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Quantitative Multiplex Methylation-Specific PCR Assay for the Detection of Promoter Hypermethylation in Multiple Genes in Breast Cancer

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ABSTRACT

If detected early, breast cancer is eminently curable. To detect breast cancer in samples with little cellularity, a high level of sensitivity is needed. Tumor-specific promoter hypermethylation has provided such a valuable tool for detection of cancer cells in biological samples. To accurately assess promoter hypermethylation for many genes simultaneously in small samples, we developed a novel method, quantitative multiplex-methylation-specific PCR (QM-MSP). QM-MSP is highly sensitive (1 in 10^4–10^5 copies of DNA) and linear over 5 orders of magnitude. For *RASSF1A*, *TWIST*, *Cyclin D2*, and *HIN1*, we observed significant differences in both the degree ($P < 0.003$) and incidence ($P < 0.02$) of hypermethylation between normal and malignant breast tissues. Evaluation of the cumulative hypermethylation of the four genes within each sample revealed a high level of sensitivity (84%) and specificity (89%) of detection of methylation. We demonstrate the application of this technique for detecting hypermethylated *RASSF1A*, *TWIST*, *Cyclin D2*, *HIN1*, and *RARB* in 50–1000 epithelial cells collected from breast ducts during endoscopy or by lavage. Such an approach could be used in a variety of small samples derived from different tissues, with these or different biomarkers to enhance detection of malignancy.

INTRODUCTION

Epigenetic alterations including hypermethylation of gene promoters are proving to be consistent and early events in neoplastic progression (1–4). Such alterations are thought to contribute to the neoplastic process by transcriptional silencing of tumor suppressor gene expression and by increasing the rate of genetic mutation (5, 6). DNA methylation is reversible because it does not alter the DNA sequence; however, it is heritable from cell to cell. Methylated genes can serve as biomarkers for early detection of cancer. Widschwendter and Jones (4) reviewed >40 genes whose expression is lost in breast cancer because of promoter hypermethylation, and we and others have studied hypermethylation of genes, including *NES-1* (7–10), *APC* (11–13), *Cyclin D2* (14, 15), *RARB* (16–18), *TWIST* (19), *RASSF1A* (15, 20, 21), and *HIN1* (22) in tissue (23) and ductal lavage fluids (19). Multigene promoter hypermethylation occurs commonly in tumors (24, 25). Because methylation changes often appear early in disease, detection of hypermethylated genes could identify tissues derived from patients with increased risk. Furthermore, the reversible nature of methylation offers the potential to revert aspects of the cancer phenotype with the appropriate therapy (26).

Tumor DNA can be found in various body fluids, and these fluids can potentially serve as diagnostic material (19, 27–29). Evaluation of tumor DNA in these fluids requires methods that are specific as well as sensitive. For example, a PCR-based technique called methylation-specific PCR (MSP) can detect 1 copy of methylated DNA in 1000 unmethylated copies of genomic DNA (30). Palmisano *et al.* (31) and others (32, 33) have modified this approach to coamplify several genes simultaneously in a nested or multiplex MSP assay. This method has been used to establish the frequency of gene promoter hypermethylation among patients with pulmonary (31) and esophageal (33) carcinoma. However, the method cannot quantitatively measure the levels of gene methylation because the read-out is gel-based and qualitative ("all or nothing"), based on the visual detection of the presence or absence of a band on a gel. The issue gains importance because benign tissues often show low levels of methylation in several genes.

To evaluate the degree of gene methylation within a single sample, quantitative MSP (Q-MSP) methods have been developed (15, 34–39). High and low levels of methylation may help to stratify different types or stages of carcinoma. The Q-MSP method is based on real-time PCR that uses fluorogenic probes to increase the assay specificity and the sensitivity; Q-MSP can detect one copy of the methylated marker gene among 10,000 unmethylated copies (36). The addition of a fluorogenic probe makes the technique more informative, quantitative, and suitable for clinical format. This technique is now becoming widely used (15, 34–39). However, analyses of multiple genes require additional quantities of template DNA. A dilemma is how best to distribute the available DNA to allow quantitative analyses of many different genes from precious small samples.

We have developed a technique called quantitative multiplex-MSP (QM-MSP) to coamplify many genes from quantities of sample previously used for just one gene. This technique combines multiplex PCR and Q-MSP in such a way that a panel of five genes can be coamplified in tissues derived from different sources, including those from ductal lavage, endoscopy, and fine-needle aspirates, in which the amount of DNA is limiting, as well as in larger samples, such as formalin-fixed, paraffin-embedded sections of core biopsies. This technique can be used to define the extent of gene promoter hypermethylation in normal tissues on a gene-by-gene basis and provides the ability to discriminate between normal/benign and malignant tissues.

MATERIALS AND METHODS

Tissues and Cells. Paired primary invasive breast carcinomas and adjacent normal tissues (frozen tissue), paraffin-embedded normal breast (routine reduction mammoplasty), and primary breast carcinoma sections were obtained from the Surgical Pathology archives of The Johns Hopkins Hospital following approval by the Institutional Review Board. The percentage of epithelial cells in each tissue section ranged from 30 to 80% on each slide. Ductal cell samples obtained by lavage of high-risk women and endoscopy of women with biopsy-proven breast cancer before surgery were provided after cytological diagnosis. Histopathological diagnosis of the resected specimen from patients undergoing endoscopy was obtained from Surgical Pathology. Human sperm was obtained from a healthy volunteer. The MDA-MB231 (231) breast cancer cell line was obtained from American Type Culture Collection (Manassas, VA) and cultured

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Table 1 *Sequences of multiplex primers used in round 1 of PCR*

Primer name	Primer sequence (5′ → 3′)	Orientation
Cyclin D2 Ext F	tatttttgtaaagatagttttgat	Forward
Cyclin D2 Ext R	tacaactttctaaaaaataaccc	Reverse
RASSF1A Ext F(2)	gttttatagttttgtatttagg	Forward
RASSF1A Ext R(2)	aactcaataaactcaaactccc	Reverse
TWIST Ext R(4)	cctcccaaaccattcaaaaac	Forward
TWIST Ext F(3)	gagatgagatattatttattgtg	Reverse
RARB Ext F	gtaggagggtttatttttgtt	Forward
RARB Ext R(2)	aattacattttccaaacttactc	Reverse
HIN1 Ext F(2)	gtttgttaagaggaagtttt	Forward
HIN1 Ext RSEQ	ccgaaacatacaaaacaaaaccac	Reverse
ACTB Ext F	tatataggttggggaagtttg	Forward
ACTB Ext R	tataaaaacataaaacctataacc	Reverse
ACTB Ext F	tatataggttggggaagtttg	Forward

as directed. Peripheral blood leukocytes were isolated from blood collected from normal volunteers after receipt of informed consent following review by the Johns Hopkins Institutional Review Board.

DNA Extraction from Tissues and Peripheral Blood Cells. For each tissue, the lesion was confirmed on a H&E-stained section. For DNA extraction, one 5-μm tissue section from the same block was deparaffinized in xylene (20 min), scraped from the slide, and extracted in 100 μl of buffer [10 mM Tris (pH 8.0), 150 mM NaCl, 2 mM EDTA, 0.5% SDS] containing 40 μg proteinase K for 16 h at 50°C. For extraction of DNA from ductal cells, the number of cells on each Papanicolaou- or Diff-Quick-stained (American Scientific Products, McGraw, IL) cytospin preparation was counted, the coverslip was removed by treatment with xylene, and cells were scraped and transferred to 50 μl of buffer [10 mM Tris (pH 8.0), 150 mM NaCl, 2 mM EDTA, 0.5% SDS] containing 40 μg/ml proteinase K and 200 ng of salmon sperm carrier DNA. After proteinase K treatment, samples were heat-inactivated at 70°C for 10 min and centrifuged at 16000 × *g* for 10 min. Fifty μl of the supernatant were used directly as a source of DNA for sodium bisulfite treatment.

For leukocytes, frozen tissues, and 231 cells, DNA was extracted with phenol–chloroform (40). Human sperm DNA (HSD) was isolated by use of the PUREGENE DNA Purification Kit (Gentra Systems, Minneapolis, MN) and stored at 4°C. One μg of purified DNA was modified by sodium bisulfite treatment.

Sodium Bisulfite Treatment of DNA. Tissue, control and cell line DNAs were treated with sodium bisulfite and analyzed by MSP as described by Herman *et al.* (30). This process converts nonmethylated cytosine residues to uracil, whereas methylated cytosines remain unchanged. Bisulfite-modified samples were aliquoted and stored at −80°C.

Probes and Primers. The sequences of primers used for multiplex and for amplifying unmethylated and methylated CpG islands by Q-MSP are shown in Tables 1 and 2. Gene-specific probes were obtained from Applied Biosystems (Foster City, CA), and primers were obtained from Invitrogen Corporation (Carlsbad, CA). For methylated *Cyclin D2* and *RASSF1A* genes, the Q-MSP primers and probes were as described in Lehmann *et al.* (15). Methylation-independent Q-MSP primers and probes for β-actin (*ACTB*) were as described by Eads *et al.* (36). All other sequences for methylation-dependent primers were designed in known regions of promoter hypermethylation in breast carcinoma; each Q-MSP primer set (forward, reverse, and probe) contained 7–12 CpG dinucleotides of the promoter sequence and numerous independent cytosine residues.

QM-MSP. The QM-MSP procedure required two sequential PCR reactions (Fig. 1). In the first PCR reaction (the multiplex step), 1 μl of sodium bisulfite-treated DNA was added to 24 μl of reaction buffer [1.25 mM deoxynucleotide triphosphates, 16.6 mM $(NH_4)_2SO_4$, 67 mM Tris (pH 8.8), 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.1% DMSO, and 2.5–5 units of Platinum *Taq* (Invitrogen)] containing 100 ng each of the forward and reverse primers. Conditions were 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 45 s, with a final extension cycle of 72°C for 5 min. The PCR products were diluted up to 125 μl with water and stored at −20°C. For this reaction, the input DNA ranged from 50 ng (purified DNA) to ~40 pg (for some ductal cell samples).

For the second round (the Q-MSP step), 1 μl of the diluted PCR product from reaction 1 was used directly or after further dilution of up to $1:10^4$ (when 50 ng DNA was used in reaction 1). The diluted DNA was added to the Q-MSP reaction buffer containing 16.6 mM $(NH_4)_2S0_4$, 67.0 mM Tris (pH 8.8), 6.7 mM $MgCl_2$, 10.0 mM β-mercaptoethanol, 0.1% DMSO, 200 μM deoxynucleotide

Table 2 *Sequences of Q-MSP[a] primers used in round 2 PCR*

Primer name	Q-MSP primer sequence (5′ → 3′)	Orientation	Status
Cyclin D2 RT-FM	tttgatttaaggatgcgttagagtacg	Forward	M
Cyclin D2 RT-RM	actttctccctaaaaaccgactacg	Reverse	M
Cyclin D2 M Probe	6FAM-aatccgccaacacgatcgacccta-TAMRA	Reverse	M
Cyclin D2 RT-FUM	ttaaggatgtgttagagtatgtg	Forward	U
Cyclin D2 RT-RUM	aaactttctccctaaaaaccaactacaat	Reverse	U
Cyclin D2 UM Probe	6FAM-aatccaccaacacaatcaaccctaac-TAMRA	Reverse	U
RASSF1A RT-FM	gcgttgaagtcggggttc	Forward	M
RASSF1A RT-RM	cccgtacttcgctaactttaaacg	Reverse	M
RASSF1A M Probe	6FAM-acaaacgcgaaccgaacgaaacca-TAMRA	Reverse	M
RASSF1A RT-FUM	ggtgttgaagttggggtttg	Forward	U
RASSF1A RT-RUM	cccatacttcactaactttaaac	Reverse	U
RASSF1A UM Probe	6FAM-ctaacaaacacaaaccaaacaaaacca-TAMRA	Reverse	U
TWIST RT-FM	gttagggttcgggggcgttgtt	Forward	M
TWIST RT-RM	ccgtcgccttcctccgacgaa	Reverse	M
TWIST M-Probe	6FAM-aaacgatttccttccccgccgaaa-TAMRA	Reverse	M
TWIST RT-FUM (3)	ggtttgggggtgttgtttgtatg	Forward	U
TWIST-RT-RUM (3)	cccacctcctaaccaccctcc	Reverse	U
TWIST UM Probe	6FAM-aaacaatttccttccccaccaaaaca-TAMRA	Reverse	U
RARB RT-FM	agaacgcgagcgattcgagtag	Forward	M
RARB RT-RM	tacaaaaaaccttccgaatacgtt	Reverse	M
RARB M Probe	6FAM-atcctaccccgacgatacccaaac-TAMRA	Reverse	M
RARB RT-FUM	ttgagaatgtgagtgatttgagtag	Forward	U
RARB RT-RUM	ttacaaaaaaccttccaaatacattc	Reverse	U
RARB UM Probe	6FAM-aaatcctaccccaacaatacccaaac-TAMRA	Reverse	U
HIN1 RT-FM	tagggaaggggtacgggttt	Forward	M
HIN1 RT-RM	cgctcacgaccgtaccctaa	Reverse	M
HIN1 M Probe	6FAM-acttcctactacgaccgacgaacc-TAMRA	Reverse	M
HIN1-RT-FUM (2)	aagtttttgaggt ttgggtaggga	Forward	U
HIN1 RT-RUM (2)	accaacctcacccacactccta	Reverse	U
HIN1UM Probe	6FAM-caacttcctactacaaccaacaaacc-TAMRA	Reverse	U
ACTB F	tggtgatggaggaggtttagtaagt	Forward	Indep
ACTB R	aaccaataaaacctactcctcccttaa	Reverse	Indep
ACTB Probe	6FAM-accaccacccaacacacaataacaaacaca-TAMRA	Reverse	Indep

[a] Q-MSP, quantitative methylation-specific PCR; M, methylated; 6FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine; U, unmethylated; Indep, methylation-independent.

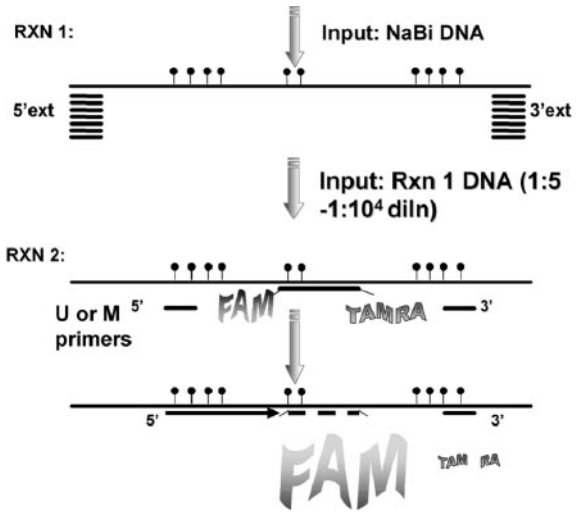


Fig. 1. Scheme for the quantitative multiplex methylation-specific PCR. In reaction 1 (*RXN 1*), a cocktail of gene-specific primer pairs is used to coamplify DNA for multiple genes independent of their DNA methylation status. In reaction 2 (*RXN 2*) quantitative real-time PCR is performed with gene-specific primers, using the DNA template derived from the end product of RXN 1 (diluted 1:5–1:10^4). DNA was analyzed in separate wells, using methylation status-specific [*U*, unmethylated; *M*, methylated) primers (forward, reverse, and probe, conjugated with the 6-carboxyfluorescein (*FAM*) label and 6-carboxytetramethylrhodamine (*TAMRA*) quencher]. The oligonucleotide probe is progressively degraded with each cycle of PCR by the 5′–3′ nuclease activity of the DNA polymerase; therefore, the fluorescence signal generated by 6-carboxyfluorescein is directly proportional to the extent of DNA amplification. % M = 100 × [no. of copies of M/no. of copies of (unmethylated + methylated)], as determined by the absolute quantification method computed against a standard curve. *NaBi*, sodium bisulfite; *ext*, external primer; *diln*, dilution.

triphosphates, 1.25 units of Platinum DNA *Taq* Polymerase (Invitrogen), and 1× ROX (Invitrogen) in a final volume of 25 μl. Six hundred nM each of two primers (forward and reverse) and 200 nM labeled probe (Applied Biosystems) were also present. The reaction was carried out in a 96-well reaction plate in an ABI Prism 7900HT Sequence Detector (Applied Biosystems). The reaction conditions were as follows: 95°C for 5 min, followed by 35 cycles of 95°C for 15 s and 60–65°C (depending on the primer set) for 1 min, with a 10-min extension at 72°C. For each gene included in the reaction plate, the following were used to create standard curves and to provide controls: (*a*) serially diluted stock multiplexed HSD/231 DNA (described below, to establish a standard curve); (*b*) 40,000 copy (40 K) standards; (*c*) no-template control; and (*d*) a known DNA ("1% M" control) to ensure consistency among runs. In addition, 100% methylated DNA (231 cell DNA), 0% methylated DNA (HSD), and a sample lacking template DNA from the first PCR reaction (diluted 1:5) were present as controls. All of the above samples were analyzed with primer sets for both methylated and unmethylated DNA. Because the 1% M control values have remained constant over a period of 1 year (data not shown) we conclude that the assay is stable and reproducible.

Preparation of Standards. A stock of multiplexed DNA was prepared as follows: PCR was performed in a reaction that contained all first-round gene primer pairs (Table 1) as well as a mixture of 50 ng each of sodium bisulfite-treated genomic 231 and HSD. Serial dilutions of this stock DNA were used to establish a standard curve in the real-time PCR reaction. To do this, the cycle threshold (C_T; the cycle in which the signal exceeds the background) of each dilution was determined during the Q-MSP reaction and then plotted against the dilution to generate a line for the standard curve. For each reaction plate, the standards were diluted from the same stock stored frozen at −80°C for all assays, and new dilutions were made each time. All assays had a correlation coefficient of the standard curve of 0.99 or higher and a slope of approximately −3.33, indicating 2-fold increases in PCR product per cycle in the linear phase of the quantitative PCR reaction.

Copy Number Standard. For the preparation of this standard, unmethylated or methylated genomic DNAs were amplified for each gene separately by use of a gene-specific pair of external primers and 50 ng of sodium bisulfite-treated genomic DNA derived from either MDA-MB231 (100% methylated) or HSD (100% unmethylated). A single band was observed by gel electrophoresis. The reaction products were then purified with the Qiaquick PCR purification kit (Qiagen Inc., Valencia, CA) and eluted in 100 μl of water. The eluate was quantitated by use of a NanoDrop spectrophotometer (NanoDrop Technologies, Montchanin, DE), and the DNA concentration (μg/μl) was determined (A_{260}). The molecular weight (μg/μmol) of the PCR product was calculated by use of Biopolymer Calculator v4.1.1 (C. R. Palmer).[5] The concentration of each gene template control was adjusted to 3×10^{10} copies/μl in 1 mg/ml salmon sperm carrier DNA, and then a cocktail of unmethylated and methylated template control was immediately prepared that contained 4×10^6 copies/μl each of the genes in 1 mg/ml salmon sperm DNA. This stock was stored at −80°C. For each reaction plate the stock was diluted 100-fold to 40,000 copies/well. We used this known quantity of standard (40,000 copies/well, denoted "40K" control), prepared as described above, to transform the standard curve to represent copy number. To accomplish this the C_T of the 40 K control was determined during the Q-MSP reaction and plotted on the line obtained for the standard curve. The copy number for each dilution was then "back calculated," based on where the 40K C_T intersected the standard curve. Sample 40K had approximately equal amounts of unmethylated and methylated DNA for each of the five genes (*RASSF1A*, *TWIST*, *Cyclin D2*, *HIN1*, and *RARB*) along with carrier salmon sperm DNA (10 μg/ml).

Calculation of Percentage of Methylation. The relative amount of methylation in each unknown sample was calculated as % M = 100 × [no. of copies of methylated DNA/(no. of copies of methylated + unmethylated DNA)]. The sum of unmethylated plus methylated DNA (U + M) was used as an approximation of the total number of copies present of a target gene. To determine the number of copies of methylated and unmethylated DNA, we mixed sample DNA with Q-MSP reaction buffer after the multiplex reaction, assayed the mixture with methylated primers and unmethylated primers (in separate wells) in the Q-MSP reaction, and then determined the C_T was for each. Using the ABI Prism SDS 2.0 software supplied by Applied Biosystems (Foster City, CA) with the 7900 HT Sequence Detector, we extrapolated the number of copies of methylated and unmethylated DNA from the respective standard curves, using the sample C_T and applying the absolute quantification method according to the manufacturer's directions. Only values falling within the range covered by the standard curve (usually 100–10,000,000 copies) were accepted.

Direct Q-MSP of Genomic DNA. For direct Q-MSP, standard curves were prepared using 10 pg, 100 pg, 1 ng, and 10 ng total genomic 231 DNA (fully methylated) or HSD (fully unmethylated), according to the absolute quantitation method described by Applied Biosystems in the 7900 HT Sequence Detector manual. The concentrations of methylated and unmethylated DNA were extrapolated from these curves, and the percentage of methylation was calculated as % M = 100 × [ng methylated gene A/(ng methylated gene A+ unmethylated gene A)], where total target gene DNA was taken as the sum of U + M. For purposes of comparing these results with methods that use β-actin (*ACTB*) as a reference DNA, we also computed the percentage of methylation using two other formulas. We calculated % M = 100 × (ng methylated gene A in tumor/ng *ACTB* gene in tumor), or essentially as described by Trinh *et al.* (37), who calculated the percentage of methylation as % M = 100 × [(ng methylated gene A in tumor/ng *ACTB* in tumor)/(ng methylated gene A in 231 controls/ng *ACTB* in 231 controls)].

Statistical Analysis and Graphical Representation of Data. Statistical analyses and plotting of data were performed with GraphPad Prism (GraphPad Software Inc., San Diego, CA) and Stata 7.0 (Stata Corporation, College Station, TX). *P* values < 0.05 were considered significant, and all tests were two-tailed. The nonparametric Mann–Whitney test was used to test whether the samples were from identical distributions, indicating that their medians were equal. Sample means were compared by use of the unpaired *t* test, assuming unequal variances (Welch's correction). For testing of means, data were transformed as a function of Ln_e(%M + 1) where stated to fulfill the assumption of normality. The Fisher's exact test was used to test whether the differences between the incidence of positivity for methylation in tumor and nontumor samples were significant.

[5] http://paris.chem.yale.edu/extinct.html.

RESULTS

Validation of the Two-Step QM-MSP Assay. Multiplex PCR was accomplished by performing two sequential PCR reactions (Fig. 1). In the first PCR reaction, a cocktail of gene-specific primers was used to coamplify *RASSF1A*, *TWIST*, *Cyclin D2*, *HIN1*, and *RARB* (Table 1). These external primer pairs were complementary to the sequences flanking the CpG island that was to be assayed in the second PCR reaction. External primers were selected to exclude CG dinucleotides, thereby rendering DNA amplification independent of the methylation status. In the second reaction, quantitative analysis of methylated and unmethylated DNA for each gene was performed separately with the primer pairs and probes shown in Table 2.

Unmethylated and Methylated Primers are Equally Efficient in Amplifying Sodium Bisulfite-Converted DNA. Primer sets for specifically amplifying methylated (M) or unmethylated (U) DNA (Table 2) were designed for comparable performance; to confirm this we plotted, the ΔC_T (C_T M − C_T U) as a function of sample dilution over a wide range of dilutions (10^{-3}–10^{-8}) of the standard stock HSD/231 DNA. Analyses were performed as shown for *RASSF1A* (Fig. 2). The ΔC_T was approximately the same for all dilutions, as shown by the horizontal nature of the line, indicating that the primer sets were equally efficient over 5 logs of template quantities (Fig. 2*A*). In addition, for both unmethylated and methylated DNA, the slopes of the standard curves were approximately −3.33, which is reflective of a 2-fold increase in PCR product per cycle during the linear phase of real-time PCR. Finally, the correlation coefficient (R^2) of 0.999 provided evidence of linearity over the entire range of template concentration (Fig. 2*B*). Similar results were obtained for each of the other genes in this study (data not shown).

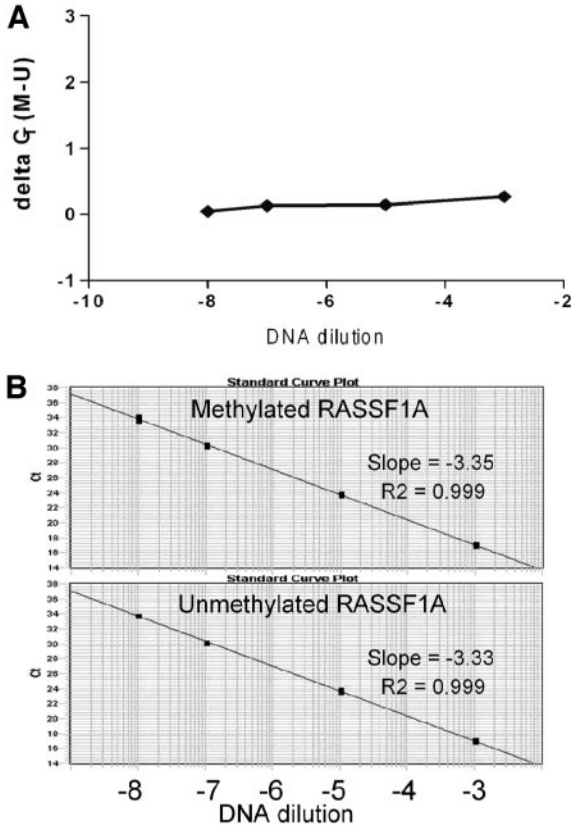


Fig. 2. Efficiency of quantitative multiplex methylation-specific PCR. For verification of the comparable function and efficient performance of the unmethylated (*U*) and methylated (*M*) primers, QM-MSP was performed using *RASSF1A* U and M primer sets to amplify serially diluted (10^{-3}–10^{-8}) fragments of multiplexed standard stock human sperm DNA/MDA-MB231 DNA. The cycle threshold (C_T) was determined for each dilution of DNA. *A*, plot of ΔC_T ($\Delta C_T = C_T$ M − C_T U) *versus* dilution. Nearly identical ΔC_T values for each DNA dilution indicate uniform primer performance over 5 logs. *B*, standard curve plots (C_T *versus* quantity) of serially diluted DNA. The slope of −3.33 reflects a 2-fold amplification of DNA per cycle (high efficiency). The correlation coefficient (R^2) of 0.999 shows the high degree of linearity over the entire range.

Table 3 *Determining sensitivity of quantitative multiplexed methylation-specific PCR*

	Methylation[a] (%)			
	RASSF1A	*TWIST*	*HIN1*	*Cyclin D2*
Copies M[b] and U template[c,d]				
1000/100,000	0.9220	2.7604	4.8024	1.5760
100/100,000	0.0602	0.2482	0.6273	0.1525
10/100,000	0.0078	0.0272	0.0024	0.0025
1/100,000	0.0072	0.0012	0.0000	0.0000
0.1/100,000	0.0000	0.0000	0.0000	0.0000
HSD (100% U control)	0.0000	0.0003	0.0000	0.0000
231 (100% M control)	99.9981	100.0000	100.0000	100.0000
Water	0.0000	0.0000	0.0000	0.0000
Copies M and U[c,e]				
300/29,700	0.7823	1.2190		
30/2,970	0.7195	0.9376		
3/297	0.2390	0.1451		

[a] The percentages of methylation of *RASSF1A*, *TWIST*, *HIN1*, and *Cyclin D2* from the quantitative multiplexed methylation-specific PCR assay are shown. The assay sensitivity is 1–10 in 100,000 for methylated DNA, and it detects as few as 1–3 copies of methylated DNA.
[b] M, methylated; U, unmethylated; HSD, human sperm DNA; 231, MDA-MB231.
[c] Purified methylated and unmethylated stock DNA template were mixed in the proportions indicated.
[d] Unmethylated DNA was kept constant as the amount of methylated DNA was decreased.
[e] A 1% M control was kept constant as the total quantity of DNA decreased.

Specificity and Sensitivity. To assess the sensitivity and specificity of the QM-MSP method, we performed a mixing experiment, using column-purified, PCR-amplified fragments of sodium bisulfite-modified DNA as template (Table 3). The amount of unmethylated DNA was kept constant (100,000 copies/well), and the amount of methylated DNA was decreased (1000−0.1 copy/well). While the general efficiency of real-time PCR is known to fall off at <100 copies, it was expected that some level of methylation would be detected at concentrations <100 copies, although probably not in a linear manner. For *RASSF1A* and *TWIST*, methylation was detected at 1 methylated copy in 100,000 unmethylated copies, and for *Cyclin D2* and *HIN1*, methylation was detected at 10 in 100,000 copies. Therefore, the overall sensitivity of the method was 1–10 in 100,000.

The highly specific performance of the methylated primers was demonstrated by the use of HSD control (100% unmethylated DNA) and the 0.1/100,000 sample (diluted to <1 copy of methylated in the presence of 100,000 copies of unmethylated DNA per well), both of which showed 0% M in the Q-MSP reaction (Table 3; Fig. 3). Likewise, no unmethylated signal (0% U) was detected in 231 methylated DNA control (100% methylated DNA). It was expected that the 1000/100,000 sample would be read as 1% methylated (1000 copies of methylated and 100,000 copies of unmethylated). That some samples were slightly higher (*e.g.*, 4.8% for *HIN1* and 2.8% for *TWIST*) probably reflects difficulty in accurately diluting samples from a starting concentration of 3×10^{10} copies/μl (diluted over 5–10 logs).

To characterize the behavior of the QM-MSP assay below the lower limit of linearity (specifically, whether a 1% M template produces a 1% M assay result as the total quantity of DNA template diminishes), we serially diluted a "1%" standard and tested samples beginning at a template quantity estimated to contain 300 copies of methylated DNA to ~ 29,700 unmethylated copies (300/29,700 per well), using column-purified DNA as a template (Table 3). The lowest quantity tested contained 3 copies of methylated DNA to ~297 copies of unmethylated DNA (3/297). The results for *RASSF1A* and *TWIST* (Table 3) revealed that at ratios of 300/29,700 and 30/2,970 total copies, the

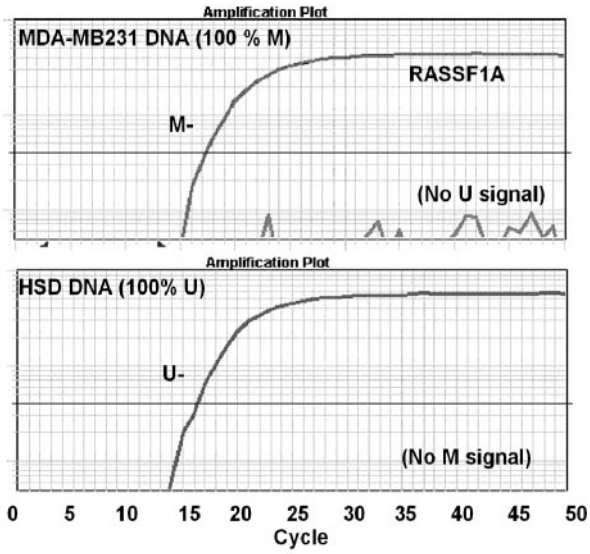


Fig. 3. Specificity of quantitative multiplex methylation-specific PCR. Shown are amplification plots of multiplexed human sperm DNA (*HSD*) or MDA-MB231 template DNA tested with unmethylated (*U*) and methylated (*M*) primers for *RASSF1A*. The unmethylated and methylated reactions were 100% specific because U primers did not cross-react with MDA-MB231 DNA (100% methylated) and M primers did not cross-react with human sperm DNA (100% unmethylated).

assay result (% M) was ~1% for each. Methylation was still detectable at the lowest ratio template quantity tested, at 3 copies of methylated DNA, consistent with the previous experiment (1 copy of methylated DNA was detected in 100,000 copies unmethylated DNA). We found a bias toward underreporting of the % M below 30 copies of methylated DNA, probably reflecting the relative lack of efficiency of the methylated reaction compared with the unmethylated reaction that contained nearly 100-fold more copies of the gene (Table 3). This result was predicted because linearity is generally known to be lost below 100 copies of DNA template in real-time PCR.

Genomic DNA is a more challenging template than PCR-amplified DNA because breakage of genomic DNA is known to occur in the process of sodium bisulfite conversion. To evaluate the sensitivity of the QM-MSP method for detecting methylated alleles when genomic DNA was used, we mixed ~40 pg of methylated DNA (~13 copies derived from 231 cell DNA) with 600–60000 pg of unmethylated genomic DNA (~200–20,000 copies of HSD), using the conversion estimate of 3 pg/copy of genomic DNA. Our data showed that 40 pg of methylated *RASSF1A* genomic DNA was easily detected even in the presence of a 1500-fold excess of unmethylated DNA (Fig. 4).

To ensure specific amplification of sodium bisulfite-modified DNA (but not nonconverted DNA; Ref. 41), each primer set (forward, reverse, and probe) covered 7–12 CpG dinucleotides pairs when used together and also covered numerous independent (presumably unmethylated) cytosines. Controls were included during the development of QM-MSP to ensure that no reactivity with nonconverted or sodium bisulfite-treated carrier (salmon sperm) DNA was detectable by QM-MSP (data not shown). Lastly, in each QM-MSP reaction, 100% unmethylated (HSD) and 100% methylated (231) sodium bisulfite-modified DNAs were analyzed as controls, and no background signals were detectable with use of real-time primers.

Comparison of QM-MSP with Direct Q-MSP. The QM-MSP method is sensitive and specific (Table 3; Fig. 3). There are two basic differences between most conventional Q-MSP methods and QM-MSP: (*a*) QM-MSP includes an additional PCR step (multiplex), which could lead to greater sensitivity but lower specificity than Q-MSP; and (*b*) in contrast to the standard use of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) or *ACTB* reference DNA, QM-MSP uses the sum of methylated and unmethylated DNA (U + M) of the same gene for determining total gene DNA present in the sample. The use of the (U + M) formula ignores the possible contribution to total DNA from partially methylated DNA and thus could potentially overestimate the percentage of methylation in each sample. We tested the impact of these differences experimentally.

QM-MSP *versus* Direct Q-MSP Using (U + M) as Total DNA. It is possible that performing a two-step multiplex PCR method could yield results that differed from those obtained with a direct one-step PCR method because of the addition of the multiplex step. We performed QM-MSP and direct Q-MSP assays on a panel of five tumor DNAs and calculated the percentage of methylation by the (U + M) method to estimate total DNA. With few exceptions, there was excellent concordance between the percentage of methylation values obtained for the *RASSF1A*, *TWIST*, *HIN1*, or *Cyclin D2* genes (Table 4). The QM-MSP readout was much more robust (as a result of the preamplification of DNA), usually appearing around cycles 12–25 compared with the readout by Q-MSP, in which the C_T signal appeared around cycles 27–37 (data not shown).

Comparison of Percentage Methylation Using Actin (*ACTB*) *versus* (U + M) Target Gene DNA to Approximate Total DNA. We first addressed the question of whether the *ACTB* reference method and (U + M) methods yielded similar data in the setting of Q-MSP, as described in the "Materials and Methods." We found that, with few exceptions, there was excellent concordance between the % M values when we used either of the *ACTB* formulas or the (U + M) formula to estimate total DNA in Q-MSP (data not shown).

We next tested the same concept in the QM-MSP setting. For the same DNA samples, discordance was observed for % M values between (U + M) or *ACTB* formulas (data not shown). The rate of amplification of *ACTB* compared with those of the other genes in the mixture was not predictable in the multiplex reaction. For example, values obtained with *ACTB* as the reference gene (as described in the "Materials and Methods") showed concordance for some genes, very high values for others, and lower methylation values in ~50% of the samples (data not shown). On the other hand, with the (U + M) method, QM-MSP values were reproducible across quadruplicate assays. For example, different tumors gave % M values for *RASSF1A* of 42.6 ± 3.5, 42 ± 6.9, 71 ± 1.5, 13 ± 6.0, 27 ± 7.5, 81 ± 1.5, and

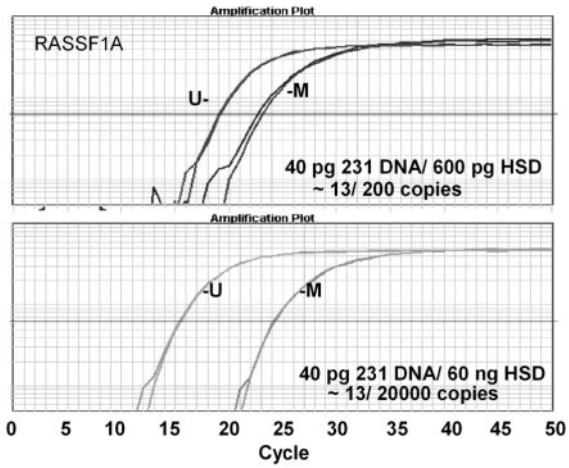


Fig. 4. Sensitive detection of hypermethylated genomic *RASSF1A* DNA. Genomic human sperm DNA (*HSD*) and MDA-MB231 (*231*) DNA were mixed as indicated before sodium bisulfite treatment (*top panel*, 600 pg of HSD and 40 pg of 231 DNA; *bottom panel*, 60 ng of HSD and 40 pg of 231 DNA). Quantitative multiplex methylation-specific PCR was performed. Shown are the amplification plots of each mixture of template. The plots show that 40 pg of genomic DNA is easily detectable even in the presence of a 1500-fold excess of unmethylated DNA.

Table 4 *Comparison between Q-MSP[a] and QM-MSP*

The percentage of methylation (*% M*) was calculated as: (the number of copies of methylated DNA divided by the number of copies of unmethylated + methylated DNA) × 100, using absolute quantitation. The positive control for unmethylated DNA was HSD, and for methylated DNA was 231.

	% M	
	Q-MSP	QM-MSP
RASSF1A		
1	95	99
2	80	81
3	39	41
4	12	21
5	28	34
HSD	0	0
231	100	100
Water	0	0
TWIST		
1	96	70
2	0	0
3	54	26
4	1	3
5	0	5
HSD	0	0
231	100	100
Water	0	0
HIN1		
1	95	82
2	81	74
3	82	48
4	41	16
5	47	43
HSD	0	0
231	100	100
Water	0	0
Cyclin D2		
1	47	25
2	0	0
3	1	0
4	19	22
5	2	0
HSD	0	0
231	100	100
Water	0	0

[a] Q-MSP, quantitative methylation-specific PCR; QM-MSP, quantitative multiplexed methylation-specific PCR; HSD, human sperm DNA.

41 ± 2.0%, whereas DNA samples that were negative for methylation yielded % M values of 0, 0, 0.4 ± 1.0, and 1.5 ± 0.5%. In QM-MSP, the sum of the unmethylated and methylated alleles of the same gene appear to serve as a reliable internal control for integrity, copy number, and method efficiency. The (U + M) formula was therefore used for the rest of the study.

Quantitation of Methylation in Invasive Breast Carcinoma and Comparison with Normal Breast Tissue. We analyzed test sets of DNA from 18 normal mammoplasty and 21 tumors specimens by QM-MSP for gene promoter hypermethylation of *RASSF1A*, *TWIST*, *Cyclin D2*, and *HIN1* (Table 5; Fig. 5). For *RASSF1A* alone, the normal breast test set was further expanded to 28 samples based on a previous report of a higher incidence of hypermethylation in benign breast tissue (15). Occasionally, PCR amplification failed for some genes within a test sample, presumably because of the fragile nature of archival DNA. *RASSF1A* hypermethylation ranged from 0 to 71% (mean, 18.5%) in carcinoma and from 0 to 56% (mean, 2.6%) in normal tissues ($P = 0.0001$), *TWIST* hypermethylation ranged from 0 to 72% (mean, 21.1%) in carcinomas and from 0 to 1.6% (mean, 0.11%) in normal tissues ($P = 0.0001$), *Cyclin D2* hypermethylation ranged from 0 to 44.5% (mean, 5.0%) in carcinomas and from 0 to 0.2% (mean, 0.02%) in normal tissues ($P = 0.02$), and *HIN1* hypermethylation ranged from 0 to 82.2% (mean, 24.5%) in carcinomas and from 0 to 18% (mean, 2.3%) in normal tissues ($P = 0.003$). When we used the Mann–Whitney test on untransformed data, the differences in the medians were highly significant for all genes tested: *RASSF1A* ($P = 0.0001$), *TWIST* ($P = 0.001$), *Cyclin D2* ($P = 0.0009$), and *HIN1* ($P = 0.003$). We also analyzed normal leukocyte DNA and found that methylation in these samples derived from the buffy coat was extremely low or undetectable ($n = 25$): median leukocyte methylation was 0% for *RASSF1A*, 0.06% for *TWIST*, 0% for *Cyclin D2*, 0.005% for *HIN1*, and 0.25% for *RARβ*. Because DNA from mammoplasty specimens and leukocytes is largely unmethylated relative to tumor samples, it is likely that the methylation signals observed in the tumors (Fig. 5) are derived largely from the carcinoma cells rather than normal ducts, stroma, and/or infiltrating leukocytes.

We chose to establish a cutoff (% M) for each gene at approximately the 10th percentile of the population, such that ~90% of normal breast tissues would be at or below the cutoff (we allowed 85–90% for *HIN1*; see above). Using cutoffs of 2% M for *RASSF1A* and *HIN1*, 0.5% M for *TWIST*, and 0.2% M for *Cyclin D2* in normal tissues, we considered values above the cutoffs "positive" for hypermethylation. Among carcinomas, 68% were positive for *RASSF1A*, 67% for *TWIST*, 57% for *Cyclin D2*, and 57% for *HIN1*. By comparison, 7–14% of normal mammoplasty samples were positive for *RASSF1A*, *TWIST*, *Cyclin D2*, and *HIN1*. Some samples had low-level methylation that was below the cutoff. Using these cutoffs, we observed a significant difference in the incidence of positivity between carcinoma and normal tissues (*RASSF1A*, $P < 0.00002$; *TWIST*, $P < 0.0002$; *Cyclin D2*, $P < 0.002$; and *HIN1*, $P < 0.02$, Fisher's exact).

Cumulative Gene Promoter Hypermethylation Scores in Primary Breast Cancer. To calculate the total amount of gene promoter hypermethylation as determined by QM-MSP, we used the sum of all

Table 5 *Quantitative multiplexed methylation-specific PCR analysis of normal breast and breast cancer DNA*

	Methylation (%)					
	Range	Median	Mean ± SE	Lower 95% confidence limit[a]	Upper 95% confidence limit	Positive for methylation,[b] n (%)
Normal breast						
RASSF1A	0–56	0[c]	2.6 ± 2.0	(0)	6.7	2/28 (7)
TWIST	0–1.6	0[d]	0.11 ± 0.09	(0)	0.29	1/18 (6)
HIN1	0–18	0[e]	2.3 ± 1.5	(0)	5.5	2/14 (14)
Cyclin D2	0–0.2	0[f]	0.019 ± 0.014	(0)	0.48	1/16 (7)
Breast carcinoma						
RASSF1A	0–71	7.0[c]	18.5 ± 4.7	8.7	28.2	13/19 (68)
TWIST	0–72	5.0[d]	21.1 ± 5.5	9.6	32.6	14/21 (67)
HIN1	0–82	9.9[e]	24.5 ± 6.1	11.8	37.3	12/21 (57)
Cyclin D2	0–44	0.26[f]	5.0 ± 2.5	(0)	10.3	12/21 (57)

[a] Values in parentheses indicate that the value is less than zero.
[b] Based on percentage of methylation cutoffs of 2% for *RASSF1A* and *HIN1*, 0.5% for *TWIST*, and 0.2% for *Cyclin D2*.
[c] $P = 0.0001$ for *RASSF1A*.
[d] $P = 0.001$ for *TWIST*.
[e] $P = 0.003$ for *HIN1*.
[f] $P = 0.0009$ for *Cyclin D2*.

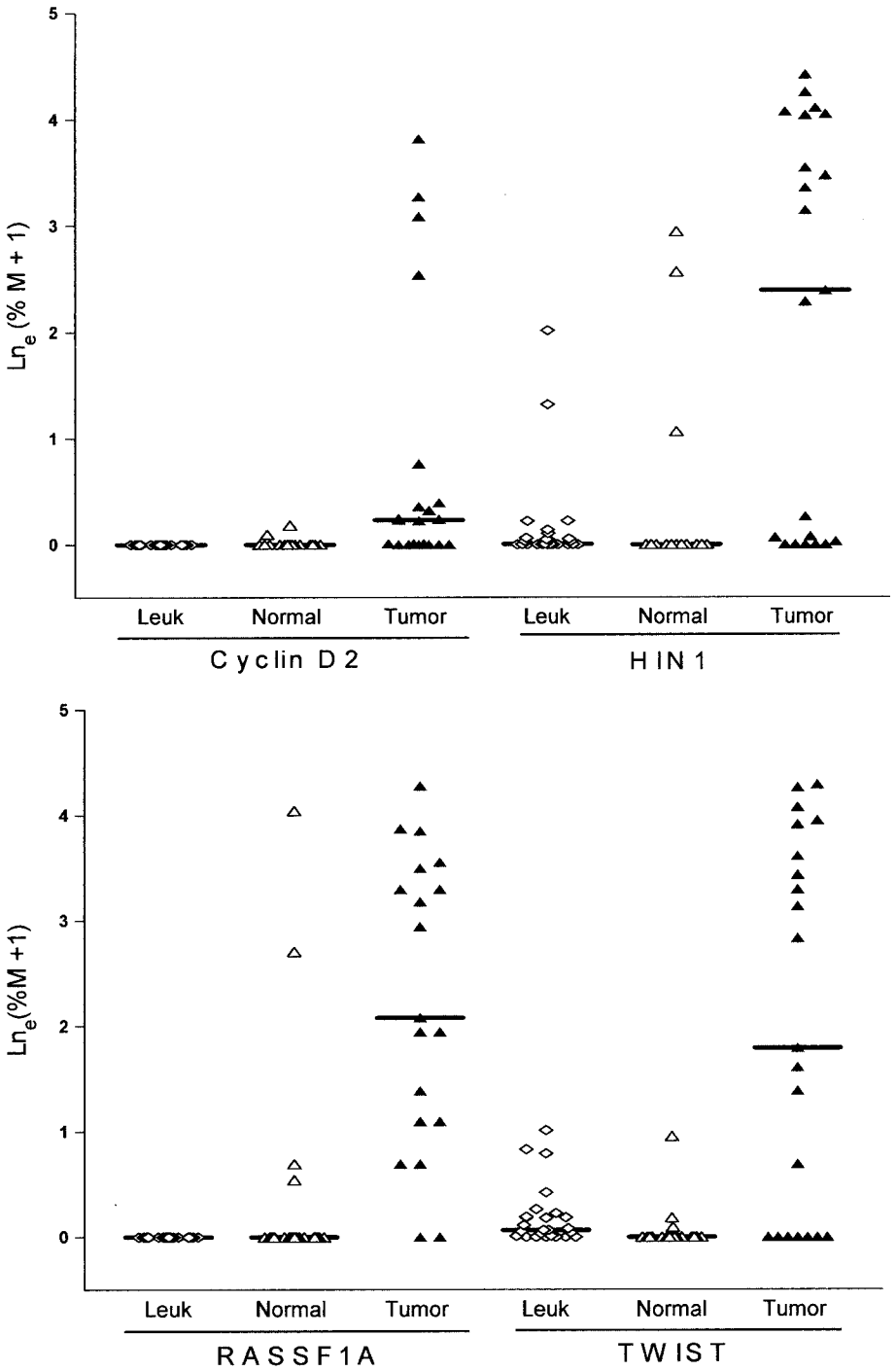


Fig. 5. Comparison of gene promoter hypermethylation in normal and malignant breast tissues. Quantitative multiplex methylation-specific PCR was performed on DNA from formalin-fixed, paraffin-embedded normal tissues, derived from patients undergoing reduction mammoplasty, for quantitation of *RASSF1A* (n = 28), *TWIST* (n = 18), *Cyclin D2* (n = 16), and *HIN1* (n = 14) gene promoter hypermethylation. In parallel, DNA derived from individuals with invasive ductal carcinoma was tested for *RASSF1A* (n = 19), *TWIST* (n = 21), *Cyclin D2* (n = 21), and *HIN1* (n = 21). Displayed is the Ln_e(% M + 1). The median of each group is indicated by a *horizontal bar*. Percentage methylation values for normal and carcinoma DNA were significantly different for all genes. Leukocyte (*Leuk*; n = 25) DNA derived from normal individuals showed low to no levels of methylation.

% M within the panel of genes to provide an overall cumulative score for each sample. In Fig. 6*A*, this is represented graphically relative to 231 DNA, which is 100% methylated for *RASSF1A*, *TWIST*, *Cyclin D2*, and *HIN1*; therefore, this control DNA had a relative score of 400. The cumulative methylation profiles of 9 normal mammoplasty samples were compared with those of 19 invasive carcinomas (Table 6; Fig. 6) in a subgroup of our test set in which results for all four markers were available. Normal tissues ranged from 0 to 18 units, and carcinomas ranged from 1 to 248 units. Among the nine normal tissues tested for four genes (36 values) the mean cumulative score was 2.61 ± 2.05 (median = 0; Fig. 6*B*). Among the 19 carcinomas tested for four genes (76 values), the mean cumulative score was 72.8 ± 15.03 units (median = 74) out of a possible 400 units (see above; Table 6; Fig. 6). The difference in log-transformed means between normal and malignant breast tissue was highly significant (P = 0.0001, unpaired t test with Welch's correction).

We explored the use of 4.7 units as a cumulative score cutoff value. This was based on the individual gene cutoffs (2% each for *RASSF1A* and *HIN1*, 0.5% for *TWIST*, and 0.2% for *Cyclin D2* = 4.7%). When we used this cutoff, 84% (16 of 19) of carcinoma samples were positive. Although 3 of 19 carcinomas fell below the cutoff, all of the "negative" carcinomas were methylated at low levels for one or more genes in this panel (Fig. 6*A*). Among normal samples, 89% (8 of 9) were negative. Thus, in this group of 28 samples (9 normal and 19 carcinoma), the sensitivity for detection of carcinoma was 0.84 and specificity was 0.89; the overall accuracy was 0.86 (24 of 28).

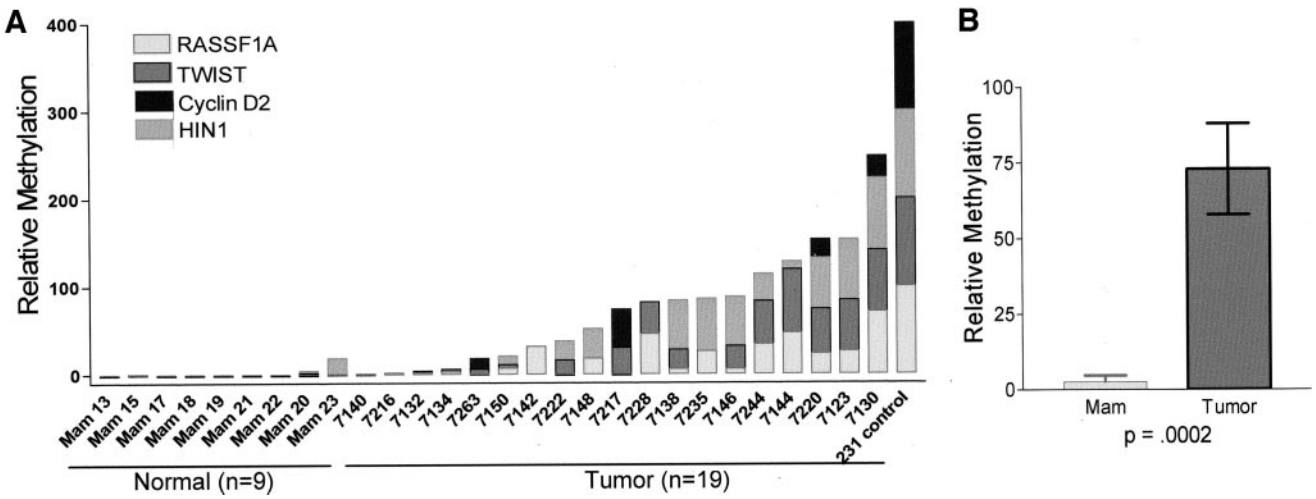


Fig. 6. Cumulative promoter hypermethylation of *RASSF1A*, *TWIST*, *Cyclin D2*, and *HIN1* in normal and malignant breast tissues. *A*, scores of cumulative methylation in normal mammoplasty and carcinoma. In a subgroup of samples from Fig. 5 for which results were available for all four genes in the panel, normal (*Mam*; $n = 9$) and carcinoma (*Tumor*; $n = 19$) samples were scored for cumulative methylation by adding the percentage of methylation for all four genes within each sample. A maximum of 400 relative methylation units was possible [*e.g.*, MDA-MB231 (*231 control*) DNA is 100% methylated for each of the four genes]. Results are plotted as *stacked bar graphs*. The *bar height* reflects total cumulative methylation, whereas the *segments* corresponding to the relative amounts of methylation of each gene are indicated. *B*, mean cumulative methylation in normal mammoplasty (*Mam*) *versus* carcinoma (*Tumor*). Plotted is the mean (± SE; *bars*) amount of cumulative methylation.

Comparison of Paired Carcinoma and Adjacent Normal Breast Epithelium. In an independent experiment, we examined six pairs of carcinoma and adjacent tissue from the surgical margins that were histologically normal to determine the cumulative amount of gene promoter hypermethylation in *RASSF1A*, *TWIST*, *Cyclin D2*, and *HIN1* (Table 6; Fig. 7). The cumulative methylation ranged from 2 to 29 units within adjacent normal tissues and from 5 to 258 units within carcinoma tissues, out of a possible 400 units (Fig. 7*B*). When we used the cutoff established for cumulative normal in mammoplasty samples (≤4.7 units; see above), all six carcinomas were positive. The adjacent "normal" tissues were also positive in four of six individuals. Although the cumulative methylation levels within carcinoma-adjacent, histopathologically normal tissues were significantly lower than in the nearby carcinoma ($P = 0.03$, Mann–Whitney), they had a significantly higher levels of methylation than normal mammoplasty samples ($P = 0.01$, Mann-Whitney; Table 6; Fig. 7*B*).

Detection of Multigene Promoter Hypermethylation in Breast Ductal Cells. To test the applicability of this method to investigate small clinical samples, we performed QM-MSP to assess *RASSF1A*, *TWIST*, *Cyclin D2*, *HIN1*, and *RARB* gene promoter hypermethylation in ductal cells derived from lavage of high-risk women ($n = 7$) or cells in the irrigation fluid obtained during breast endoscopy in four patients with biopsy-proven cancer, before resection of the lesion. The total number of epithelial cells present in the samples was estimated to be 50–1000 cells. The level of gene promoter methylation was quantitated for each sample, and the cumulative promoter methylation profile was established. Six of seven samples from women with a high risk of developing breast cancer had no detectable hypermethylation in the five genes tested, whereas one of seven displayed low-level methylation in *RASSF1A*. Cytological diagnosis of all of the specimens in this group was "benign." Of interest, cells obtained during endoscopy from the two women with invasive carcinoma had high-level multigene promoter hypermethylation (samples 10 and 11 in Table 7 and Fig. 8). The samples from the two women with ductal carcinoma *in situ* displayed benign cytology and lacked detectable promoter hypermethylation (samples 8 and 9).

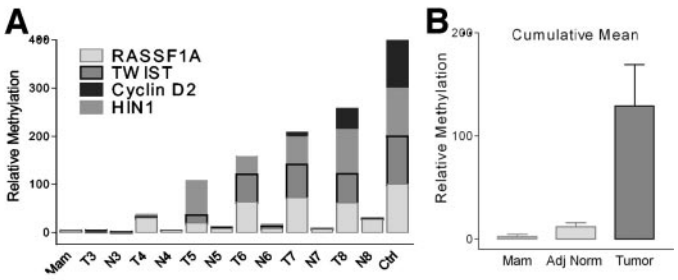
DISCUSSION

Cytological analysis of breast ductal cells could aid in assessing risk and in the early detection of breast cancer, particularly in young women. To provide a robust and objective method to analyze these cells, in this report we describe a technique called QM-MSP. We show that QM-MSP evaluates the level and incidence of promoter hypermethylation of several genes in samples where DNA is limiting. Scoring the cumulative methylation of these genes within a sample provided a high level of sensitivity and specificity of detection of cancer. We tested this method in clinical settings where samples yield

Table 6 *Cumulative promoter hypermethylation in normal, adjacent "normal" and malignant breast tissues*

Tissue	Methylation (units)[a] Range	Median	Mean ± SE	Lower 95% confidence limit[b]	Upper 95% confidence limit	Positive for methylation,[c] n (%)	n
Normal mammoplasty and malignant breast tissues							
Normal	0–18	0[d]	2.61 ± 2.05[e]	(0)	7.35	1/9 (11)	9
Carcinoma	0–248	74[d]	72.8 ± 15.03[e]	41.3	104.4	16/19 (84)	19
Paired malignant breast tissues and their adjacent normal breast tissues							
Adjacent normal	2–29	9[f]	11.7 ± 4.07	1.2	22.1	4/6 (67)	6
Carcinoma	5–258	133[f]	129.2 ± 39.9	26.5	231.8	6/6 (100)	6

[a] Relative units of methylation is the sum of percentage methylation for each of four genes in the panel.
[b] Value in parentheses indicates that the value is less than zero.
[c] Based on a cutoff of ≤4.7 units.
[d] $P = 0.0001$.
[e] $P = 0.0002$.
[f] $P = 0.03$.



Gene	3 T	3 N	4 T	4 N	5 T	5 N	6 T	6 N	7 T	7 N	8 T	8 N	Ctrl
	% Methylation												
RASSF1A	1	0	30	4	19	8	63	8	72	7	61	27	100
TWIST	3	1	2	0	17	2	58	5	69	1	61	2	100
Cyc D2	1	1	0	0	0	0	0	0	9	0	44	0	100
HIN1	0	0	6	0	72	0	37	4	58	0	92	0	100
Total	5	2	38	4	108	10	158	17	208	8	258	29	400

Fig. 7. Cumulative promoter hypermethylation of *RASSF1A*, *TWIST*, *Cyclin D2*, and *HIN1* in adjacent normal and malignant breast tissues. *A*, cumulative methylation in adjacent normal and carcinoma tissues. In an independent experiment, paired samples ($n = 6$) of adjacent normal and carcinoma tissue were quantitated by quantitative multiplex methylation-specific PCR, and the extent of cumulative methylation of the gene panel was determined, as described in the legend for Fig. 6. Shown are results of each sample as well as positive control MDA-MB231 DNA (*Control*; 400 units). For purposes of comparison, also shown at the *far left* is the average cumulative methylation of normal mammoplasty ($n = 9$) samples (*Mam*) from Fig. 6*B*. Raw scores are shown in the *table*. *B*, mean cumulative methylation in adjacent normal (*Adj Norm*) *versus* carcinoma (*Tumor*) compared with normal mammoplasty (*Mam*). Differences between normal mammoplasty samples (median = 0) and adjacent normal tissue samples (median = 9 units) were significant ($P = 0.01$, Mann–Whitney test). Plotted is the mean (± SE; *bars*) amount of cumulative methylation found above in adjacent normal tissues (mean, 11.7 ± 4.07) and the nearby carcinoma (mean, 129 ± 39.9) compared with normal mammoplasty (mean, 2.61 ± 2.05; as shown in 6*B*). Actual percentage of methylation values are listed in the *table* for tumor (*T*), adjacent normal (*N*), and MDA-MB231 positive control (*Ctrl*).

very limited amounts of DNA, such as those from ductal lavage and ductal irrigation fluid collected during endoscopy. In the process, we more clearly defined the extent of gene promoter hypermethylation in normal breast cells and showed the feasibility for adapting this method to clinical testing. Although we applied this technique to breast tissues, it can be used to evaluate gene promoter hypermethylation in a wide variety of tissues.

The expression of >40 genes is reportedly lost in breast cancer because of promoter hypermethylation (4). Recent work in our laboratory and by others has shown that some of the genes most frequently hypermethylated (30–90%) in breast carcinomas, but not in normal breast epithelium or circulating blood cells, are *Cyclin D2* (14, 15), *RARB* (16–18), *TWIST* (19), *RASSF1A* (15, 20, 21), and *HIN1* (22). In a study of 103 cases of breast cancer we recently reported that 100% of cases of invasive carcinoma and 95% cases of ductal carcinoma *in situ* were hypermethylated for one or more gene promoters in a panel of these five genes (23). In fact, the vast majority of carcinomas (80%) were hypermethylated for two or more of these five genes. From our work we developed support for the concept that profiling the cumulative methylation of multiple genes would serve to better distinguish benign from malignant tissues and would provide a more powerful approach than characterizing the status of only one gene marker.

Studies in our laboratory have also demonstrated the feasibility of assessing gene promoter hypermethylation in ductal lavage samples (19). We found *TWIST*, *Cyclin D2*, or *RARB* gene promoter hypermethylation in cells derived from patients with ductal carcinoma. Because the ductal cell samples most often contained 50–1000 epithelial

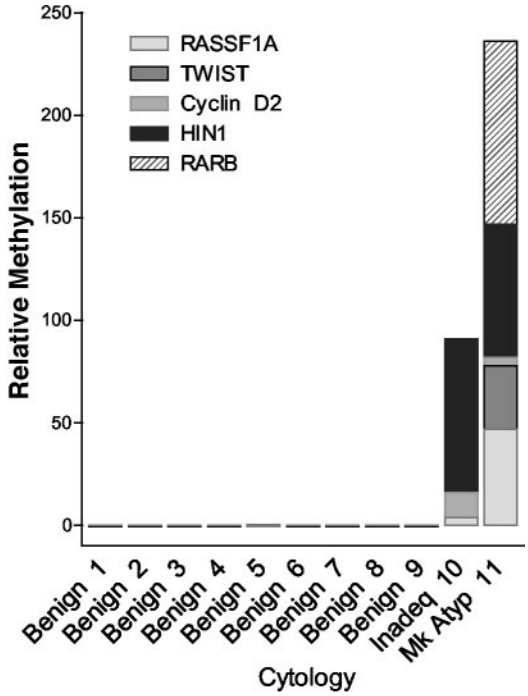


Fig. 8. Cumulative promoter hypermethylation in ductal fluid. *RASSF1A*, *TWIST*, *Cyclin D2*, *HIN1*, and *RARB* genes were coamplified, and promoter hypermethylation levels were quantitated by quantitative multiplex methylation-specific PCR in ductal cells derived from lavage of high-risk women ($n = 7$) or from cells present in the irrigation fluid obtained during breast endoscopy in patients with biopsy-proven cancer ($n = 4$), before resection of the lesion. *Inadeq*, inadequate sample available; *Mk Atyp*, markedly atypical.

Table 7 *Quantitative multiplexed methylation-specific PCR analysis of ductal breast cells*

ID[a]	Methylation (%) *RASSF1A*	*TWIST*	*Cyclin D2*	*HIN1*	*RARB*	Cytology	Mammography	Histopathology
Ductal lavage cells from high-risk women[b]								
1	0	0	0	0	0	Benign	Normal	
2	0	0	0	0	0	Benign	Normal	
3	0	0	0	0	0	Benign	Normal	
4	0	0	0	0	0	Benign	Normal	
5	0	0	0	0	0	Benign	Normal	
6	0	0	0	0	0	Benign	Normal	
7	0.4	0	0	0	0	Benign	Normal	
Ductal lavage cells from women with carcinoma[c]								
8	0	0	0	0	0	Benign		DCIS
9	0	0	0	0	0	Benign		DCIS
10	4	0	12	75	0	Inadequate		Invasive carcinoma
11	47	31	4	65	89	Markedly atypical		Invasive carcinoma

[a] ID, identification; DCIS, ductal carcinoma *in situ*.
[b] High-risk women with no mammographic abnormality.
[c] Ductal cells retrieved from irrigation fluid during endoscopy of women with biopsy-proven cancer. Histopathological diagnosis of the resected lesion from these breasts showed ductal carcinoma in situ or invasive ductal carcinoma.

cells, assessment of the status of more than three genes in replicate assays could be extremely difficult. There is clearly a need for new strategies to better evaluate methylation in samples where DNA is limited (*e.g.*, ductal lavage, plasma, fine-needle or core biopsy, or nipple aspiration fluid).

The QM-MSP method combines the principles of M-MSP (30, 31, 33) with quantitative real-time MSP (15, 34–39). We have shown that this method can detect as few as 1–10 methylated copies of DNA in a mixture of ~100,000 copies of unmethylated DNA (Table 3; Fig. 3) and 40 pg of methylated genomic DNA in up to 1500-fold excess unmethylated DNA (Fig. 4). This compares favorably with Q-MSP, which has a sensitivity of 1:10,000 (36), and conventional MSP, which has a sensitivity of 1:1000 (30). Reactions were specific: no cross-reactivity was observed between methylated and unmethylated primers even in mixtures containing a $>10^5$-fold excess of one or the other DNA (Fig. 3).

Most often the real-time PCR technology used for absolute quantification of DNA uses β-actin (*ACTB*) or *GAPDH* as reference DNA (34, 36, 37). We have demonstrated that by assessing the levels of unmethylated and methylated product for each gene, it is possible to quantitate the percentage of methylated gene product. Lo *et al.* (35) and Wong *et al.* (42) used a similar approach for Q-MSP, although they also considered the contribution of any unconverted bisulfite-treated DNA. However, two potential pitfalls of the QM-MSP method needed to be addressed. One matter of concern is how much bias is introduced into the estimation of gene methylation by the addition of the multiplex reaction to the Q-MSP procedure, as described here. A second concern is that QM-MSP does not take into account the existence of variable fractions of partially methylated DNA in the tissue samples. Therefore, samples could appear to contain higher levels of methylated DNA in the test genes than are present.

By testing the first question experimentally, we showed that, with few exceptions, there was excellent concordance between QM-MSP and Q-MSP for all four genes when we used (U + M) as the measure of total gene DNA (Table 4). Because QM-MSP and Q-MSP give essentially the same readout, significant bias is not likely in QM-MSP. To address the second question, using direct Q-MSP we calculated methylation as done with *ACTB*-based Q-MSP by Trinh *et al.* (37) and as % M by (U + M) (see the "Results"). There was concordance in percentage of methylation calculated by the two *ACTB*-based formulas and the (U + M) formula. Such concordance would be unlikely if partially methylated DNA formed a substantial component of the DNA. In contrast, in the two-step QM-MSP assay, we observed that *ACTB* does not perform predictably. The reasons could be as follows: In the first step, that of multiplex PCR amplification, the efficiency of amplification of each of the genes was not identical, however well optimized. In some samples, *ACTB* did not seem to amplify as well as some of the genes in the mixture. Because the strength of the second Q-PCR reaction depends on the efficiency of the first, differences are magnified in the second reaction. Thus, for QM-MSP we decided to calculate percentage of methylation using the (U + M) formula.

In addition, in QM-MSP, a gene controls for itself. In this assay, the sum of unmethylated and the methylated alleles of the same gene serve as a reliable internal control for integrity, copy number, and method efficiency. Among these, copy number is an important consideration because allelic losses and amplifications can vary among different areas of the genome and between samples. For example, for *RASSF1A* two simultaneous methods of gene inactivation have been observed: loss of one allele and methylation of the other (43). This has also been reported for *FHIT* (44), *APC*, and *CDH1* (13). Wang *et al.* (45) reported that nearly every breast tumor has an individual pattern of allelic imbalance or loss of heterozygosity at multiple loci, which constitutes its "fingerprint."

The QM-MSP technique is applicable to frozen or archival paraffin-embedded clinical tissues (Figs. 5–7) as well as to ductal lavage material (Fig. 8). In a study of 14–28 tissue samples/group, we observed significant differences in the level of promoter hypermethylation between normal and carcinoma samples for each of four genes, based on comparison of mean and median normal values (Fig. 5; Table 5). QM-MSP enabled definition of the normal range for the percentage of methylation in the genes in normal breast tissue (Table 5; Fig. 5). Techniques that give higher sensitivity usually also give higher "background," picking up signals that are missed by other methods. This was also observed in our present study, in which we found a higher incidence of methylation in normal mammoplasty than we did previously (23) using gel-based nonquantitative MSP. Nevertheless, with QM-MSP, the median for normal tissues was 0% M for all genes. By setting an upper threshold for normal, we acknowledge the occasional low-level methylation that occurs in some normal tissues and set criteria that define "positive" in carcinoma. By determining that peripheral blood cells contain little or no methylation of the genes tested and that normal breast tissue, which is rich in stroma, is for the most part negative, we were able to deuce that the methylation signal is derived largely from the epithelial cells (Fig. 5). The incidence of positivity among carcinomas was 68% for *RASSF1A*, 67% for *TWIST*, 57% for *Cyclin D2*, and 57% for *HIN1* (Table 5) according to this stringent criterion. However, all carcinomas showed some degree of methylation of one or more of the genes in our panel.

Studies of cumulative multigene promoter hypermethylation revealed striking differences between normal and malignant tissues (Fig. 6; Table 6). There was a highly significant ($P = 0.0002$) difference between levels of cumulative gene promoter hypermethylation in normal tissues compared with malignant tissues. Cumulative methylation profiling of four genes was able to detect 84% of carcinomas, whereas single-gene analyses yielded positive results in only 57–68% cases, depending on the gene analyzed (Figs. 5 and 6). To our knowledge, this is the first study to describe quantitation of cumulative methylation and to show its importance in distinguishing between normal and carcinoma tissues.

Molecular alterations in histologically normal-appearing breast tissue adjacent to carcinomas have been reported previously (46–48). Promoter hypermethylation analysis of *RASSF1A*, *TWIST*, *Cyclin D2*, and *HIN1* (Table 6; Fig. 7) in six pairs of carcinomas and histologically normal adjacent tissues showed that all six carcinomas were positive. Four of six adjacent normal tissues were also positive, although the levels were considerably lower than in the carcinoma ($P = 0.01$, Mann–Whitney test). More detailed studies are needed to determine whether methylation in histologically normal tissue adjacent to carcinoma represents a "field effect" presaging cancer or are normal, age-related changes. That this may represent a field effect is suggested by our observation that in 25 samples of normal breast tissue, including those reported here, we have not observed a correlation between age and methylation in these five genes.[6]

That it is possible to apply the QM-MSP successfully to samples with little cellularity was demonstrated by our pilot study with ductal cells retrieved by lavage or endoscopy (Fig. 8; Table 7; Ref. 19). In the seven DL samples from high-risk, but mammographically normal breasts, no promoter hypermethylation was detectable. In contrast, ductal lavage samples obtained during endoscopy of two women with invasive carcinoma had high-level multigene promoter hypermethylation, consistent with the histological diagnosis of the resected tissue. Interestingly, samples from the two women with ductal carcinoma *in situ* demonstrated benign cytology and lacked detectable promoter

[6] Our unpublished data.

hypermethylation (samples 8 and 9). The sample size is small, and an ongoing clinical trial collecting ductal cells from both diseased and uninvolved breasts of cancer patients will allow us to address the utility of QM-MSP in greater detail.

With the QM-MSP approach, it is possible to put together several gene panels consisting of scores of genes that are designed for early detection or to provide intermediate markers or endpoints for clinical protocols. For example, when retinoids or demethylating agents are being used as chemopreventive agents, a panel can be designed to query pathway-specific genes for their use as intermediate markers in clinical trials (26). Furthermore, the QM-MSP method is applicable to all types of cancer and evaluation of methylated tumor DNA in other small clinical samples, such as prostatic fluid, bile duct washings, and fine-needle aspirates.

In summary, we describe a method that assesses the gene promoter hypermethylation status of multiple genes, using only picograms of DNA. We demonstrate the advantages of a cumulative score of promoter hypermethylation among multiple genes and how this approach may better distinguish normal/benign from malignant tissues. With QM-MSP it is possible to objectively define the range of normal/abnormal gene promoter hypermethylation in a manner that could translate to a larger clinical setting. Further studies should examine cumulative hypermethylation in benign conditions and as a predictor of breast cancer risk.

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Quantitative Multiplex Methylation-Specific PCR Assay for the Detection of Promoter Hypermethylation in Multiple Genes in Breast Cancer

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