Gene Promoter Hypermethylation in Tumors and Plasma of Breast Cancer Patients

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Purpose: To measure the hypermethylation of four genes in primary tumors and paired plasma samples to determine the feasibility of gene promoter hypermethylation markers for detecting breast cancer in the plasma.

Materials and Methods: DNA was extracted from the tumor tissues and peripheral blood plasma of 34 patients with invasive breast cancer, and the samples examined for aberrant hypermethylation in _cyclin D2,_ retinoic acid receptor β (_RARβ,_ twist and high in normal-1 (_HIN-1_) genes using methylation-specific PCR (MSP), and the results correlated with the clinicopathological parameters.

Results: Promoter hypermethylation was detected at high frequency in the primary tumors for _cyclin D2_ (53%), _RARβ_ (56%), _twist_ (41%) and _HIN-1_ (77%). Thirty-three of the 34 (97%) primary tumors displayed promoter hypermethylation in at least one of the genes examined. The corresponding plasma samples showed hyperme

thylation of the same genes, although at lower frequencies (6% for _cyclin D2_, 16% for _RARβ_, 36% for _twist,_ and 54% for _HIN-1_). Overall, 22 of the 33 (67%) primary tumors with hypermethylation of at least one of the four genes also had abnormally hypermethylated DNA in their matched plasma samples. No significant relationship was recognized between any of the clinical or pathological parameters (tumor size, axillary lymph node metastasis, stage, or Ki-67 labeling index) with the frequency of hypermethylated DNA in the primary tumor or plasma.

Conclusion: The detection of aberrant promoter hypermethylation of cancer-related genes in the plasma may be a useful tool for the detection of breast cancer. (_Cancer Res Treat._ 2005;37:233-240)

Key Words: Methylation, Plasma, Breast neoplasms

INTRODUCTION

Breast cancer is the most common malignancy in women in Korea and Western countries, accounting for 16.8% of all cancers in Korean female patients (1). The chance of being cured is greatest for those individuals whose primary tumor or tumor relapse are detected at an early stage, which permits curative surgery. Standard techniques for the detection and monitoring of breast cancer rely on palpation or radiological images, but many tumors escape these detection methods until they reach a relatively advanced stage. Therefore, it is important that new methods be developed to provide more sensitive approaches to the detection of new breast cancers or their recurrences.

One possible approach to the detection of breast cancer detection is via analysis of the circulating DNA from cancer cells. Nanogram quantities of DNA circulating in the blood are present in healthy individuals, while cancer patients have an average of 219 ng DNA/ml plasma (10~1,200 ng/ml plasma) (2). In seeking more specific markers, several studies have shown that it is possible to identify microsatellite alterations in the plasma and serum DNA of patients with head and neck (3) and small cell lung carcinomas (4). Additionally, _p53_ and _ras_ gene mutations have been detected in the plasma and serum of patients with colorectal and pancreatic carcinomas (5,6).

Another DNA abnormality detected in the plasma and serum of cancer patients is that of gene promoter hypermethylation. The presence of gene promoter hypermethylation in the serum and plasma DNA has also been demonstrated in patients with cancers of the lung (7), head and neck (8), liver (9), colon (10), stomach (11) and breast (12~15).

In all types of human cancers studied to date, hypermethylation of the CpG islands of genes is associated with transcriptional gene silencing. In the case of breast cancer, genes previously reported to be hypermethylated include several genes involved in DNA repair (_BRCA1_ and _GSTP1_), cell cycle

regulation (*p16INK4A* and *cyclin D2*), cell adhesion (*E-cadherin*), hormone- and receptor-mediated cell signaling (*ER*, *RARβ* and *THRβ*) and regulation of cell transcription (*HOX5A*), as well as other functions (*RASSF1A, Twist,* and *HIN-1*) (16).

On the basis of our previous study (16), four genes (*cyclin D2, RARβ twist,* and *HIN-1*) commonly methylated in invasive breast carcinomas were chosen for this study. *Cyclin D2* is a member of the D-type cyclin group, consisting of cyclins implicated in cell cycle regulation, differentiation and malignant transformation. In contrast to *cyclin D1*, it has been suggested that *cyclin D2* is involved in a vital tumor suppressor function in normal breast tissue, and that its loss may be related to tumorigenesis (17). *RARβ* is known as a tumor suppressor gene through its mediation of the growth-inhibitory effects of retinoic acids. The frequency of methylation of *RARβ* in invasive breast carcinomas was 41%, while the gene was rarely methylated in benign breast tissue (16,18). 'Twist' belongs to the basic-helix-loop-helix family of transcription factors, and has been implicated in lineage-specific cellular differentiation and survival. Little is known about the role of *twist* in breast cancer development. The high frequency of *twist* methylation (50~56%) in breast cancer, coupled with no methylation in normal mammary epithelial cells, suggests it plays an important role in carcinogenesis (16,18). *Hin-1* is a putative cytokine, recently discovered by SAGE, to be highly expressed in the normal terminal duct-lobular unit (TDLU) and downregulated by promoter hypermethylation in 74% of primary breast tumors (19).

To determine the feasibility and the clinical significance of detecting gene promoter hypermethylation in the plasma of patients with breast cancer, 34 patients with invasive breast cancer were examined, using the sensitive MSP technique, for abnormal promoter hypermethylation in *cyclin D2, RARβ twist* and *HIN-1* of primary tumors and their paired plasma samples.

MATERIALS AND METHODS

1) Sample collection and DNA extraction

Matched primary tumor specimens and peripheral blood samples were collected from 34 patients diagnosed with breast cancer, between November 2002 and May 2003. Fresh tumor samples were obtained immediately after surgical resection of the breast and stored at -70°C before DNA extraction. Pathological diagnosis and clinical evaluation disclosed no evidence of metastatic dissemination in any patient. In order to avoid the possible clearance of plasma DNA after removal of the primary tumor, a blood sample was collected from each patient on the day of surgery, prior to the mastectomy or breast-conserving surgery. The sample was later discarded if the histological diagnosis did not conclusively indicate the presence of a malignant lesion. Blood specimens were centrifuged at 3,000 rpm, in EDTA-containing tubes, for 20 minutes at room temperature, and plasma was then stored at either -20 or -70°C until DNA extraction. Plasma samples from 10 age-matched normal individuals without breast disease were used as controls. Tumor and plasma DNAs were prepared using the QIAamp Tissue Kit (Qiagen, Hilden, Germany) and the QIAamp Blood

Kit (Qiagen, Hilden, Germany), respectively, according to a modified protocol of the manufacturer. One ml of plasma was used for DNA extraction, with subsequent sodium bisulfite treatment in both the cancer patients and normal controls. To obtain a higher plasma DNA concentration, one column was used repeatedly until the entire sample had been processed. When spectrophotometrically quantified, 1 ml of plasma yielded an average of 330 ng of DNA (178~630 ng). All samples were collected in accordance with institutional guidelines for protection of human subjects, and the informed consents were obtained from all patients prior to obtaining their blood and tumor samples.

2) Sodium bisulfite treatment of DNA

DNA from the tumor and plasma specimens were treated with sodium bisulfite and analyzed using MSP, as previously described (16). This process converts all unmethylated cytosine to uracil, which is recognized as thymidine by Taq polymerase; this process does not affect methylated cytosines. Total extracted DNA from 1 ml of plasma, and 1μg of DNA from tumor tissue, was used for this step. DNA, in 50μl of water, was denatured with 5.5μl of 2 M sodium hydroxide for 10 min at 37°C. Thirty μl of 10 mM hydroquinone (Sigma-Aldrich, Inc., St. Louis, MO) and 520μl of 3 M sodium bisulfite (Sigma-Aldrich, Inc., St. Louis, MO) were subsequently added, with mixing. The DNA was overlain with several drops of mineral oil, and the sample incubated at 50°C for 16 hr. Bisulfite-modified DNA was purified using Microcon® YM-30 centrifugal filter devices (Millipore, Bedford, MA), according to the instructions of the manufacturer. DNA was eluted from the column in 50μl of distilled water into a 1.5 ml microcentrifuge tube, desulfonated with 5.5μl of 3 M sodium hydroxide for 10 min at room temperature, and then neutralized with 17μl of 10 M ammonium acetate. DNA was precipitated with 700μl of absolute ethanol and 1μl of glycogen, at -70°C, washed twice with 70% ethanol, air-dried and then resuspended in 20μl of distilled water. Samples were stored at -20°C until used. DNA from normal breast tissue was treated *in vitro* with SssI methyltransferase (New England Biolabs Inc. Beverly, MA), in order to generate completely methylated DNA at all CpGs, which was included in each round of sodium bisulfite treatment as a quality control of the bisulfite conversion process and subsequent methylation reactions.

3) Methylation-specific PCR (MSP)

PCR amplification was performed to detect the presence of hypermethylation within the promoter CpG islands of *cyclin D2, RARβ twist* and *HIN-1* genes, using primers specific for methylated or unmethylated DNA. The primer sequences were based on previous reports, and are listed in Table 1. For these reactions, 1~2μl of bisulfite-treated DNA was added to bring the reaction volume up to 25μl, containing 1.25 mM dNTP, 16.6 mM $(NH_4)_2SO_4$, 67 mM Tris, pH 8.8, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1U RedTaq genomic DNA polymerase (Sigma-Aldrich, Inc., St. Louis, MO) and 300 ng each of the forward and reverse primers specific to the methylated and unmethylated DNA sequences. Methylated and unmethylated primers were tested in separate reactions. Amplification was carried out in a PTC-200 thermal cycler (MJ

Table 1. Primer sequences used in MSP

| Gene	Direction	Sequence	bp	References
Unmethylated				
Cyclin D2	Forward	5'-AGA GTA TGT GTT AGG GTT GAT T	106	18
	Reverse	5'-ACA TCC TCA CCA ACC CTC CA		
RARβ	Forward	5'-GGA TTG GGA TGT TGA GAA TGT	163	16
	Reverse	5'-CAA CCA ATC CAA CCA AAA CAA		
Twist	Forward	5'-TTT GGA TGG GGT TGT TAT TGT	193	16
	Reverse	5'-CCT AAC CCA AAC AAC CAA CC		
HIN-1	Forward	5'-GGT ATG GGT TTT TTA TGG TTT GTT	136	16
	Reverse	5'-CAA AAC TTC TTA TAC CCA ATC CTC A		
Methylated				
Cyclin D2	Forward	5'-GGC GGA TTT TAT CGT AGT CG	101	18
	Reverse	5'-CTC CAC GCT CGA TCC TTC G		
RARβ	Forward	5'-GAA CGC GAG CGA TTC GAG T	142	16
	Reverse	5'-GAC CAA TCC AAC CGA AAC G		
Twist	Forward	5'-TTT CGG ATG GGG TTG TTA TC	200	16
	Reverse	5'-AAA CGA CCT AAC CCG AAC G		
HIN-1	Forward	5'-GGT ACG GGT TTT TTA CGG TTC GTC	136	16
	Reverse	5'-AAC TTC TTA TAC CCG ATC CTC G		
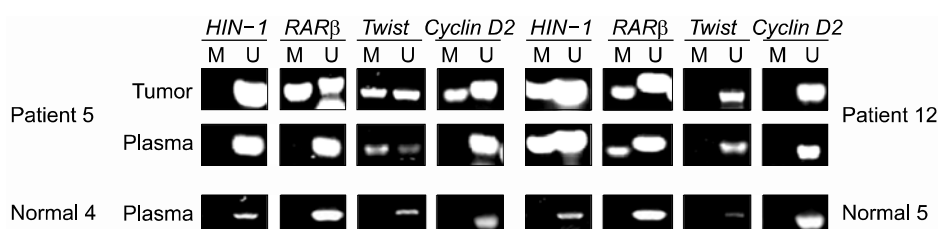
Fig. 1. Representative MSP results in breast cancer tissues, their paired plasma samples and normal controls. Lanes labeled "M" represent reactions using primers specific for bisulfite-treated DNA products with methylated CpG sites, and lanes labeled "U" represent reactions using primers specific for bisulfite-treated DNA products with unmethylated CpG sites. Some normal controls showed no amplified signals for methylated or unmethylated reactions. In those cases, the MSP was repeated and the absence of a methylated signal, in contrast to the presence of an unmethylated signal, confirmed.

Research, Inc., Waltham, MA), with a total of 35 and 45 cycles for tumor and plasma DNA, respectively. The conditions were: $95^{\circ}C$ for 5 min, followed by repetitions of $95^{\circ}C$ for 30 sec, $56^{\circ}C$ for 30 sec and $72^{\circ}C$ for 45 sec, with a final extension step of $72^{\circ}C$ for 10 min. *In vitro* methylated DNA, with SssI methyltransferase, was used as a positive control for the methylated products. As a negative control for each set of PCR, distilled water was used instead of DNA. Ten μl of PCR products were loaded onto a 2% agarose gel mixed, with GelStar® nucleic acid gel stain (Biowhittaker Molecular Applications, Rockland, MA), and visualized under UV illumination. Images were obtained using the Electrophoresis Document and Analysis System (EDAS290, Kodak, Rochester, NY). Samples were scored as methylated DNA when there was clear visible band on the gel when the methylated primers were used (Fig. 1).

Patient No.	Age (yrs)	Type	TNM	Ki-67	HIN-1 Tumor	HIN-1 Plasma	RARβ Tumor	RARβ Plasma	Twist Tumor	Twist Plasma	Cyclin D2 Tumor	Cyclin D2 Plasma
1	64	Ductal	$T_2N_1M_0$	Low								
2	41	Mucinous	$T_1N_0M_0$	Low								
3	37	Ductal	$T_2N_0M_0$	Low								
4	39	Ductal	$T_2N_2M_0$	Low								
5	47	Ductal	$T_2N_3M_0$	High								
6	35	Ductal	$T_2N_1M_0$	High								
7	43	Ductal	$T_1N_0M_0$	Low								
8	46	Ductal	$T_1N_0M_0$	Low								
9	38	Ductal	$T_1N_1M_0$	Low								
10	56	Ductal	$T_1N_0M_0$	Low								
11	46	Medullary	$T_2N_0M_0$	High								
12	46	Ductal	$T_2N_1M_0$	Low								
13	38	Ductal	$T_2N_0M_0$	Low								
14	44	Ductal	$T_3N_1M_0$	High								
15	42	Ductal	$T_1N_0M_0$	Low								
16	49	Ductal	$T_1N_1M_0$	High								
17	57	Ductal	$T_1N_0M_0$	High								
18	55	Ductal	$T_2N_2M_0$	Low								
19	47	Ductal	$T_2N_2M_0$	Low								
20	46	Ductal	$T_2N_0M_0$	High								
21	41	Ductal	$T_1N_1M_0$	Low								
22	61	Ductal	$T_1N_0M_0$	Low								
23	49	Ductal	$T_2N_2M_0$	Low								
24	59	Ductal	$T_2N_3M_0$	Low								
25	47	Ductal	$T_2N_0M_0$	High								
26	55	Papillary	$T_2N_0M_0$	Low								
27	35	Ductal	$T_1N_0M_0$	Low								
28	51	Ductal	$T_1N_0M_0$	Low								
29	49	Ductal	$T_2N_1M_0$	High								
30	40	Micropap	$T_2N_3M_0$	High								
31	39	Ductal	$T_2N_2M_0$	High								
32	49	Ductal	$T_1N_0M_0$	Low								
33	43	Ductal	$T_2N_0M_0$	High								
34	54	Ductal	$T_1N_1M_0$	High								

Fig. 2. Clinicopathological data and MSP results of breast cancer patients. Black and white boxes indicate methylated and unmethylated samples, respectively. Criteria for high Ki-67 is nuclear staining in more than 30% of tumor cells.

4) Statistical analysis

Fisher's exact and χ^2 tests (SPSS 12.0 for windows) were used to analyze the correlation between the gene promoter hypermethylation and clinicopathological features of the breast cancer. Spearman rank correlation was used to find concordant methylation between two different genes. $p \leq 0.05$ was considered statistically significant.

RESULTS

1) Patients and tumor characteristics

Thirty-four patients with breast cancer were examined. The clinicopathological features of the patients were obtained from the medical records and pathological reports. Among these 34 patients, there were 30 invasive ductal carcinomas, 1 mucinous carcinoma, 1 medullary carcinoma, 1 papillary carcinoma and 1 micropapillary carcinoma. The mean age of the patients was 46.7 years (range $35 \sim 64$ years), and that of normal individuals used as controls was 49.6 years (range $33 \sim 64$ years). The tumor sizes were 2 cm or less in 14 cases (41%), larger than 2 cm, but less than 5 cm, in 19 cases (56%), and larger than 5 cm in one case (3%). Seventeen of the 34 cases (50%) had axillary lymph node metastasis at the time of surgery. High Ki-67 expression ($>30\%$ nuclei positive) was observed in 13 of the 34 cases (38%).

2) Gene hypermethylation frequency in primary breast carcinoma

The methylation stati of the *cyclin D2, RARβ twist* and *HIN-1* gene promoters in 34 invasive breast cancer tissues were analyzed using the MSP technique (Fig. 2). Thirty-three of the 34 (97%) primary breast tumors exhibited abnormal promoter hypermethylation in at least one of the four genes studied (Fig. 3). This frequency was very similar to that (100%) of a previous report, which studied five genes in invasive breast carcinomas (18). Twenty-six (76%) invasive breast carcinomas displayed hypermethylation in two or more genes (Fig. 3). The individual genes were hypermethylated in 53, 56, 41 and 77% of the *cyclin D2, RARβ twist* and *HIN-1* genes, respectively (Table 2). Significantly concordant methylation was observed for *HIN-1* and twist (r=0.41, p=0.04), but methylation among other genes in the primary tumors appeared to occur independently. For each gene, there was no difference between the mean ages of the patients in the groups with the methylated versus the unmethylated genes. The clinicopathological data of the patients was then correlated with the results of MSP. As
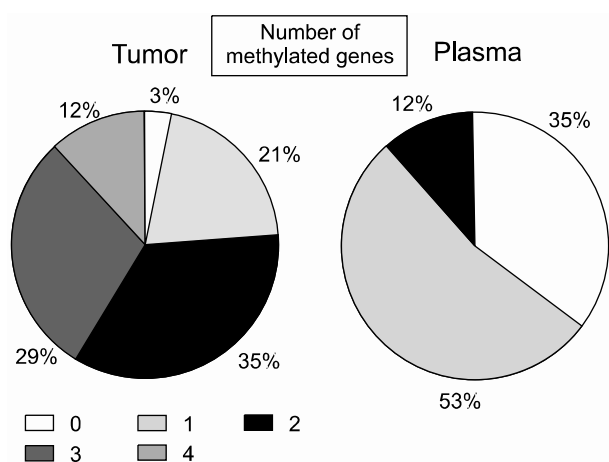
Fig. 3. Distribution of patients according to the number of methylated genes in the primary breast tumor and the plasma. The numerals in the box indicate the number of methylated genes.

Table 2. Frequency of promoter hypermethylation of the *cyclin D2, RARβ twist* and *HIN-1* genes in tumor tissues and plasma samples of breast cancer patients

	Frequency of hypermethylation	
	Tumor (%)	Plasma (%)
Cyclin D2	18/34 (53)	1/18 (6)
RARβ	19/34 (56)	3/19 (16)
Twist	14/34 (41)	5/14 (36)
HIN-1	26/34 (77)	14/26 (54)

seen in our previous data (16), the presence of hypermethylation in invasive breast carcinomas was not associated with tumor size, axillary lymph node metastasis, stage or Ki-67 labeling index (Table 3).

3) Promoter hypermethylation in plasma DNA from breast cancer patients

The frequency of hypermethylation for the aforementioned gene promoters was then analyzed in the 34 matched plasma samples from the breast cancer patients to determine the feasibility of MSP analysis for detecting tumor-derived DNA in plasma samples, and the concordance of the methylation profiles between the two specimen types. Promoter hypermethylation in one or more genes was detected in the plasma of 22 (65%) of the breast cancer patients (Fig. 3). In contrast, no hypermethylated DNA was detected in any of the 10 age-matched control plasma samples, or in the plasma from the single patient with a medullary carcinoma that showed no methylation of any of the four genes in the primary tumor.

The relative frequencies of hypermethylation of all four genes were lower in the plasma than in the primary tumors, but these relative frequencies were not uniform for the four

Table 3. Clinical and pathological features of breast cancer patients with promoter hypermethylation in primary tumors and plasma

	Primary tumors												Plasma		
	HIN-1			RARβ			Twist			Cyclin D2			No. genes hypermethylated		
	M	U	p	M	U	p	M	U	p	M	U	p	≥1	0	p
Mean age (yrs)	45	47		47	47		46	47		47	46		47	46	
Tumor size (cm)															
≤ 2	12	2		7	7		3	11		10	4		11	3	
2 <	14	6	0.42	12	8	0.73	11	9	0.08	8	12	0.09	11	9	0.28
Stage															
I	9	1		5	5		2	8		7	3		8	2	
II	10	5		8	7		6	9		7	8		8	7	
III	7	2	0.4	6	3	0.74	6	3	0.12	4	5	0.43	6	3	0.38
ALN* metastasis															
Absent	14	3		11	6		6	11		9	8		12	5	
Present	12	5	0.69	8	9	0.49	8	9	0.73	9	8	1.0	10	7	0.72
Ki-67															
Low	18	3		12	9		9	12		12	9		13	8	
High	8	5	0.17	7	6	0.71	5	8	0.54	6	7	0.39	8	5	0.63

*axillary lymph node.

genes. Hypermethylation of *HIN-1* was detected in the plasma from 14 of the 26 patients (54%) with methylation of the gene in the primary tumor; the other relative frequencies were 6% (1 of 18), 16 % (3 of 19) and 36% (5 of 14) for *cyclin D2*, *RARβ* and *twist,* respectively (Table 2). By combining the results for the 4 genes, hypermethylation in the plasma DNA was detected in 22 of the 33 patients (67% sensitivity) with confirmed methylation of the genes in the primary tumor. In the plasma samples, 18 of the 22 patients were found to have one methylated gene, while four patients showed concurrent methylation in two genes. In only one case, a faint band, indicating hypermethylation for *HIN-1* gene, was detected in the plasma when it was not detected in the corresponding tumor.

The potential correlations between the presence of hypermethylated genes in the plasma and the clinicopathological parameters of the breast cancers were also studied. A statistical analysis revealed there was no significant difference in the clinicopathological parameters between the subgroups of patients with and without hypermethylated genes in their plasma (Table 3).

DISCUSSION

The finding of tumor-derived DNA in the circulation of cancer patients has inspired further efforts to develop DNA-based assays capable of detecting evidence of cancer using serum or plasma samples. Several previously tested approaches for the detection of cancer-derived DNA in serum or plasma in other types of cancers; however, have shown little promise for the detection of breast cancers. For example, microsatellite shifts (low-level instability) and loss of heterozygosity were detected in the plasma or serum DNAs from patients with head and neck (3) and small cell lung carcinomas (4), but breast cancers rarely exhibited microsatellite instability, with the detection of LOH requiring the majority of the DNA to be tumor-derived (20). Mutant *K-ras* and *p53* DNAs have also been identified in the plasma or serum of patients with colorectal and pancreatic carcinomas (5,6), but *K-ras* mutations rarely occur in breast cancers, and *p53* mutations in only 20∼40% (21).

The goal of our study was to determine the feasibility of detecting breast cancers through the measurement of another common class of cancer-specific alterations of DNA, gene promoter hypermethylation, in plasma samples. Although serum and plasma have often been used interchangeably for methylation studies, plasma samples were used in this study because serum may contain higher extra normal DNA due to cell lysis during coagulation (22).

Individual hypermethylation of the *cyclin D2, RARβ twist* and *HIN-1* genes were found at high frequencies in the tumor samples (41∼77%), similar to those previously reported for the same genes in other studies evaluating invasive breast cancers (16,18,23). Furthermore, reasonable complementation was observed among the four genes of the panel for detecting hypermethylation in breast cancer tissues, with almost all (97%) of the invasive cancers used in our study showing methylation of at least one of these markers.

This panel of genes was somewhat less effective at detecting hypermethylated DNA in the peripheral circulation of breast

cancer patients; however, an overall sensitivity of 67% was observed for the 33 patients with tumors showing hypermethylation for at least one of the genes compared to two other panels (*APC, RASSF1A, DAP-K*) (14) and (*p16* and *CDH1*) (15), with 76 and 82% sensitivities, respectively. This can likely be attributed to differences in PCR amplification efficiencies between the primer sets and laboratories, as two panels-(*cyclin D2, RARβ twist* and *HIN-1*) and (*APC, RASSF1A, DAP-K*)-provided similar diagnostic coverage (97% and 94%, respectively) in the corresponding tumors. It is expected that the detection sensitivity is enhanced by the addition of other genes that are frequently hypermethylated in breast cancers to the panel. Our previous study established no significant differences between two demographically distinct populations or among the different histological breast cancer types with regard to the overall frequency of gene methylation or frequency of methylation for any specific genes, with the exception of the high frequency of *BRCA1* methylation observed in mucinous cancers (16). Through MSP analysis, Dulaimi et al. (14) detected methylation in the serum DNA from patients with preinvasive lesions (ductal CIS and lobular CIS). Both of these studies have offered promising suggestion that, if a panel, with a large number of genes specific for breast cancer, was constructed, and the MSP primers or PCR technology optimized, the methylation-based screening of the plasma or serum could cover all races, histological types and stages for the detection of breast cancers. However, it may not be possible to identify hypermethylated genes in the plasma or serum in all patients with hypermethylation of the same gene in the primary tumor, as not all tumors shed DNA into the blood and because; in cancer patients, the tumor DNA fraction varies from 3 to 93% of the total circulating DNA (2). Additionally, circulating DNA fragments have limited stability, and hypermethylation is not a fixed event (24). This would partially explain the differences in the methylation frequencies of the same gene between tumor tissues and plasma, which has been shown in this study as well as other reports (7∼11,14,15).

Another issue with using hypermethylated genes as markers for the detection of breast cancer in plasma is that of achieving good specificity. Methylation of our markers was not detected in the normal controls. The sample size of the control group in our study; however, was not large enough to validate 100% specificity. Evron et al. (23) demonstrated low methylation frequency (4%) for *RARβ* in white blood cells. In our study, methylation of the *HIN-1* gene was detected in the plasma of one patient not showing methylation in the same gene in the corresponding tumor tissue. A similar phenomenon was also observed in another group (25). The investigators suggested the presence of other undetected organ malignancies, or irritation by environmental factors, as possible explanations for the positive results. Thus, examination of gene methylation patterns between different normal tissues and the effects of aging, inflammatory diseases of major organs and life styles, such as smoking and air pollution, on the methylation patterns can further validate the specificity of a methylation-based study.

Another potentially important finding of our study was the detection of methylated DNA in the plasma of breast cancer patients, irrespective of tumor stage. This contrasts with the findings of Muller et al. (13) and Hu et al. (15), who reported

a strong relationship between the presence of hypermethylated DNA in the serum and clinicopathological features of the underlying breast cancer. Their results appear more convincing due to the presence of tumor-derived DNA in the plasma was related to cellular turn-over, necrosis and apoptosis of tumor cells (2), and also because the presence of tumor-specific hypermethylated genes in the plasma or serum indicates a release of sufficient amounts of tumor DNA into the circulation, which is likely to be correlated with the degree of invasiveness. However, several previous studies, which investigated stomach, lung, liver, and head and neck cancers, also failed to demonstrate any correlation between the detection of promoter hypermethylation in the serum and the tumor stage (7∼9,11). The findings of a recently published study support our results, as hypermethylation of *APC, RASSF1A* and *DAP-K* were detected by an MSP analysis of the serum DNA from patients with preinvasive breast cancer (ductal CIS and lobular CIS), and from patients with all grades and stages of invasive breast cancer; additionally, positive detection of hypermethylation in the serum was not associated with the tumor stage (14). These apparent discrepancies could also be related to the specific gene markers used, and it would clearly be advantageous for panels of markers to be developed that are both highly sensitive to cancers at all stages, as well as useful for the clinical staging in breast cancer management.

Our study provides encouragement that measurements of gene promoter hypermethylation using sensitive methods, such as MSP, could be used to develop sensitive and relatively specific testing methods for detecting and monitoring breast cancer, although further investigation is needed to confirm the sensitivity and specificity of this and other procedures. The selection of an optimal panel of markers will most likely be critical for increasing the sensitivity of this approach, which will require the location of DNA sequences are commonly methylated in the serum or plasma DNA from cancer patients, as well as in breast tumor tissue.

CONCLUSIONS

The hypermethylation of four genes (*cyclin D2, RARβ twist,* and *HIN-1*) were measured in primary tumors and paired plasma samples of breast cancer patients. Almost all of the primary breast tumors (97%) showed abnormal promoter hypermethylation in at least one of these genes. Hypermethylation in the plasma DNA was detected in 67% of the patients with confirmed methylation of the genes in the primary tumor. Detection of aberrant promoter hypermethylation of cancer-related genes in the plasma could be a useful method for detecting and monitoring breast cancer, although further studies are needed to confirm the sensitivity and specificity of the procedure.

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