA washings by MSP analysis is feasible, but the selected gene panel does not discriminate between benign and malignant breast lesions.

INTRODUCTION

Breast cancer is the most common malignant neoplasm in women in developed countries, and it is estimated that 182,800 new cases were diagnosed in the United States in 2000 (1). For the same year, the IARC estimated an incidence of 73.84/100,000 for this malignancy in Portuguese women. Despite improvements in diagnosis and treatment over the last decade, breast cancer remains a leading cause of cancer related mortality (1). Thus, novel approaches that might increase the rate of early detection are likely to contribute to a better management of this highly prevalent and lethal neoplasm.

Currently, FNA cytology is an integral part of the diagnostic evaluation of suspicious breast lesions (2–5). In many cases, this minimally invasive procedure allows the collection of representative material for cytological evaluation. However, the accuracy of FNA in the diagnosis of breast malignancy depends on the epigenetic alterations both in performing the aspirate and the cytomorphological analysis (4, 5). As a consequence, false negative rates for this procedure range from 5 to 30% (2, 3, 5), and this could represent a major limitation for the identification of small preinvasive lesions and well-differentiated tumors (6). This problem could be overcome by coupling the evaluation of cellular morphology with analyses of tumor-associated DNA alterations. Indeed, several studies have demonstrated that some genetic and epigenetic alterations are frequent and specific enough to augment the accuracy of malignant disease detection (7–11).

Changes in the status of DNA methylation are among the most common molecular alterations in human neoplasia (12–14). Several studies have suggested that promoter CpG islands of many genes involved in tumorigenesis are abnormally methylated in multiple types of cancer, whereas they are usually unmethylated in normal human tissues (12–14). Furthermore, it is acknowledged that the profile of promoter hypermethylation for these genes differs for each cancer type (14). Therefore, hypermethylation, which is often associated with gene silencing, seems to be a tumor-specific alteration prone to be used to identify and/or characterize a tumor. In breast cancer, gene hypermethylation profiling analysis revealed that CDH1, GSTP1, BRCA1, and RARβ are among the most commonly silenced genes (14, 15).

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4 The abbreviations used are: FNA, fine needle aspiration; MSP, methyl-specific PCR; CDH1, E-cadherin; GSTP1, glutathione S-transferase P1; BRCA1, breast cancer 1; RARβ, retinoic acid receptor β. 
The aim of this study was to evaluate whether the use of hypermethylation analysis by MSP for a panel of genes (comprising **CDH1**, **GSTP1**, **BRCA1**, and **RARβ**) was feasible on fine needle washings and whether it objectively distinguishes malignant from benign breast disease.

**MATERIALS AND METHODS**

**Patients**

One hundred and twenty-three female patients suspected to harbor breast cancer and consecutively submitted to FNA at the Department of Pathology of the Portuguese Oncology Institute–Porto, from June 2001 to March 2002, were enrolled in this study.

**Cytological Preparations**

FNA biopsy was performed using a 23-gauge needle attached to a 10-ml syringe and inserted into a syringe holder. The aspirates were smeared on microscope slides and routinely stained for cytopathological evaluation. Samples for methylation analysis were produced by washing the needle and syringe aspirates were smeared on microscope slides and routinely stained for cytopathological evaluation. Samples for methylation analysis were produced by washing the needle and syringe with 250 μl of PBS. The solution was spin-dried, and the pellet was collected in an Eppendorf tube and stored at −80°C.

**Tissue Sample Collection**

Paired surgical specimens were available for additional analysis in 34 cases (27 malignant and 7 benign lesions). For control purposes, 12 samples of normal breast tissue were also collected from mastectomy specimens. Precautions were taken to select areas distant from the primary tumor (opposite quadrant). Fresh tissue, snap-frozen in liquid nitrogen and stored at −80°C, or paraffin-embedded material was collected from each of these cases. Sections were cut for the identification of the areas of interest. These areas were then carefully microdissected from 12-μm thick sections, and an average of 20 sections with an enrichment (>70%) in epithelial cells were used for DNA extraction. Paraffin-embedded tissue was similarly microdissected, but was placed in xylene for 3 h at 48°C to remove the paraffin. DNA was extracted using the method described by Ahrendt et al. (8).

**Methylation Analysis**

**Bisulfite Treatment.** Sodium bisulfite conversion of 2 μg of genomic DNA was performed by a modification of a method described previously (16). Briefly, NaOH was added to denature DNA (final concentration 0.2 M) and incubated for 20 min at 50°C. A volume of 500 μl of freshly made bisulfite solution [2.5 M sodium metabsulfite and 125 mM hydroquinone (pH = 5.0)] was added to each sample, and incubation was continued at 50°C for 3 h in the dark. Modified DNA was purified using the Wizard DNA purification resin according to the manufacturer (Promega Corp., Madison, WI) and eluted in 45 μl of water at 80°C. After treatment with NaOH (final concentration, 0.3 M) for 10 min at 37°C, isolation was continued with 75 μl of 7.5 M ammonium acetate followed by an incubation step of 5 min at room temperature. Finally, the modified DNA was precipitated by adding 2.5 volumes of 100% ethanol and 2 μl of glycogen (5 mg/ml). The pellet was washed with 70% ethanol, dried, and eluted in 30 μl 5 mM Tris (pH 8.0).

**MSP Analysis.** For PCR amplification, 2 μl of bisulfite-modified DNA was added in a final volume of 25 μl PCR mix containing 1× PCR buffer [16.6 mM ammonium sulfate, 67 mM Tris (pH 8.8), 6.7 mM MgCl2, and 10 mM 2-mercaptoethanol], deoxynucleotide triphosphates (each at 1.25 mM), 1 unit Platinum TaqDNA polymerase (Life Technologies, Inc., Rockville, MD), and primers (300 ng each per reaction). The primer sequences for either methylated or modified unmethylated genes have been described elsewhere (17–20). The annealing temperatures were 64°C for **GSTP1**, 62°C for **CDH1**, 60°C for **BRCA1**, and 57°C for **RARβ** (17–20). All of the PCR amplifications were carried out using the following conditions: 1 cycle at 95°C for 5 min; and 38 cycles of 95°C for 1 min, the specific annealing temperature for each gene for 1 min, and 1 min at 72°C. Additionally, a final extension for 5 min at 70°C was performed. As a positive control for methylated alleles, DNA from normal lymphocytes treated *in vitro* with SsIl bacterial methylase (New England Biolabs Inc., Hitchin, Hertfordshire, United Kingdom) was used. DNA from normal lymphocytes was used as a negative control for methylated genes. The PCR products were directly loaded onto a nondenaturing 6% polyacrylamide gel, stained with ethidium bromide, and visualized under UV illumination.

**Statistical Analysis**

The frequencies of gene promoter methylation in benign and malignant lesions were compared using the Yates’ corrected χ² test. Significance was set at *P* < 0.05.

**RESULTS**

For this study, 123 women (median age, 52 years; range, 18–92) with palpable breast lesions clinically evaluated at the Portuguese Oncology Institute–Porto were enrolled. The FNAs were cytomorphologically diagnosed as benign (25 cases), malignant (76 cases), and suspicious for malignancy (6 cases). The remaining 16 cases were reported as unsatisfactory.

The results of the methylation analyses are summarized in Table 1, and representative cases displayed in Fig. 1. Considering FNA washings from benign lesions, aberrant promoter
hypermethylation at any of the genes studied was detected in 22 of 25 (88%) cases (Table 1). The frequency of hypermethylation in this group for each gene was 60% (15 of 25) for CDH1, 52% (13 of 25) for GSTP1, 32% (8 of 25) for BRCA1, and 16% (4 of 25) for RARβ.

Concerning the FNA washings of malignant tumors, only 10 (13.2%) cases did not exhibit aberrant promoter hypermethylation for any of the genes studied (Table 1). The most frequently methylated genes were CDH1 in 65.8% (50 of 76) and GSTP1 in 57.9% (44 of 76), followed by BRCA1 in 39.5% (30 of 76) and RARβ in 34.2% (26 of 76) of the cases.

No statistically significant differences in the frequencies of gene promoter methylation in FNA washings were found between the malignant and the benign lesions ($P = 0.78$, $P = 0.78$, $P = 0.67$, and $P = 0.14$, for CDH1, GSTP1, BRCA1, and RARβ, respectively).

In cytologically suspicious lesions, hypermethylation was only found for CDH1 gene and in 4 of the 6 cases (66.7%). Regarding the percentages of methylation in FNA reported as unsatisfactory by cytology, the same trend was seen as in the conclusive FNA, i.e., the more frequently methylated genes were CDH1 (56.3%) and GSTP1 (31.3%), followed by RARβ (25%) and BRCA1 (18.8%).

In 7 cases of benign lesions, we were able to analyze the respective tissue sample. All of these cases corresponded to fibroadenomas, and the results of the hypermethylation analyses were entirely concordant with those obtained in the respective FNA washing.

From the 76 cases of malignancy diagnosed by FNA, we had access to tissue samples of 27 cases. The comparisons between the hypermethylation results in FNA washings and respective tissue are depicted in Table 2. Sixty-seven % (18 of 27) of FNA washings displayed hypermethylation patterns identical to the malignant paired tissue. Nine cases displayed hypermethylation in the tissue sample that was not detected in the paired FNA washing. Overall, discrepant results were observed in 10 of 108 MSP assays (9.3%).

Promoter methylation of the screened genes was not found in any of the 12 normal breast tissue samples.

**DISCUSSION**

Using FNA biopsy, a minimally invasive method, suspicious breast lesions can be evaluated morphologically. However, because morphological features of malignancy are more ambiguous in small tumors and premalignant lesions, this pro-
Gene Promoter Methylation in Breast FNA Washings

Promising results of molecular studies in FNA washings from breast lesions have been reported recently by Guðlaugsdóttir et al. (21). Indeed, those authors demonstrated that FNA needle rinsing yielded sufficient material for seeking TP53 mutations, but the many individual TP53 mutations that can be present in breast tumors limits its usefulness in a cost-effective early detection approach (22). Comparatively, detection of promoter CpG island hypermethylation offers several advantages. A major one is that abnormal promoter hypermethylation profiling of cancer-associated genes may be a tumor-specific marker, and this alteration is often enhanced during disease progression, thus making it readily detectable in clinical specimens (10, 12–14, 23). Additionally, CpG island hypermethylation constitutes a positively detectable signal with a very high degree of specificity, even in presence of a vast excess of unmethylated DNA (23). These findings suggest that the use of these genetic markers for cancer diagnosis may become increasingly important and standard of care. In this work, we demonstrated that abnormal promoter hypermethylation of key genes in breast carcinogenesis can be easily detected in FNA washings collected from women with palpable lesions suspicious of cancer.

Promoter hypermethylation in benign and malignant lesions was more commonly found in CDH1 and GSTP1 than in RARβ and BRCA1. Moreover, hypermethylation was frequently not limited to one target gene, but was often found in at least two genes (52% of benign lesions and 71.1% of malignant lesions). Globally, the promoter methylation rates found in our study for CDH1 and RARβ are similar to those reported previously in primary breast carcinomas (14, 15, 24) but are higher for the remainder genes (14, 17, 19). A possible explanation for this finding would be clonal enrichment that underlies the FNA sampling, presumably because cancer cells are more easily dislodged than normal cells by this procedure, whereas tissue analysis entails a greater degree of heterogeneity because of a wider sampling. However, we consider this hypothesis unlikely given the high sensitivity of the assay (23). We believe that the hypothesis of low specificity of the MSP conditions can also be safely disregarded, because the very same conditions, namely the number of cycles, were used for all of the genes. Moreover, we tested lower and higher numbers of amplification cycles, and there was complete agreement in the results. In addition, the annealing temperatures used for each gene in our study were identical to those indicated in the aforementioned reports (17–20). Finally, no methylation was found in any of the 12 normal breast tissue samples.

Our data demonstrate that MSP analysis of CDH1, GSTP1, RARβ, and BRCA1 genes does not distinguish benign from malignant breast lesions, because aberrant promoter hypermethylation was found to be equally prevalent in both groups. Indeed, previous reports emphasized the detection of genetic alterations in breast tissue samples containing hyperplastic epithelium, papillomas, and other proliferative lesions, although in a different set of genes (15, 21, 25, 26). Furthermore, during the elaboration of our study, Lehmann et al. (26) reported the detection of promoter methylation for other genes in tissue samples from hyperplastic lesions, as well as in papillomas, but not in normal breast epithelium. Moreover, using a quantitative approach, those authors clearly showed that there were major gene-specific differences in the extent of methylation between benign breast lesions and breast carcinomas (26). It is presently unknown whether benign proliferative lesions with gene promoter hypermethylation harbor an increased risk of malignant transformation. Moreover, genetic alterations such as loss of heterozygosity have already been reported in normal breast tissue close to invasive carcinoma, and these alterations were similar to those found in hyperplastic, preneoplastic, and neoplastic breast lesions (27, 28). Interestingly, in normal breast tissue distant to invasive carcinoma no loss of heterozygosity was observed (27). Thus, it is likely that benign and malignant breast lesions also share common epigenetic alterations, although quantitatively different. Taking these data in consideration, it is tempting to speculate that quantitative hypermethylation analysis in FNA washings could increase the diagnostic precision of cytological diagnosis in breast lesions.

To a large extent, the epigenetic alterations observed in the tumor tissue samples were evident also in the corresponding FNA. However, in 9 malignant cases the promoter aberrant methylation detected in the tissue sample was not found in the corresponding FNA washing. In most of the cases, only one of the genes studied displayed such a discordant result. Because FNA renders a more limited sample of neoplastic cells, these findings suggest that breast cancer may display heterogeneous gene promoter methylation profiles within the same tumor.
Additional studies in tissue samples are necessary to confirm this hypothesis. Additionally, it is noteworthy that even in unsatisfactory cytological cases we were able to obtain good quality DNA, thus enabling MSP analysis of those cases.

In summary, we demonstrated that hypermethylation by MSP analysis of FNA washings is feasible, but the methylation pattern of the chosen gene panel does not augment standard cytomorphological methods in the differential diagnosis of breast lesions. A quantitation-based approach for the determination of gene promoter methylation is more likely to yield clinically relevant results.

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