Very High Frequency of Hypermethylated Genes in Breast Cancer Metastasis to the Bone, Brain, and Lung

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ABSTRACT

Purpose: **Most often it is not the primary tumor, but metastasis to distant organs that results in the death of breast cancer patients. To characterize molecular alterations in breast cancer metastasis, we investigated the frequency of hypermethylation of five genes (*Cyclin D2*, *RAR*-β, *Twist*, *RASSF1A*, and *HIN-1*) in metastasis to four common sites: lymph node, bone, brain, and lung.**

Experimental Design: **Methylation-specific PCR for the five genes was performed on DNA extracted from archival paraffin-embedded specimens of paired primary breast cancer and its lymph nodes (LN) metastasis ($n = 25$ each); in independent samples of metastasis to the bone ($n = 12$), brain ($n = 8$), and lung ($n = 10$); and in normal bone, brain, and lung ($n = 22$).**

Results: **No hypermethylation was detected in the five genes in the normal host tissues. In paired samples, LN metastasis had a trend of higher prevalence of methylation compared with the primary breast carcinoma for all five genes with significance for *HIN-1* ($P = 0.04$). Compared with the primary breast carcinomas, all five genes had higher methylation frequencies in the bone, brain, and lung metastasis, with *HIN-1* and *RAR*-β methylation being significantly higher ($P < 0.01$) in each group. Loss of expression of all five genes correlated, with a few exceptions, to hypermethylation of their promoter sequences in metastatic carcinoma cells microdissected from LNs.**

Conclusion: **The frequent presence of hypermethylated genes in locoregional and distant metastasis could render them particularly susceptible to therapy targeted toward gene reactivation combining demethylating agents, histone deacetylase inhibitors, and/or differentiating agents.**

INTRODUCTION

Metastatic breast cancer remains essentially incurable, and almost all women diagnosed will eventually die from their disease (1, 2). Therefore, important goals of current therapy have been to palliate symptoms and prolong patient survival. Molecular analysis of metastatic lesions is slowly leading to an understanding of the events underlying distant spread of breast cancer cells from the site of origin (1, 3, 4). Comparative large-scale gene expression analysis by SAGE (serial analysis of gene expression) of breast cancer and normal breast epithelial cells has led to the discovery of several genes that are differentially expressed between the two tissues (5–7). This knowledge might, in the near future, provide potent targets for therapy.

Work from our laboratory (8–10) and others (11–13) have shown that the genes that are expressed in normal breast epithelium but not in the carcinoma cells are frequently silenced by promoter methylation. Promoter hypermethylation is now recognized as a common method for cancer-specific repression of gene transcription. Some of the genes most frequently methylated (30–90%) in breast carcinomas, but not in normal breast epithelium are 14.3.3 sigma (8), *Cyclin D2* (9, 10, 14), *RAR*-β (the P2 promoter; Refs. 10, 11, 14), *Twist* (10, 14), *RASSF1A* (10, 15, 16), and *HIN-1* (10, 17). Our recent study has shown that hypermethylation of these genes is a feature common to both lobular and invasive breast carcinomas (10).

In this report, we investigated the incidence of methylation of the five genes, *Cyclin D2*, *RAR*-β (the P2 promoter), *Twist*, *RASSF1A*, and *HIN-1* in breast cancer metastasis in the lymph node (LN), bone, lung, and brain. We report that when compared with primary invasive ductal carcinomas, there is a striking increase in the incidence of tumor-specific methylation in breast cancer metastasis to all four sites. We demonstrate that hypermethylation of gene sequences correlates with loss of gene expression. The significance of these findings is discussed in the context of their therapeutic potential.

MATERIALS AND METHODS

Tissues. Paraffin-embedded samples of paired primary invasive ductal carcinomas of the breast and their LN metastasis; breast cancer metastasis to the bone, brain, and lung; and normal samples of bone, brain, and lung, and snap-frozen LNs containing metastatic cells were obtained from the Surgical Pathology archives of the Johns Hopkins Hospital after obtaining approval from the institutional review board. A hema-

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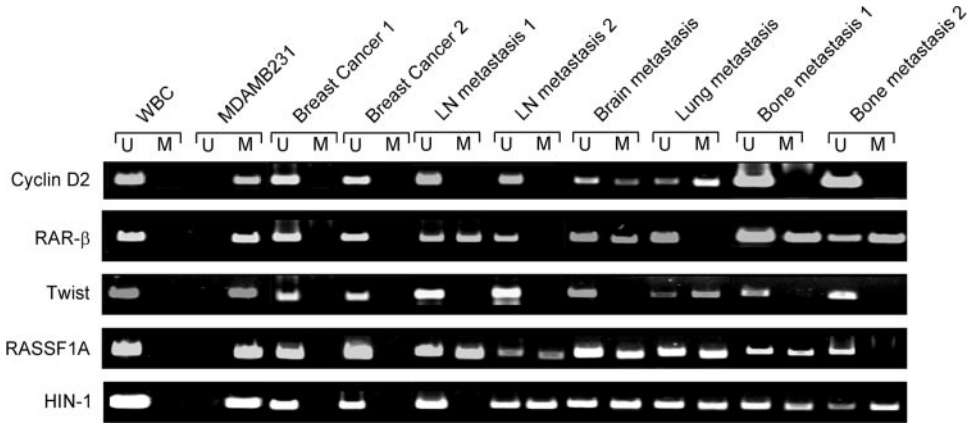

Fig. 1 Incidence of hypermethylation in primary breast cancer, local metastasis [lymph node metastasis (*LN metastasis*)], and distant metastasis (*Brain metastasis*, *Lung metastasis*, *Bone metastasis*). Methylation-specific PCR (MSP) analysis was performed using primers specifically amplifying *Cyclin D2*, *RAR*-β, *Twist*, *RASSF1A*, and *HIN-1* gene promoters. Peripheral WBCs (*WBC*) and MDA-MB231 cells served as controls for unmethylated (*U*) and hypermethylated (*M*) genes, respectively. PCR products were visualized using ethidium bromide after electrophoresis on a 2% agarose gel.

toxylin-eosin-stained section was examined for each tissue before entry into the study. The percentage of carcinoma cells in the tumor sections varied from 20 to 50%. Normal bone, brain, and lung tissues were obtained from surgeries for disease not related to cancer.

DNA Extraction. One to two 5-μm tissue sections were deparaffinized with xylene and scraped into tubes containing 100 μl of Tris/NaCl/EDTA/SDS plus proteinase K (10, 18). The tubes were incubated in a rotary shaker overnight at 50°C. The tubes were then heated at 65°C for 10 min to inactivate the enzyme, and were centrifuged at 14,000 rpm for 10 min. The supernatant was used directly for sodium bisulfite treatment (10, 18).

Bisulfite Treatment of DNA. Fifty μl of the cell lysate (see above) or 1 μg of purified DNA was treated with sodium bisulfite for 16 h as described previously (10, 18). After the DNA purification step, the DNA was resuspended in 20 μl of water. PCR was performed for each gene under the conditions previously described, using primers for *Cyclin D2*, *RAR*-β-P2 promoter, and *Twist* described in (10, 14) and primers for *RASSF1A* and *HIN-1* described in (10). For each reaction, 1 μl of sodium bisulfite-treated DNA was added to 24 μl of reaction buffer [1.25 mM dNTP, 16.6 mM $(NH_4)_2SO_4$, 67 mM Tris (pH 8.8), 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.1% DMSO, and 1.25 units RedTaq (Sigma, St. Louis, MO)] containing 100 ng each of forward and reverse primers specific to the unmethylated and methylated DNA sequences. Conditions for amplification of DNA 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.

Table 1 Distribution of risk factors across patients with primary breast cancer and lymph node metastasis

Risk factor	*n* (%)
Age >50	16 (64)
Age ≤50	9 (36)
Grade 2[a]	7 (33)
Grade 3	14 (67)
ER−/PR−[b]	17 (68)
ER+/PR+	8 (32)
Stage 2[a]	6 (30)
Stage 3	14 (70)

[a] Grade and stage not available for grade/stage four and grade/stage five samples, respectively.
[b] ER, estrogen receptor; PR, progesterone receptor.

Statistical Analysis. To assess the association between risk factors (age, tumor grade, stage, and estrogen receptor/progesterone receptor (ER/PR) status) and methylation of samples, proportions were compared. Significance was determined by Fisher's exact test. The association between tumor type and methylation was also assessed by Fisher's exact test. For comparison of methylation in paired samples from primary breast tumors and from LN metastasis, odds ratio were calculated and evaluated using McNemar's test. All 95% confidence intervals are calculated using exact methods.

Laser Capture Microdissection, RNA Isolation, and Reverse Transcription-PCR. Cryosections (5–8-μm) of LN metastasis samples ($n = 7$) were immediately fixed in 70% ethanol for 30 s, briefly stained with H&E before laser capture microdissection of malignant epithelial cells using a PixCell II laser capture microdissection system (Arcturus Engineering, Mountain View, CA) as per manufacturer's instructions. RNA from microdissected samples was isolated using RNeasy Mini kit (Qiagen Inc., Valencia, CA) and was reverse transcribed to cDNA using Superscript II (Invitrogen). Primers sequences for PCR were as follows: RAR-β2: CTTCCTGCATGCTCCAGGA (sense), CGCTGACCCCATAGTGGTA (antisense); Cyclin D2: CATGGAGCTGTGCCACG (sense), GTCCAGGTAATTCATGGCC (antisense) RASSF1A: GGCGTCGTGCGCAAAGGCC (sense), GGGTGGCTTCTTGCTGGAGGG (antisense) Twist: AGTCTCGCCGGCCGACGACA (sense), CGCGCTGCGCCTGCTGCTG (antisense); HIN-1: GGCCCTGAAGGCCCTGCTG (sense), TTTTGCTCTTAACCACGTTTATTGA (antisense);

Table 2 Prevalence of methylated *Cyclin D2*, *RAR*-β, *Twist*, *RASSF1A*, and *HIN-1* in breast cancer metastasis
Prevalence is compared in bone, brain and lung metastasis to prevalence in primary breast cancer using Fisher's exact test.

Tissue	*n*[a]	*Cyclin D2* (%)	*P*	*RAR*-β (%)	*P*	*Twist* (%)	*P*	*RASSF1A* (%)	*P*	*HIN-1* (%)	*P*
Primary breast cancer	25	10/25 (40)		9/25 (36)		8/25 (32)		14/25 (56)		10/25 (40)	
Bone	12	6/12 (50)	0.73	9/10 (90)	<0.01	5/9 (56)	0.25	7/9 (78)	0.42	9/10 (90)	<0.01
Brain	8	7/8 (88)	0.04	6/7 (86)	0.03	5/7 (71)	0.09	4/6 (67)	0.99	7/7 (100)	<0.01
Lung	7	6/9 (67)	0.25	7/9 (78)	0.05	6/9 (67)	0.12	10/10 (100)	0.01	8/8 (100)	<0.01

[a] *n*, number of cases.

Table 3 Differential methylation in primary breast and its paired lymph node metastasis

Odds ratios (ORs) less than one indicate that lymph nodes (LNs) tend to have higher prevalence of methylation than the primary tumor. ORs are determined by McNemar's test and 95% confidence intervals (CI) are calculated using exact method.

	Primary +, LN −[a]	Primary −, LN +[a]	Matched OR	95% CI	*P*
Cyclin D2	0	1	0	0, 39	1.00
RAR-β	1	1	1	0.01, 78.5	1.00
Twist	1	4	0.25	0.005, 2.53	0.38
RASSF1A	1	2	0.50	0.008, 9.60	1.00
HIN-1	1	8	0.125	0.003, 0.93	0.04

[a] Number of samples methylated (+) or unmethylated (−) in primary breast carcinoma and its paired lymph node metastasis.

36B4: GATTGGCTACCCAACTGTTGCA (sense), CAGGGGCAGCAGCCACAAAGGC (antisense). For PCR, 1 μl of cDNA was used in a 25-μl reaction volume, which for RAR-β, Cyclin D2, RASSF1A, Twist, and 36B4, contained 12.5 μl of 2× PCR mix (Life Technologies, Inc.), 200 nM each primer, and 1.25 units of RedTaq (Sigma, St. Louis, MO); and for HIN-1, 24 μl of reaction buffer [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, 1.25 mM dNTP, and 1.25 units RedTaq, and 400 nM each primer]. A touch down PCR was used for HIN-1, RASSF1A. and Twist: 95°C for 3 min, 5 cycles of 95°C for 30 s and 68°C for 2 min, 5 cycles of 95°C for 30 s, 64°C for 1 min, and 70°C for 1 min, then 35 cycles of 95°C for 30 s, 61°C for 1 min, and 70°C for 1 min, followed by 70°C for 5 min. RAR-β and Cyclin D2 were amplified by regular PCR for 40 cycles at 58°C and 60°C annealing temperatures, respectively. 36B4 was amplified for 35 cycles at 56°C annealing temperature.

***In Situ* Hybridization for HIN-1.** Paraffin-embedded sections were deparaffinized and processed as described by Porter *et al.* (19). Six primary tumors, six LN-metastases, and five samples of each of the distant metastases to bone, brain, and lung were tested. The slides were scored for intensity of staining after examining at least 10 fields in each slide.

RESULTS AND DISCUSSION

Frequent Incidence of Hypermethylated Genes in Distant Metastasis. Sodium bisulfite-treated DNA from 8 to 12 samples each from breast cancer metastasis to the bone, brain, and lung were evaluated by methylation-specific PCR (MSP) for the presence of hypermethylated promoter sequences in the *Cyclin D2*, *RAR*-β, *Twist*, *RASSF1A*, and *HIN-1* genes. These genes were selected for analysis because normal breast epithelium, stroma, as well as peripheral WBCs are most often negative for methylation (9, 10, 14, 17). Evaluable results were obtained from 7 to 12 samples in each group. The incidence of hypermethylated genes in the three distant organ-metastases varied between 50 and 100% (Table 2). A representative MSP analysis is shown in Fig. 1.

To determine whether there was a significant increase in the frequency of hypermethylation of the five genes in distant metastases compared with their primary site, we analyzed the DNA from 25 LN-positive primary breast carcinomas (Tables 1 and 2). Compared with the LN-positive primary breast carcinomas, there was a statistically significant increase in the incidence of hypermethylation in *RAR*-β and *HIN-1* genes in lung, brain, and bone metastasis ($P < 0.01$). An increased incidence (67–100%) of methylation in *Cyclin D2*, *Twist*, and *RASSF1A* was also observed; however, the differences were statistically significant for only *Cyclin D2* ($P = 0.04$) in the brain, and *RASSF1A* ($P = 0.01$) in the lung metastases (Table 2).

We next asked whether hypermethylation in these five genes correlates uniquely only with the ability of tumor cells to metastasize to distant organs, or whether it is a change occurring in local metastasis as well. To address this question, DNAs from 25 primary tumors and LN metastases from the same patients were also examined by MSP (Fig. 1). When analyzing differential methylation in paired samples, we observed a trend of higher prevalence of methylation for all five genes in LN metastasis than in the primary tumor, reaching statistical significance ($P = 0.04$) for only *HIN-1* (Table 3).

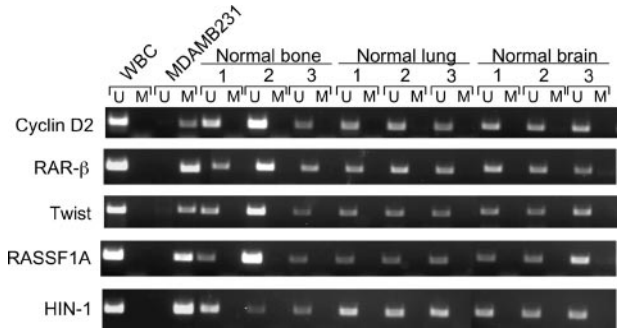

Fig. 2 Incidence of hypermethylation in normal bone, lung, and brain tissue. Methylation-specific PCR (MSP) analysis was performed using primers specifically amplifying *Cyclin D2*, *RAR*-β, *Twist*, *RASSF1A*, and *HIN-1* gene promoters. Peripheral WBCs (*WBC*) and MDA-MB231 cells served as controls for unmethylated (*U*) and hypermethylated (*M*) genes, respectively. PCR products were visualized using ethidium bromide after electrophoresis on a 2% agarose gel.

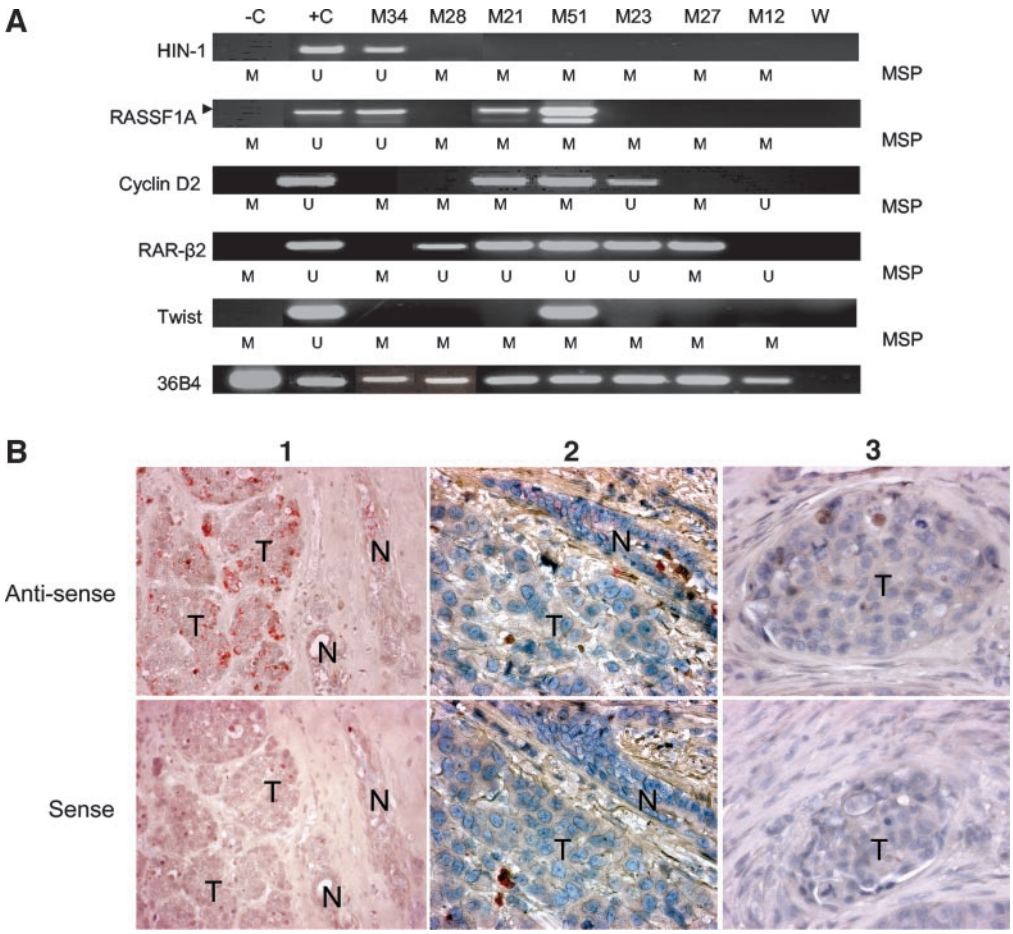

Fig. 3 Correlation of hypermethylation of genes with expression of their mRNA: *A,* reverse transcription-PCR analysis of expression of HIN-1, RASSF1A, Cyclin D2, RAR-β2, Twist, and 36B4 (a ribosomal protein gene) in microdissected epithelial cells from lymph node (LN) metastasis (*M34, M28, M21, M51, M23, M27, M12*). The breast cancer cell line, MDA-MB231, which is hypermethylated for all five genes and lacks detectable expression, served as a negative control (*-C,*) and a primary breast carcinoma sample with unmethylated DNA that shows expression for all five genes served as a positive control (*+C*). Water (*W*) was a no cDNA control. *36B4* served as a positive control for the presence of cDNA in each of the samples. Two bands are coamplified with the primer set for RASSF1 cDNA, RASSF1A (*top band*) and RASSF1F (*bottom band*). Methylation status of each gene, determined by methylation specific PCR (*MSP*), is reported below each sample for direct comparison as methylated (*M*) or unmethylated (*U*). *B, in situ* hybridization (ISH) analysis for expression of HIN-1 mRNA; *T,* tumor; *N,* normal. ISH was performed on paraffin-embedded sections of human primary breast tumor tissue (1), metastasis to the lung (2), and metastasis to the bone (3) using HIN-1 sense and antisense digoxigenin-labeled riboprobes. Sections were counterstained with hematoxylin and were visualized by light microscopy. *Panel 1, ×200; Panels 2 and 3, ×400.*

We also determined the association of risk factors, *i.e.*, age, tumor grade, stage, and ER/PR status with methylation status of five genes in either primary breast tumor or its paired LN metastasis. No significant association of risk factors with methylation status of the five genes was observed except for ER+/PR+ status, which, in the primary breast tumor, was significantly associated with *HIN-1* methylation ($P = 0.03$; data not shown).

Hypermethylated Genes Are Not Detectable in Adjacent Normal Host Tissue. Our previous investigations using MSP analysis have confirmed the absence, in general, of hypermethylation of these five genes in normal breast samples and in peripheral WBCs (9, 10, 14). However, one could argue that the high frequency of hypermethylation in distant metastasis is a characteristic of the normal host cells rather than the metastasis. To resolve this question, we investigated DNA from normal tissues of the site of origin of the distant breast metastases: normal bone, brain, and lung. MSP was performed on histopathologically normal tissues obtained at autopsy, or other surgical procedures for noncancer-related diseases. DNA from 6 to 10 samples from each site was analyzed by MSP. All of the DNAs were negative for the presence of hypermethylated gene sequences (representative data in Fig. 2). Thus, the adjacent normal tissues are most likely not the source of the frequent hypermethylation observed in distant metastasis. However, without performing MSP analysis on microdissected carcinoma cells and the adjacent stroma, we cannot exclude the possibility that the host cells immediately adjacent to the metastatic lesion contain hypermethylated genes, and that they are influenced by the epigenetic status of DNA and histone levels in metastatic cells.

In summary, hypermethylated genes were more frequently found in the local and distant metastasis compared with the primary breast carcinomas. Adjacent normal tissue from the host site may not be the source of the hypermethylation observed in these tissues. These results suggest that the increase in frequency of hypermethylated genes in distant metastasis may be an important event in the progression of breast cancer. Such a marked increase in the frequency of promoter hypermethylation also suggests that these losses may confer survival advantage to the disseminated cells at the distant site.

Correlation of Hypermethylation of Genes with Expression of Their mRNA. Dysfunctional epigenetic control by aberrant methylation of DNA is now well accepted as an important mechanism for shutting down gene expression and leading to loss of tumor/growth suppressor function. We sought to determine whether hypermethylation resulted in the loss of gene expression, as has been previously shown for Cyclin D2, RAR-β2, RASSF1A, and HIN-1 (9, 11, 15, 17). We performed reverse transcription-PCR to test the expression of all five genes on RNA extracted from microdissected epithelial cells from seven LN metastasis samples. MDA-MB231 cells served as control for DNA that is hypermethylated for all five genes, and shows loss of expression of their mRNA, and a primary carcinoma sample as a control that shows unmethylated DNA and the presence of detectable mRNA (Fig. 3*A*). With few exceptions (8 of 35), expression of the mRNA correlated with hypermethylation of the promoter sequences. Lack of perfect correlation in these cases is not unexpected, because control of gene expression is a complex process, in which the chromatin conformation, availability of cofactors, and repressor and enhancer molecules all play a part, and methylation status alone does not determine gene expression. Our data show that methylation is one of the important determinants, because in 77% of the cases, expression of the five genes in microdissected breast carcinoma metastasis in the LNs correlates with hypermethylation of the promoter sequences.

To visualize the expression of HIN-1 at the cellular level, we performed mRNA *in situ* hybridization for HIN-1 expression in the primary tumor and distant metastasis. HIN-1 expression was lost in four of six tumors with methylated *HIN-1* genes. Although HIN-1 is expressed in the primary tumor shown in Fig. 3*B*, there is complete loss of expression in its LN metastasis (not shown) and in each of the distant metastases to the lung, bone (Fig. 3*B*), and brain (not shown). Here again, for HIN-1, there is a fairly direct correlation between promoter hypermethylation and loss of mRNA expression. Loss of the cytokine HIN-1, which occurs very early and very frequently in breast tumorigenesis, could have profound biological effects that are yet to be elucidated.

In conclusion, we show for the first time, the methylation patterns of *Cyclin D2*, *RAR*-β, *Twist*, *RASSF1A*, and *HIN-1* genes in distant metastasis to common sites of breast cancer dissemination: the LNs, bone, brain, and lung. A high frequency of hypermethylated genes was revealed in locoregional and distant metastasis compared with primary breast carcinoma. This report provides information that may be important to the biology of distant metastasis and that may assist in the design of therapeutic modalities. It is already recognized that methylated genes could provide unique targets for therapy (20). Re-expression of these genes, in particular RAR-β genes, can lead to growth inhibition and death of cancer cells in the presence of retinoic acid (RA). In particular, RA-induced RAR-β gene expression in cells is mediated through a RA response element (β-RARE; Ref. 21). As already shown (22), histone deacetylase inhibitors appear to relieve the repressive chromatin conformation at the β-RARE-containing promoter of *RAR*-β and restore RA responsiveness. Endogenous up-regulation of RAR-β by RA is very important because it plays a critical role in amplifying the RA response, and is required for RA-induced growth inhibition and apoptosis in breast cancer. This was demonstrated by treating human breast cancer cells and xenografts carrying a methylated *RAR*-β promoter with a combination of the re-acetylating agent Trichostatin A and RA, which resulted in a significant increase in the degree of apoptosis and tumor-growth inhibition as compared with treatment with either agent alone (22). On the basis of preclinical studies, it is tempting to speculate that differentiation therapy for distant metastasis could be improved through reversing the silenced state of *RAR*-β by using a combination of chromatin remodeling agents and retinoids. Many genes, including each of the five tested in this study, can be reactivated in metastatic breast cancer cell lines using demethylating agents, histone deacetylase inhibitors, or a combination of the two.[6] Our molecular data provide reinforcement for studies aimed at the design and development of therapeutic strategies targeting specific epigenetic changes in metastatic breast cancer.

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