Abstract

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Results: DNA methylation in the core RASSF1A promoter was low in reduction mammoplasty tissues ($P = 0.0001$) when compared with primary tumors. The adjacent normals had an intermediate level of methylation. The regions surrounding the core were highly methylated in all sample types. Microsatellite markers showed AI/LOH in tumors and some of the adjacent normals. Concurrent AI/LOH and DNA methylation in RASSF1A promoter occurred in two of six tumors. Global methylation screening uncovered genes more methylated in adjacent normals than in reduction mammoplasty tissues. The methylation status of four genes was confirmed by quantitative methylation-specific PCR.

Conclusions: Our findings suggest a field of methylation changes extending as far as 4 cm from primary tumors. These frequent alterations may explain why normal tissues are at risk for local recurrence and are useful in disease prognostication.

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Previous studies have confirmed that genetic alterations exist in histologically normal breast tissues immediately adjacent to invasive cancers (3). Additional studies have suggested a preponderance of alterations in the upper outer quadrant of the breast where the majority of cancers occur (4). These normal epithelial cells frequently harbor loss of heterozygosity

Human Cancer Biology

Mapping Geographic Zones of Cancer Risk with Epigenetic Biomarkers in Normal Breast Tissue

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LOH; refs. 3, 5–7), microsatellite and chromosomal instability (8), and gene mutations (9, 10). In addition to genetic changes, altered DNA methylation has recently been implicated in preneoplastic lesions associated with various tumor types (11–13). This epigenetic alteration, by adding a methyl group to the cytosine residues of CpG dinucleotides, is frequently observed in 1- to 2-kb CpG islands located at the promoter/first exon of genes (14). Promoter hypermethylation contributes to the reorganization of chromatin structure and plays a role in the transcriptional inactivation in cancer (15). Epigenetic dysregulation is shown to be responsible for the silencing of genes that encode cellular proteins responsible for growth suppression, genomic stability and repair, apoptosis, and cell cycle regulation in many tumor types (15).

We have recently reported that increased methylation density within promoter CpG islands can be linked to tumor progression (11, 16). The prevalence of this epigenetic event occurring in histologically normal epithelium surrounding the tumor sites remains to be determined. Because the density of DNA methylation can be progressively accumulated during neoplastic transformation from premalignant lesions to invasive cancers (12, 17–19), we have hypothesized that frequent promoter hypermethylation of tumor suppressor loci in normal breast tissue is a harbinger for local recurrence.

The goal of this study is to identify epigenetic biomarkers frequently hypermethylated in normal breast tissue immediately adjacent to, and up to 4 cm from, tumor sites. We evaluated four zones of normal breast tissue in the ipsilateral and contralateral breasts of patients undergoing unilateral or bilateral mastectomy for invasive breast cancer. The previously developed methylation-specific oligonucleotide (MSO; refs. 20, 21) microarray was used to comprehensively map a 4-kb promoter/first exon region of RASSF1A in a large series of breast tumors, adjacent normal tissues, and breast samples of unrelated healthy individuals. The promoter/first exon of this gene is densely methylated in primary breast tumors (11), but our present study further shows that this event may be initiated via methylation spreading from outer flanks to the promoter/first exon core in normal adjacent tissues. The increased presence of epigenetic alterations in adjacent tissues may signify a greater risk of local recurrence. We therefore extended the study to identify novel loci frequently hypermethylated in normal epithelia adjacent to cancer sites by a second microarray technique, differential methylation hybridization (22, 23). This microarray study suggests that a more extensive field of epigenetically altered breast epithelia is present in breast cancer patients than previously thought. The identified epigenetic markers are useful for predicting future local recurrence.

**Materials and Methods**

**Tissue collection, histology, and DNA extraction.** Tissues were collected in accordance with the protocols approved by the Institutional Review Boards of the University of South Florida and the Ohio State University. Breast tissues from patients that underwent mastectomy at various stages of breast carcinoma were collected. Mastectomy specimens were marked for tumor and its four surrounding zones (1, 2, 3, and 4 cm away from the grossly visible tumor boundary). Samples from each zone were collected and frozen in liquid nitrogen. In bilateral and prophylactic mastectomy cases, normal breast tissues were taken from the four quadrants of the breast. Tissues were also obtained from healthy individuals undergoing breast reduction mammoplasty. Samples were embedded in Tissue-Tek optimum cutting temperature compound and 5-um sections were cut and mounted on Mercedes Platinum StarFrost Adhesive slides. The slides were stained using a standard H&E protocol and the tissue boundaries were marked. Using the marked slide as a "map," tissues were then microdissected. Adipose tissues were trimmed and the tumor and normal tissues were separated and stored in liquid nitrogen. Histologic examination of all tissue sections and microdissection of samples were conducted in close collaboration with a pathologist to ensure consistency in the clinical diagnoses. To ensure that the DNA methylation observed in the tumor-associated normals was contributed mainly by the epithelial components, the marked sections of H&E-stained slides from the adjacent normal sections were further quantified for infiltrating inflammatory cells by careful microscopic examination.

DNA was isolated from breast tissues using the Puregene DNA Purification method (Centra Systems, Inc., Minneapolis, MN). Briefly, tissues were ground in liquid nitrogen, resuspended in 10 mL of lysis buffer, and incubated for 20 minutes at room temperature. RNase was added (50 mL of 10 mg/mL) and incubated at 37 °C for 30 minutes. Proteinase K (100 mL of 10 mg/mL) was added and incubated at 55 °C for 1 hour. Then, samples were added with 3 mL of protein precipitation solution and mixed well for 3 minutes. The samples were centrifuged at 11,500 × g for 15 minutes at 4 °C and the supernatants were collected. Genomic DNA was precipitated by adding 10 mL of isopropanol and mixing gently. DNA was pelleted by centrifuging at 10,000 × g for 10 minutes. The pellet was washed with 75% ethanol and resuspended in 100 mL of deionized water. The amount of DNA was quantified by measuring A260. Due to limited amount of material available from microdissected tissues, different sets of samples were used for some of the analyses described below.

**MSO microarray.** Preparation of MSOs was carried out as described (11, 21). These oligonucleotides were used to interrogate bisulfite-treated DNA covering the various regions of RASSF1A. Of note, the oligonucleotides used in the promoter/first exon region are the same as those previously used. Each oligonucleotide was suspended in microspotting solution (Telechem, Sunnyvale, CA) and printed in triplicate as microdots on the superaldehyde-coated glass slides (Telechem) using a GMS 417 microarray. Slide processing was done following the protocol of the manufacturer. For DNA target preparation, bisulfite-treated DNA was amplified by PCR from regions 1 to 5 located in the RASSF1A promoter/first exon region and its flanking area. The primer sequences and PCR conditions are available on request. Purified PCR products were labeled at the 3' terminus with Cy5-dCTP (Amersham Pharmacia, Piscataway, NJ) by terminal transferase (New England Biolabs, Beverly, MA). These labeled products were resuspended in the Unihybridization Solution (Telechem), denatured at 95 °C for 5 minutes, and applied to MSO slides. Hybridization was conducted in a moist chamber at 50 °C for 4 to 5 hours. Slides were rinsed and washed once at 50 °C with 2× SSC-0.2% SDS for a total of 15 minutes, followed by washing once with 2× SSC at room temperature for 5 minutes and drying by centrifugation at 500 rpm for 5 minutes. These microarray slides were scanned with a GenePix 4000A scanner (Axon Instruments, Union City, CA). The fluorescent images were analyzed with GenePix Pro 6.0 software. Signal intensities of individual spots were obtained and exported to Excel spreadsheets for further analysis. MSO data for each probe pair were first normalized according to their standard curve. The normalized signals were used to derive the intensity ratio of M/(M + U) (M, methylated allele; U, unmethylated allele) for each probe set. Statistical analyses were conducted using SigmaStat 2.0 software (Jandel Scientific, San Rafael, CA).

**Allelic imbalance/LOH analysis.** Microsatellite markers for allelic imbalance (AI)/LOH analysis were selected from Genethon and Marshfield genetic maps according to their physical location on the chromosome 3p and analyzed as previously described (24). Twenty-nine polymorphic microsatellite markers were selected to span the entire short arm of chromosome 3 from 1 to 87.5 Mb (telomere to
centromere). The average intermarker distance is 2.98 Mb. However, a denser coverage was implemented for 3p21.3, in which RASSF1A lies, using six microsatellite markers between 47 and 54 Mb with an average intermarker distance of 1.17 Mb.

After standard PCR, the amplicons were separated by capillary electrophoresis and the signal was detected with an Applied Biosystems semiautomated DNA sequencer 3700 (Applied Biosystems, Perkin-Elmer Corp., Norwalk, CT). The results were analyzed by automated fluorescence detection using the GeneScan collection and analysis software (GeneScan, Applied Biosystems, Perkin-Elmer). Scoring of AI/LOH was done by inspection of the GeneScan analysis output. Normal breast tissue from the contralateral breast or tissue of greatest distance to the tumor (which corresponds to normal breast tissue obtained at least 4 cm away from the tumor margin) was used as the normal control tissue (corresponding germ line). A ratio of peak heights of alleles between normal tissue and tumor DNA of 0.67 was employed to define AI/LOH as described by others and us previously (25–27).

Data were also analyzed by a more stringent cutoff of 0.5 to ensure the AI/LOH findings.

Differential methylation hybridization using CpG island microarray.

Differential methylation hybridization was done according to the detailed protocol published by Yan et al. (16), with a slight modification of using McrBC (New England Biolabs), a methylation-cutting enzyme, to interrogate DNA samples. Briefly, DNA isolated from various tissue types was digested with MspI, ligated to linkers, and half of each sample was digested with McrBC and the other half was mock digested without the enzyme and the cofactor GTP. The digested and mock-digested, linker-ligated DNAs were used as templates for PCR amplification. The amplified products were tagged with fluorescent dyes using the indirect labeling method. The mock-digested samples were coupled to Cy5 and the digested samples were coupled to Cy3. The two halves of each sample were combined and cohybridized to the CpG 12K array from the University Health Network Microarray Centre (Toronto, Ontario, Canada). The identity of the arrayed CpG island clones can be accessed from an online CGI Library Browser9 and a detailed description of this array was recently presented by Heisler et al. (28). The dye-coupled DNA was hybridized and washed as previously described (22). The washed slides were scanned with a GenePix 4000A scanner (Axon Instruments) and the acquired microarray images were analyzed with GenePix 6.0 software. The microarray data will be deposited in GEO9 for public access.

Microarray data analysis. The quality of differential methylation hybridization experiments was assessed with ArrayQuality (an R package, part of the Bioconductor open sources software).10 Data from good quality experiments were background corrected and normalized using the mitochondrial clones as described by Nouzova et al. (29), as well as clones without the methylation-cutting restriction sites. Targets differentially methylated between breast tumors, histologically normal tissues adjacent to tumors, and uninvolved normal breast tissues were selected using the Prediction Analysis for Microarray (PAM) (Prentice Hall, Upper Saddle River, NJ) that uses an artificial neural network approach to identify gene expression differences. Briefly, the training set is used to build a surrogate model (PAMnet) that identifies genes that are differentially expressed between the two classes. The prediction accuracy of the PAMnet is calculated on an independent test set. The genes that are differentially expressed in the test set are considered to be differentially methylated and are used to build a microarray test set. The microarray test set is used to test the prediction accuracy of the PAMnet and to identify genes that are differentially methylated between the two classes.

Results

Loss of methylation demarcation in the RASSF1A promoter/first exon in primary breast tumors. We conducted methylation mapping on a 4-kb region (2 kb upstream and downstream from the transcription start site) of the RASSF1A gene in breast tumors using MSO microarray (Fig. 1A). A panel of 88 oligonucleotides (17- to 25-mer) were designed and arrayed on microscopic slides for microarray hybridization (Fig. 1B). Each pair was used to evaluate the methylation status of one to three CpG sites in a stretch of DNA (20). Standardization curves, generated from a series of SsI-treated control samples (0%, 33%, 66%, and 100% methylation), were used to calculate the degrees of methylation in the interrogating sequences. Of note, the oligonucleotides used to interrogate the methylation status of RASSF1A promoter/first exon were the same used in a previous study. As such, the sensitivity and the effectiveness of this subset of oligonucleotides in quantifying methylation status had been verified by COBRA analysis and bisulfite sequencing in breast tumors, normal breast tissues, and breast cancer cell lines. Figure 1C shows a detailed methylation mapping of the 4-kb region in 23 invasive ductal carcinomas and 17 control breast tissues of healthy individuals. The outer flanking regions (regions 1 and 5) are heavily methylated (>80% methylation) in tumor and control samples. Approaching the immediate flanking regions (regions 2 and 4) of the RASSF1A promoter/first exon core, the overall levels (~40% on average) of methylation in these zones is decreased relative to those of regions 1 and 5. This trend becomes significant (P = 0.0001) in the RASSF1A core (region 3), whereas high-level methylation (40-90%) is present in 74% (16 of 23 samples) of primary tumors analyzed and low-level methylation (~20%) is present in control samples. These MSO results show that the sharp demarcation in methylation level along the border of the RASSF1A core and its outer flanks is lost in the majority of primary tumors.

Promoter hypermethylation of RASSF1A is frequently observed in normal tissues adjacent to breast cancers. To study DNA methylation in gene promoters as a marker of field defect surrounding primary breast cancer, we had to ensure that the microdissected sections in these adjacent normals did not contain many infiltrating inflammatory cells. The H&E-stained slides marked originally as maps for microdissection purposes were carefully evaluated under a microscope. Adjacent normals had an average of 2.1% inflammatory cell infiltration (range, 0-20%) and primary tumors had an average of 4% infiltration (range, 0-10%). Thus, our samples had negligible contamination of inflammatory components.
The MSO analysis was extended to determine the methylation status of RASSF1A in normal tissues adjacent to breast cancers. In this regard, samples of four successive zones, each 1 to 4 cm (N1, N2, N3, and N4) from the tumor site, were obtained in the ipsilateral breast (Fig. 2A). Unremarkable breast ducts of these samples were identified and confirmed by histologic examination for microdissection (Fig. 2B). In two cases (4974 and 4768), four quadrants (Q1, Q2, Q3, and Q4) from the contralateral breast where no tumor was visible were also procured for the analysis. MSO results show that primary tumors from the six sets of samples exhibit hypermethylation in region 3 (i.e., the RASSF1A promoter/first exon core; Fig. 2C). We also observed extensive methylation of this region in adjacent tissues closest to the cancer site (1 cm or N1) than those 2 to 4 cm away from the sites (Figs. 2C and 3B). This gradient is not obvious in the adjacent samples from the remaining patients, suggesting an absence of well-defined epigenetic margins for the other four tumors even at 4 cm away from the tumor site. Interestingly, extensive methylation of region 3 also occurred in the contralateral breast of patients 4974 and 4768.

AI/LOH of chromosome 3p is observed in normal tissues adjacent to breast cancers. The RASSF1A gene is located on chromosome 3p, a region previously reported to exhibit genetic abnormalities in normal tissues adjacent to breast cancers (8, 31, 32). We therefore determined whether AI/LOH occurred in our studied panel and whether both genetic and epigenetic alterations could be concurrently detected in adjacent tissues. A total of 29 microsatellite markers spanning the entire 3p region...
were genotyped in 8 breast tumors in conjunction with 22 corresponding adjacent normal tissues (Fig. 4). Thirteen of these 29 markers showed AI/LOH in these tumors. Specifically, one tumor, 4822, showed an obvious loss of the entire 3p region, whereas five of the remaining seven tumors showed AI/LOH in several locations, including D3S1581 (telomeric to RASSF1A), D3S3118 (centromeric to RASSF1A), and D3S663 (3p21.31). When the corresponding adjacent tissues were examined among the 13 positive markers, in 5 instances AI/LOH of the same loci were found in one or more zones near the tumor sites. Of note, we found 11 additional instances of AI/LOH in normal tissues surrounding tumors in which no AI/LOH was detected in these markers. Also of note, the AI/LOH finding using the 0.67 cutoff (less stringent) was very similar to those identified by the 0.5 cutoff (more stringent), providing confidence to these findings.

We further compared the frequency of genetic versus epigenetic events in the RASSF1A region (3p21.3-31.1) in six tumors sets (4768, 4810, 4879, 4974, 5037, and 4817). Promoter hypermethylation of this gene was detected in adjacent tissues of these tumors (see Fig. 2C). AI/LOH around the RASSF1A region (D3S1581 and D3S3118) was only detected in adjacent tissues of 4810 and 4974 tumors, suggesting that both genetic and epigenetic alterations corroborated to silence this gene in these two samples. Adjacent normal tissues of the remaining four patients showed no detectable AI/LOH. Although this study was conducted in limited sample sets, the initial results seem to indicate that promoter hypermethylation of RASSF1A is more frequent than AI/LOH of the same region in normal tissues adjacent to breast cancers.

**Global profiling identifies novel loci frequently hypermethylated in normal tissues adjacent to breast cancers.** Next, we used differential methylation hybridization to globally screen CpG islands for methylation alterations in a separate set of paired tumor/adjacent normal samples (12 invasive ductal carcinomas and 25 adjacent normal tissues). An additional 12 breast

![Fig. 2. MSO microarray analysis of DNA samples obtained from primary breast tumors and histologically normal adjacent tissues. A, breast tissues from patients undergoing mastectomy were collected. Mastectomy specimens were marked for tumor (T) and its surrounding zones N1 (1 cm), N2 (2 cm), N3 (3 cm), and N4 (4 cm) from the grossly visible tumor boundary. In bilateral and prophylactic mastectomy cases, normal breast tissues were taken from the four quadrants of the breast: Q1, Q2, Q3, and Q4. B, examples of H&E sections of tissues taken from tumor sites and adjacent area. Breast tissues were embedded in Tissue-Tek optimum cutting temperature compound and 5-μm sections were cut and mounted on Mercedes Platinum StarFrost Adhesive slides. The slides were stained using a standard H&E protocol and tissue boundaries marked. Images were captured at ×400 magnification. C, methylation status of regions flanking the RASSF1A promoter/first exon in breast tumors and adjacent histologically normal breast epithelia. Methylation level is expressed as %M [%M = M/(M+U) × 100, and the resultant value adjusted by its respective standard curve] and is depicted by the gradient blue scale (right). Each block represents %M data from one patient with methylation status of the primary tumor (T) indicated on top followed by information from histologically normal epithelia from mastectomy specimens (N) and bilateral prophylactic mastectomy normal epithelia (Q). Each column represents %M data from one set of oligonucleotides. White boxes with dotted line strike through indicate data not acquired. Genomic location of regions 1 to 5 is depicted in Fig. 3A. Region 3 contains oligonucleotides that interrogate both the promoter and the first exon region of RASSF1A. R, right breast; L, left breast.]
samples obtained from healthy individuals were used as controls. Amplicons were prerestricted with McrBC, an enzyme known to cut methylated [5′-(G/A)C(N55-103)(G/A)mC] sequences (29, 33). Therefore, methylated DNAs were restricted away and could not be amplified by linker-mediated PCR whereas unmethylated fragments remained uncut and thus amplified. The McrBC-cut (test) and uncut (control) amplicons from each sample were labeled with Cy3 and Cy5, respectively.

Fig. 3. Methylation status of regions flanking RASSF1A promoter/first exon. Genomic location of regions 1 to 5 is depicted in Fig. 1A. A, the average probe set %M of each sample within the three sample types is presented for each studied regions. Gray line, average %M within samples from each group. The probe sets of regions 2 to 4 are distributed across the RASSF1A promoter/first exon and %M from these probe sets showed the greatest separations between the different sample types as indicated by statistical comparison (*, P < 0.05; **, P < 0.01). B, the %M for individual sample set of tumor (T)/mastectomy normal tissue (N)/bilateral prophylactic mastectomy (Q) for region 3 (promoter and exon) are shown. The %M was evaluated across samples obtained from each individual patient. Samples from patients 4810 and 4817 clearly show a stepwise decrease in %M as samples moved away from the gross margin of the primary tumor (*, P < 0.05; **, P < 0.01).
respectively, and cohybridized to a microarray panel of 12,000 CpG island tags previously used to identify differentially methylated loci in solid tumors (16, 22). CpG tags that hybridized predominately with the control amplicon, but not with the test amplicon, appear as red spots. These are methylated loci, which are restricted and therefore absent in the test DNA, but present in the control DNA. The methylation data are normalized using mitochondrial clones and clones without McrBC restriction sites. Subsequently, to analyze CGI loci that best characterized differences between the tumor, adjacent normals, and the reduction normal mammoplasty samples, the shrunken centroid analysis was conducted in the microarray data set, using Prediction Analysis for Microarray software in R. Tenfold cross-validation was used to assess loci commonly methylated in tumor and adjacent groups, but not in the normal breast. Relative to the overall predicted centroids as shown in Fig. 5A as horizontal units (representing the log ratios of average DNA methylation in the analyzed samples), a positive value indicates that more methylation occurs in a locus whereas a negative value indicates less or no methylation. Collectively, this analysis identifies a subset of putative loci preferentially hypermethylated in tumor and adjacent groups (Fig. 5A; a subset of these loci is presented in Supplementary Table S1). The shrunken centroid map also reveals higher levels of methylation in tumor group than in adjacent group. Initial methylation assessment of nine loci by nonquantitative methylation-specific PCR further substantiates these microarray findings (Fig. 5B).

Putative loci, including RASSF1A, were selected for detailed analysis using real-time quantitative methylation-specific PCR (Fig. 6). Methylation-specific PCR primers for ZNF582 showed a single distinct product in the gel-based experiment, which was found to produce two PCR product peaks in a subset of the samples in quantitative methylation-specific PCR experiments. Modifying the annealing temperature and the PCR reaction mix did not resolve this issue. As such, other gene loci were tested in its place. Consistent with the MSO finding, the overall methylation level of the RASSF1A promoter/first exon is significantly higher in primary tumors than in normal controls (P = 0.05). Hypermethylation of this gene can be seen in adjacent normal tissues of 6320, 4974, and 4993. Promoter hypermethylation of three newly identified loci, CYP26A1, KCNAB1, and SNCA, is detected in 47%, 41%, and 38% of breast tumors, respectively; no or low methylation is detected in normal breast samples. This independent confirmation of
global methylation profiles of breast tumors and their adjacent normal tissues uncovers a remarkable finding. We notice in these four studied genes that ~70% of the time, the genes are methylated in the adjacent normal tissues whenever they are found to be hypermethylated in the primary tumors. In some instances, the levels of methylation in adjacent tissues (N1 and N4 of 6320 and N3 of 6316 for CYP26A1) are higher than those of their corresponding tumors. Treatment of the two breast cancer cell lines with the demethylating drug 5′-aza-

2′-deoxycytidine results in a decrease in methylation and an increase in expression in these four genes (see Supplementary data).

Discussion

Convincing evidence indicates that histone modifications precede promoter hypermethylation to initiate transcriptional silencing of tumor suppressor genes (15, 34–37). Dysregulation of signal transduction in neoplastic cells can lead to transcriptional silencing of downstream target genes (38). As a result, repressive histone modifications are first established, and over time, an increased density of DNA methylation creeps into the promoter region of target genes to produce a permanent mode of epigenetic silencing. Epigenetic changes evolve over time and the compilation of methylation profiles from tumors captured at different stages of tumor development can recreate this slow cumulative process. Therefore, we used the MSO microarray to map the methylation status of a 4-kb region surrounding the RASSF1A promoter/first exon core. The composite profiles suggest that progressive accumulation of DNA methylation occurs in the core region during breast cancer development. This reasoning is based on the narrowing of the unmethylated zone in the core region across tumors with increasing methylation level in this gene. In addition, we observe that the extent of methylation in the RASSF1A promoter core region of normal adjacent tissues is usually higher than those of unrelated controls but lower than their corresponding tumors (see Fig. 2C). We speculate that there is a localized “field” of RASSF1A hypermethylation in these tumors and their associated normal tissues. This phenomenon is consistent with the so-called methylation spreading theory in which the flanking methylation barriers of a CpG island promoter are broken down in neoplastic cells, resulting in gradual invasion of methylation to the promoter core (11, 34, 39, 40).

In addition to the spreading of DNA methylation within a promoter CpG island, we are also interested in another “geographic” event, the “field defect” of carcinogenesis. It is known that breast cancer can recut/self-metastasize locally as well as to distant sites. We are interested, as are other researchers (41, 42), in discovering a biomarker of field defect, as it will be useful in risk assessment and in early disease detection. Epigenetic modifications are believed to be early events in cancer development. This coupling with the heritable nature of DNA methylation marks renders them more traceable and thus good candidates to study the field defect of cancerization. Shen et al. (41) proposed that such a defect, which is not observed in normal specimens, should be frequently encountered in cancer samples and observed in subjects that are at risk of development cancer. Once established in premalignant tissues, the extent of such defect will accumulate as the disease progresses. It is not certain how field defect arises. Norton (43) proposed the self-metastasis model whereby a small tumor becomes a large tumor by becoming a conglomerate mass of tiny tumors, each may be started by a seed/stem, progenitor cell. This seeding concept may also explain the field defect around the primary tumor. Maekita et al. (42) also discussed the possibility of stem cell methylation profile (due to H. pylori infection) influencing the methylation in gastric mucosa long after the infection is discontinued. Here we
Fig. 6. Validation of differential methylation status of promoter CpG island loci by SYBR-based quantitative methylation-specific PCR. Standard methylation-specific PCR primers detect the methylated alleles in each sample types for a specific test locus and the resultant threshold cycle numbers are adjusted against a standard curve generated by in vitro methylated DNA. Primers representing bisulfite-converted DNA but devoid of CGs quantify the input bisulfite-converted DNA. The percent relative methylation is the ratio of the adjusted methylated alleles to the adjusted COL2A1 DNA found in the same sample. The relative methylation data for RASSF1A and three promoter CpG islands, CYP26A1, KCNAB1, and SNCA, selected from methylation microarray analysis are shown. Sample designations (see Fig. 2A) are shown below the x axis.
observed a phenomenon in a subset of the studied tumor/adjacent normals that suggests the primary tumor may serve as an epicenter from which methylation density progressively diffuses outwards to surrounding tissues (similar to the seeding process previously described). Sample sets 4810 and 4817 exhibited a clear methylation gradient that was not evident in the remaining four tumor sets (4974, 4768, 4879, and 5037). It is possible that samples showing an epicentric effect are caught early in this seeding process whereas the methylation patterns not showing such an effect may have a wider sphere of methylation gradient (>4 cm away from the epicenter). However, more samples need to be analyzed to provide credence to such an observation.

Survey of normal tissues from the contralateral breast of two of these patients (4974 and 4768) also showed the presence of higher RASSF1A methylation relative to the control samples. This finding corroborates the previous notion that promoter hypermethylation of critical tumor suppressor genes may arise early during neoplastic transformation (44, 45). In this regard, methylation spreading of RASSF1A is already evident in precancerous cells with hypermethylation progressively infiltrating the core region during the development of neoplastic lesions. This results in gradual attenuation of gene transcription. However, tumorigenesis requires multiple genetic and epigenetic abnormalities in the affected area to produce invasive carcinomas. We therefore used differential methylation hybridization to screen additional promoter loci, the hypermethylation of which may occur early during breast cancer development. Using this methylation microarray method, we have uncovered a group of loci frequently hypermethylated in normal tissues adjacent to tumors. Among these, hypermethylation of three promoters (CYP26A1, KCNAB1, and SNCA) was commonly detected in adjacent tissues (~70%) of the tumor sets analyzed by quantitative methylation-specific PCR. This high degree of concordant promoter methylation in primary tumors and adjacent normal tissues suggests that the prevalence of promoter hypermethylation in normal tissue adjacent to tumors is more extensive than previously noted (12, 41).

The present study has practical implication for predicting the future risk of local recurrence in breast cancer patients undergoing lumpectomy. As part of the breast conservation strategy, lumpectomy is a surgical procedure preferred by some patients with small, early tumors whereby only the tumor-containing breast tissue is removed while sparing the residual breast tissue (1). Because most recurrences (86%) occur in tissues near or in the same quadrant as the lumpectomy site, an issue of critical importance is to ensure that the surgical margins are cancer-free (2). Currently, this evaluation relies on pathologic examination of margins free of gross tumor tissues. The use of epigenetic markers can be an important adjunct for predicting local recurrence in histologically normal margins. In this regard, simultaneous screening of multiple methylation markers can be done by the aforementioned differential methylation hybridization technique. Because amplicons are prepared from small amounts of DNA, sufficient DNA samples can be obtained from cytology touch imprints (46) made by gently pressing the corresponding margin to the glass slide. Methylation profiling will be used to determine the prevalence of promoter hypermethylation in the margin. This type of analysis will provide oncologists useful information in determining whether patients should undergo radiological therapy or other treatments to prevent future recurrence.

In summary, the present study provides, for the first time, a comprehensive analysis of promoter hypermethylation in normal tissues adjacent to breast cancers. The results indicate that premalignant epigenetic changes radiating from the tumor epicenter could be widespread. Future studies will be oriented to the analysis of whether increased density of methylation within a promoter as well as increased number of hypermethylated loci in these adjacent normal tissues is a factor of local recurrence. As such, hypermethylation of multiple gene promoters can be used as a surrogate biomarker to define the molecular margin of lumpectomy in the future.

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