

Protein Acetylation and Histone Deacetylase Expression Associated with Malignant Breast Cancer Progression

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Abstract Purpose: Excess histone deacetylase (HDAC) activity can induce hypoacetylation of histone and nonhistone protein substrates, altering gene expression patterns and cell behavior potentially associated with malignant transformation. However, HDAC expression and protein acetylation have not been studied in the context of breast cancer progression.

Experimental Design: We assessed expression levels of acetylated histone H4 (ac-H4), ac-H4K12, ac-tubulin, HDAC1, HDAC2, and HDAC6 in 22 reduction mammoplasties and in 58 specimens with synchronous normal epithelium, ductal carcinoma *in situ* (DCIS), and invasive ductal carcinoma (IDC) components. Differences among groups were tested for significance using nonparametric tests.

Results: From normal epithelium to DCIS, there was a marked reduction in histone acetylation ($P < 0.0001$). Most cases showed similar levels of acetylation in DCIS and IDC, although some showed further reduction of ac-H4 and ac-H4K12 from DCIS to IDC. Expression of HDAC1, HDAC2, and HDAC6 was also significantly reduced but by a smaller magnitude. Greater reductions of H4 acetylation and HDAC1 levels were observed from normal to DCIS in estrogen receptor – negative compared with estrogen receptor – positive, and in high-grade compared with non – high-grade tumors.

Conclusion: Overall, there was a global pattern of hypoacetylation associated with progression from normal to DCIS to IDC. These findings suggest that the reversal of this hypoacetylation in DCIS and IDC could be an early measure of HDAC inhibitor activity.

It is commonly thought, but not yet clinically proven, that epigenetic silencing of tumor suppressor genes through the mechanism of histone deacetylation and DNA methylation is an early hallmark of malignancy. Since the 1996 discovery of the first mammalian histone deacetylase (1), 11 different zinc-dependent histone deacetylases (HDAC) have been identified and nearly a dozen different HDAC inhibitors have emerged into clinical development (2). Intracellular overexpression of HDACs can cause hypoacetylation of histones (H4, H3, H2A, H2B) and nonhistone proteins (e.g. α -tubulin, p53, MyoD, E2F, ataxia-telangiectasia mutant, and heat shock protein 90),

altering gene expression and cell phenotype. In 2006 the first HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA, vorinostat), was approved by the Food and Drug Administration for the treatment of cutaneous T-cell lymphoma. There are now more than 30 clinical trials in the United States evaluating the clinical efficacy of this HDAC inhibitor against various malignancies including breast cancer. However, a major challenge to broadening oncology applications for this and other HDAC inhibitors is the identification of cancer biomarkers predictive of HDAC inhibitor efficacy.

Among class I HDACs (HDAC1, HDAC2, HDAC3, HDAC8), nuclear localized HDAC1 and HDAC2 are believed to regulate most of the observed changes in histone acetylation, which largely involves H4 lysine (K) residues 5, 8, 12, and 16, with K12 acetylation seeming to be one of the more sensitive indicators of HDAC inhibition (2–6). Among class IIA (HDAC4, HDAC5, HDAC7, HDAC9), class IIB (HDAC6, HDAC10), and class IV (HDAC 11) HDACs, the best studied is HDAC6, which is unique among all zinc-dependent HDACs because of its novel protein structure and primary cytoplasmic localization. Such localization serves to deacetylate substrates like the microtubule component α -tubulin, thereby regulating cytoskeletal and dynein motor-controlled cell migration, protein trafficking, and misfolded protein accumulation within the aggresome (7).

A few reports have correlated HDAC expression levels with clinical prognosis in patients with invasive cancers including breast cancer (8–22), whereas others have compared histone

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Translational Relevance

Tumor-based biomarkers that can indicate response to histone deacetylase (HDAC) inhibitors have not yet been identified. Further, the association between histone acetylation and breast cancer progression is not known. In this study, baseline levels of candidate markers for assessment of tumor response to HDAC inhibitor treatment are evaluated in invasive and *in situ* breast cancer. This is the first study to show significant changes in histone acetylation levels in the course of tumor progression from normal breast epithelium to *in situ* and invasive ductal carcinoma. Based on our findings, HDAC inhibitors will be expected to reverse hypoacetylation levels observed even at the early stages of breast cancer progression. Because estrogen receptor – negative cases also showed hypoacetylation changes during histologic progression from normal epithelium to cancer, our data support that HDAC inhibitors might also be a treatment option for estrogen receptor – negative breast cancer patients.

acetylation patterns with expression of individual cancer genes (23, 24). To date, however, there has not been any work to elucidate how global patterns of histone acetylation or HDAC expression levels might differ between normal epithelium and either preneoplastic (e.g. *in situ*) or invasive malignant epithelial lesions arising in the same organ. In order to assess the time course of acetylation and HDAC expression changes during tumor progression, the present study was undertaken to assess cellular levels of histone H4 and nonhistone (α -tubulin) protein acetylation, as well as nuclear and cytoplasmic HDAC (HDAC1, HDAC2, HDAC6) protein expression in archived breast surgical samples containing synchronous normal mammary epithelium, ductal carcinoma *in situ* (DCIS), and invasive ductal carcinoma (IDC).

Materials and Methods

Case selection. As part of an Institutional Review Board – approved research protocol, 61 formalin-fixed paraffin-embedded breast tissues with synchronous normal breast epithelium, DCIS, and IDC were selected from pathology archives at the University of California, San Francisco. Three cases were excluded because of inadequate quality of the samples, reducing the study number to 58 cases. All cases contained normal epithelium, DCIS, and IDC in the same tissue block. Histologic grade was determined by Scarff-Bloom-Richardson grade system for IDC, and by nuclear grade for DCIS. In addition, 22 normal breast samples from reduction mammoplasties were stained as controls with all antibodies but HDAC1, because sufficient reagent of the same lot was not available for this antibody.

Immunohistochemistry assay development. Three breast cancer cell lines, MCF7, SKBR3, and MDA231, were treated with 5 μ mol/L SAHA for 24 h. These cell lines were used for immunohistochemistry assay optimization. Whole cell lysates used for Western blotting were prepared by harvesting cells in a buffer containing 50 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, and 2% SDS followed by a brief sonication to shear chromatin. Equal protein aliquots were combined with 2 \times Laemmli buffer (20 mmol/L Tris pH 6.8, 2% SDS, 20% glycerol, and 40 mmol/L β -mercaptoethanol), heated to 95°C for 5 min, electrophoresed using 4% to 12% gradient gels (Invitrogen),

transferred to nitrocellulose (Amersham Bioscience), and probed with specific antibodies at 1:1,000 dilution in hybridization buffers of 20 mmol/L Tris pH7.5, 130 mmol/L NaCl, 0.05% Tween-20 with 5% non-fat milk. Horseradish peroxidase coupled goat antimouse (BioRad) was used for detection of bound antibodies by the manufacturer's chemiluminescence enhancement procedure. Antibodies used included α -tubulin (Sigma), acetylated α -tubulin (Sigma), acetylated H4 (Upstate), or K12-acetylated H4 (Cell Signaling Technology).

Immunohistochemistry assays to detect protein acetylation and HDAC expression levels were developed using cell buttons prepared from three breast cancer cell lines described above. The cell lines treated with SAHA showed marked increased acetylation levels for ac-H4, particularly in MCF7, and for ac-H4K12 in all three cell lines to the same degree. Tubulin acetylation level was also increased with SAHA treatment of all three cell lines. Expression levels of all three HDACs did not show any change after SAHA treatment in any cell lines. SAHA-treated MCF7 cells were chosen as the standard positive control, and the same cells unexposed to primary antibody were used as the standard negative control. Immunohistochemistry results with cell lines were validated by Western blotting (Fig. 1).

Immunohistochemistry staining. Staining was performed on 5 μ m formalin-fixed paraffin-embedded tissue sections using the streptavidin-biotin peroxidase method. Antibodies and dilutions used were determined as assays were developed: anti-acetyl-Histone H4 (Upstate 06-598) at 1:100 dilution; Histone H4 (acetyl K12; Abcam ab1761) at 1:75; anti-acetylated tubulin (Sigma-Aldrich T7451) at 1:400; anti-HDAC1 (Upstate 06-720) at 1:50; HDAC2 (H-54; Santa-Cruz Biotechnology sc-7899) at 1:50; HDAC6 (H-300; Santa-Cruz Biotechnology sc-11420) at 1:100. Paraffin sections were dewaxed with xylene and passed through series of ethanol. Antigen retrieval was achieved using pressure-cooking in 10 mmol/L citrate buffer at pH 6.0 for all six antibodies. Slides were blocked in 3% H₂O₂ in PBS and avidin-biotin blocking reagent for ac-H4K12 and ac-tubulin, and then incubated with the primary antibodies. Binding of primary antibodies was detected by incubation with a biotinylated secondary antibody followed by avidin-biotin-horseradish peroxidase. Staining was completed with incubation with 3,3'-diaminobenzidine/H₂O₂, counterstained with hematoxylin, and mounted.

Immunohistochemistry scoring. Immunohistochemistry slides were evaluated blindly by two authors (JS and YYC) without clinical and pathologic information, or treatment status for cell lines. For staining with all six antibodies, "H-score" was used to evaluate the intensity and fraction of positive cells as previously described (25). Intensity was scored from 0 to 3, with 0 representing no staining, 1 weak, 2 moderate, and 3 strong staining. The intensity and fraction of positive cells in each slide were recorded and the H-score was calculated as a sum of the intensity of staining multiplied by percentage of stained cells for each intensity, where 0 indicated complete absence of staining and 300, the highest score, showing the highest intensity of staining in all cells (Supplemental Table S1).

Estrogen receptor/progesterone receptor/human epidermal growth factor receptor 2 status. Staining for estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (HER2) was performed in the University of California, San Francisco clinical pathology laboratory. Dichotomous scores for estrogen receptor and progesterone receptor status were assessed as positive when \geq 1% of tumor nuclei were stained regardless of intensity. Estrogen receptor and progesterone receptor were also scored using H-score for normal epithelium, DCIS, and IDC components separately. Estrogen receptor – negative or progesterone receptor – negative cases in which <1% of cells were stained could be scored >1 by H-score. HER2 was scored by criteria established by the HercepTest (DAKO), using a 0-3 scale, based on staining intensity in at least 10% of cells. Tumor cells with staining intensity 0 and 1 were considered negative for HER2 protein overexpression; those with staining intensity 3 were considered positive. All cases scoring 2 by immunohistochemistry were considered indeterminate, and fluorescence *in situ* hybridization analysis was

done. Cases showing a ratio of HER2:centromere 17 copy number >2.2 were considered positive for gene amplification, 1.8 to 2.2 were considered equivocal, and <1.8 were considered negative.

Statistical analysis. Regression analysis and nonparametric Spearman rank correlation tests were used to evaluate the correlation between antibodies. All data were stratified by estrogen receptor status (positive versus negative), progesterone receptor status (positive versus negative), HER2 status (positive versus negative), Scarff-Bloom-Richardson grades for IDC (1/2 versus 3), nuclear grades for DCIS (low/intermediate versus high), and age (≤ 50 versus >50 y), in each component for each antibody. The Mann-Whitney U test was used to detect differences in H-scores between clinical subgroups, and also between 22 normal cases from reduction mammoplasty and DCIS in 58 study cases. The Wilcoxon signed rank test was used to compare normal versus DCIS and DCIS versus IDC for all paired samples. Significance was established at $P < 0.05$. All statistical analyses were performed using StatView version 5.0.1 (SAS Institute Inc).

Results

Clinical and pathologic characteristics. The clinical and pathologic characteristics of 58 cases, selected for concurrent normal epithelium, DCIS, and IDC, are shown in Table 1. The mean age was 53 years (range, 25-81 years). Twenty-one cases were negative for estrogen receptor, progesterone receptor, and HER2. Invasive cancer and DCIS showed the same grade in 35 cases, and in all but two of the remaining cases, DCIS was a single grade higher than the paired IDC.

Substrate acetylation. Antibodies specific for acetylated lysine residues on histone H4, histone H4K12, and α -tubulin were used to assess levels of nuclear and cytoplasmic protein acetylation. As expected, staining patterns for ac-H4 and ac-H4K12 were nuclear, whereas ac-tubulin staining was cytoplasmic. Overall, normal ductal epithelium showed stronger staining than either DCIS or IDC for all three antibodies (Fig. 2). The majority of cases showed similar levels of staining in DCIS and invasive cancer, although some showed a further reduction from DCIS to invasive cancer (Table 2, Figs. 3 and 4). For ac-H4 the median H-score was reduced from 280 to 180 (36% reduction from normal to DCIS), and further reduced to 150 in IDC (46% reduction from normal to IDC). For H4K12, there was an even greater reduction (240 to 73 to 53, for 70% and 78% reduction, respectively). For α -tubulin, the changes associated with breast cancer progression were of much smaller magnitude (288 to 250 to 233, for 14% and 19% reduction). These reductions were all significant by Wilcoxon signed rank test.

In order to determine whether the changes seen between tumor cells and normal epithelium remained significant when comparing tumor cells against normal epithelium in patients without breast cancer, acetylation levels in tumor cells were compared with those of normal breast epithelium from reduction mammoplasties ("pure normal" epithelium). The differences between DCIS and pure normal cells were similar to those between DCIS and synchronous normal epithelium,

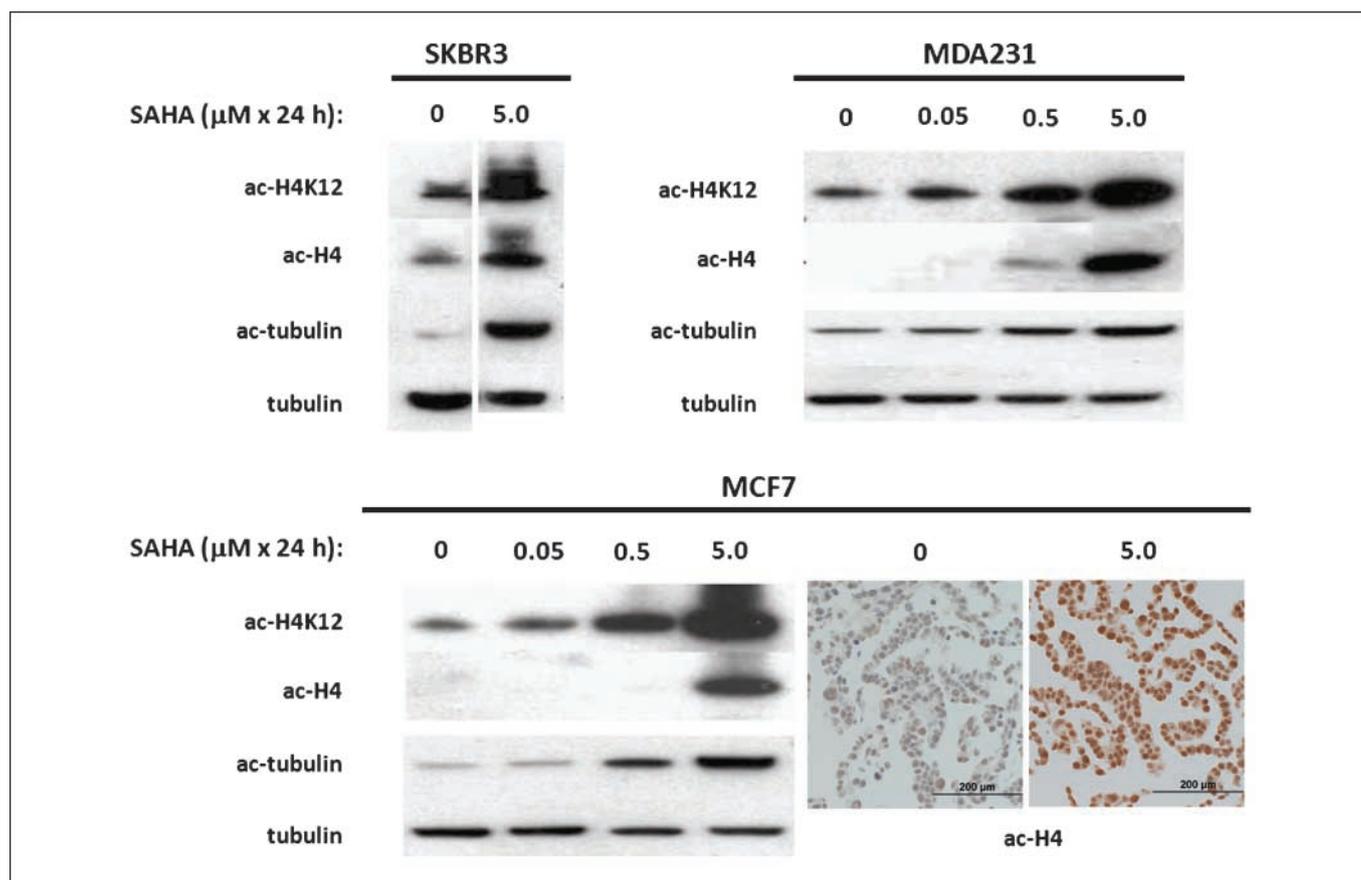


Fig. 1. Inhibition of intracellular histone H4 and α -tubulin de-acetylation by SAHA. Human breast cancer cell lines SKBR3, MDA231, and MCF7 were treated for 24 h with indicated doses of the pan-HDAC inhibitor, SAHA. Cell lysates and cell button sections were probed for total and K12 specific H4 acetylation as well as acetylated α -tubulin as described in Materials and Methods. Dose-dependent increases in ac-H4, ac-H4K12, and ac-tubulin as detected by Western blotting are shown in the three cell lines. Increased expression of ac-H4 in MCF7 (after SAHA treatment) is shown by immunohistochemistry (original magnification, $\times 200$).

Table 1. Clinical and pathological characteristics of study cohort ($n = 58$)

	No. of patients (%)
Age (y)	
≤ 50	24 (41)
> 50	34 (59)
Estrogen receptor	
Positive	35 (60)
Negative	23 (40)
Progesterone receptor	
Positive	32 (55)
Negative	26 (45)
HER2	
Positive	5 (9)
Negative	52 (89)
Equivocal	1 (2)
IDC grade*	
1	15 (26)
2	23 (40)
3	20 (34)
DCIS grade	
Low	5 (9)
Intermediate	21 (36)
High	32 (55)
Invasive cancer size	
T_1 (≤ 2 cm)	44 (76)
T_2 ($> 2, \leq 5$ cm)	14 (24)
Lymph node metastasis	
Negative	41 (71)
Positive	17 (29)
Stage	
I	36 (62)
II	19 (33)
III	3 (5)

*Tumor grade for invasive component by the Scarff-Bloom-Richardson system.

although the pure normal tissue showed slightly lower H scores for all three acetylation markers. As in comparisons between adjacent normal epithelium and DCIS, H-scores in these pure normal breast cases were significantly higher than in DCIS for all three acetylation markers.

HDAC expression. Antibodies specific for HDAC1, HDAC2, and HDAC6 were used to assess total cellular expression levels. As expected, HDAC1 and HDAC2 showed nuclear staining, whereas HDAC6 showed cytoplasmic staining in all samples (Fig. 2). Overall, HDAC expression also showed stronger staining for normal epithelium than for either DCIS or IDC (Table 2, Figs. 3 and 4). The magnitude of the reduction in HDAC expression was generally less than that for histone acetylation. HDAC2 and HDAC6 showed greater reductions from synchronous normal (median 17% and 14% for DCIS, 32% and 28% for invasive cancer) than HDAC1 (5% and 6%). In all three HDACs, there was a significant reduction in expression from normal to DCIS and from DCIS to IDC.

Normal epithelium from reduction mammoplasties showed strong staining for HDAC2 and HDAC6, similar to the synchronous normal epithelium (HDAC1 staining was not available for normal mammoplasty specimens). Although HDAC6 was significantly lower in DCIS cases than in the pure normal samples, for HDAC2 the reduction in staining from pure normal to DCIS did not reach significance (median, 245 in pure normal epithelium to 223 in DCIS; $P = 0.26$).

Within-tumor correlations. Within individual cases, H-scores for ac-H4 and ac-H4K12 were correlated in normal epithelium, DCIS, and IDC ($R^2 = 0.129, 0.199,$ and 0.359 , respectively; all $P < 0.01$ by Spearman rank correlation; see also Supplemental Table S2). Ac-tubulin staining showed no correlation with either ac-H4 or ac-H4K12. Interestingly, there was no correlation among HDAC1, HDAC2, and HDAC6 expression within cases. There was a weak but significant correlation between H-scores for ac-H4 and HDAC1 among all epithelial components ($R^2 = 0.045, 0.133,$ and 0.216 respectively, for normal epithelium, DCIS, and IDC; all $P < 0.005$).

Association with phenotype. Other phenotypic features (hormone receptor status, invasive grade, DCIS grade, and HER2 status) were tested for interaction with alterations in acetylation and HDAC staining. We tested whether cases with these additional markers showed differences in patterns of acetylation or HDAC expression compared with cases without these markers.

Within synchronous tumors, the magnitude of reduction in markers associated with cancer progression (normal versus DCIS/IDC) differed according to tumor grade and ER/PR status. Significantly greater reductions in substrate acetylation and HDAC1 expression were seen with cancer progression in high-grade IDC versus low/intermediate grade, ER-negative versus ER-positive, and PR-negative versus PR-positive tumors (Supplementary Table S3; Supplementary Fig. S1). In contrast, reduction of HDAC2 and HDAC6 expression from normal to DCIS/IDC was independent of ER/PR status or tumor grade. Because the initial set of tumors included only five HER2-positive cases, we collected an additional 17 HER2-positive tumors (10 ER-positive and 7 ER-negative) for analysis. This showed that in cancer progression, HER2 status did not affect reduction in substrate acetylation or HDAC expression, with the exception of ac-tubulin, which showed a greater reduction in HER2-positive versus HER2-negative cases (Supplementary Table S4). The magnitude of changes in H-scores with cancer progression did not differ by age group (≤ 50 versus < 50 years) for all six markers.

Discussion

In recent years, there has been growing interest in and appreciation for the role of epigenetic regulation in cancer development. One critical epigenetic pathway involves the modulation of gene expression through the control of histone acetylation, with the extent of acetylation determined by the balance between the activities of HDACs and histone acetyltransferases (HAT). In some cancers lacking any genetic mutations, abnormal gene silencing has nevertheless been observed in conjunction with gene promoter methylation and histone deacetylation (26). This observation provides the rationale for studying histone deacetylase inhibitors as potential novel anticancer agents. In breast cancer models, HDAC inhibitors can reactivate estrogen receptor α transcription in the presence of a methylated and transcriptionally silenced estrogen receptor gene (27). However, there are likely many estrogen-independent pathways affected by HDAC inhibitors because estrogen receptor-negative breast and other cancers respond clinically to HDAC inhibitors (28).

The goal of our study was to determine whether the acetylation of both histone and nonhistone substrates of HDACs as well as HDAC expression were altered with breast cancer progression. The present study focused on three

representative HDACs (HDAC1, HDAC2, and HDAC6) and two of the most abundantly expressed substrates of these HDACs (ac-H4 and ac-tubulin). There has been very limited assessment of any of these biomarkers either in *in vitro* or

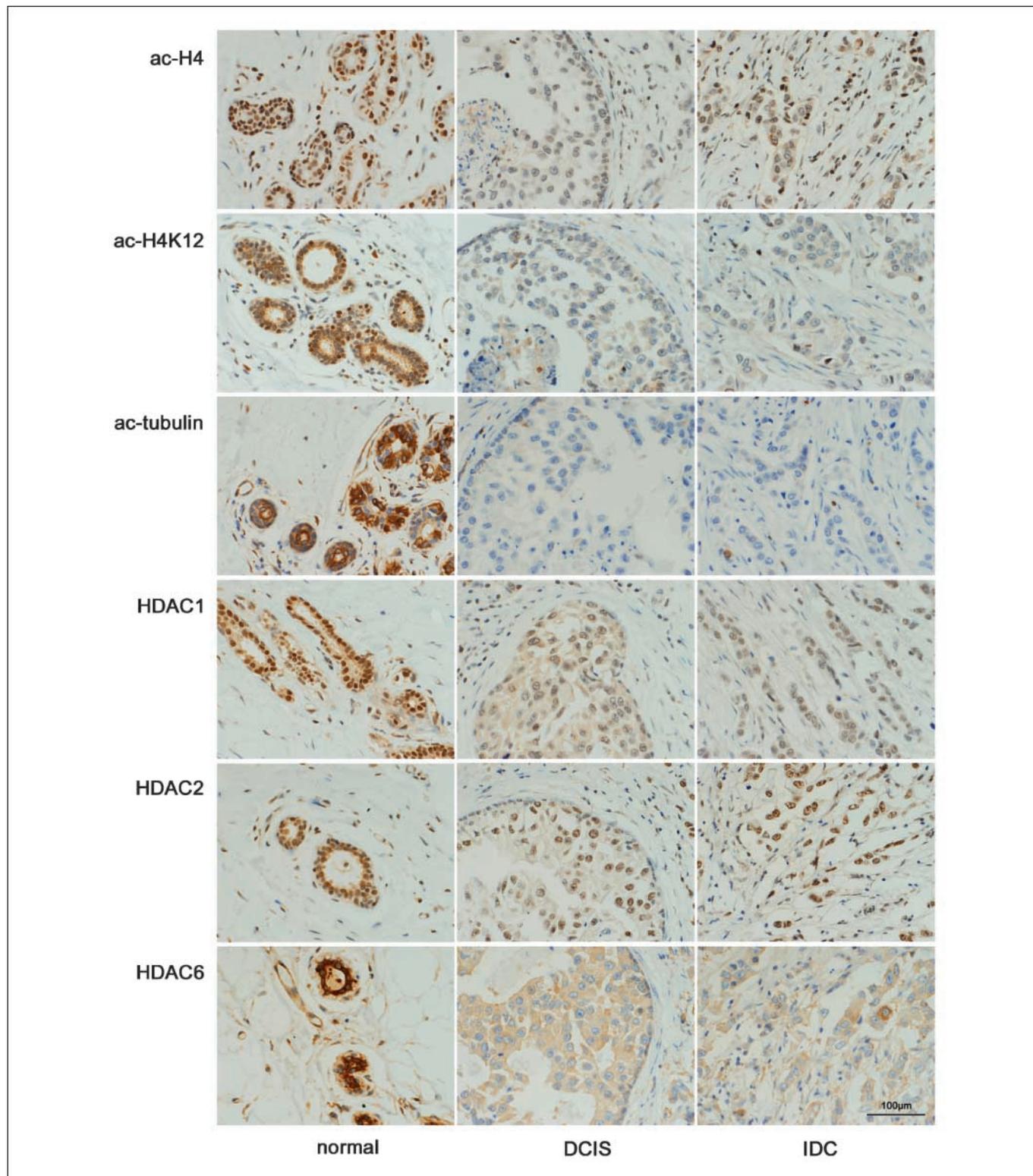


Fig. 2. Acetylation and HDAC expression in normal breast epithelium, DCIS, and IDC. The three different histologic components with the six antibodies are shown for one representative case (#38). Ac-H4, ac-H4K12, HDAC1, and HDAC2 show nuclear staining, whereas ac-tubulin and HDAC6 show cytoplasmic staining with substantial heterogeneity in background staining (original magnification, $\times 400$).

Table 2. Acetylation and HDAC expression by immunohistochemistry

	ac-H4		ac-H4K12		ac-tubulin		HDAC1		HDAC2		HDAC6	
	Score*	P†	Score	P	Score	P	Score	P	Score	P	Score	P
Normal	280	<0.0001	240	<0.0001	288	<0.0001	285	<0.0001	280	<0.0001	290	<0.0001
DCIS	180	0.0211	73	0.0003	250	0.0005	270	0.0046	233	0.0033	250	<0.0001
IDC	150		53		233		268		190		210	

*Median H-score.

† Wilcoxon signed rank test for difference between paired components.

in vivo studies. Further, there have been no prior evaluations of these biomarkers in the context of breast cancer progression and with regard to estrogen receptor status and other clinical factors.

In the present study, acetylation of H4, H4K12, and α -tubulin were markedly reduced in DCIS and IDC relative to synchronous normal breast epithelium. Hypoacetylation of both H4 and H4K12 were highly correlated, confirming that nonspecific hypoacetylation was conferred, given that the anti-ac-H4 antibody detects acetylation at any of four lysines (K5, K8, K12, and K16) in the NH₂-terminus of histone H4, whereas anti-ac-H4K12 antibodies specifically detect acetylation of the lysine 12 residue of H4. It has also been suggested that specific H4 lysines show ordered acetylation and deacetylation (3–6). Although the present study was not designed to detect preferential or ordered acetylation of specific lysines on H4 or other histones, this would be an interesting area for further research into specific epigenetic changes accompanying breast cancer progression. Taken together, the present results show that histone H4 hypoacetylation accompanies breast cancer progression from normal epithelium to invasive cancer.

Moreover, the greatest change in H4 hypoacetylation occurs at the transition from normal to DCIS, suggesting that this is an early event in the cancer pathway.

Because it is possible that epigenomic changes occur early and may be present even in histologically normal epithelium, additional comparisons of DCIS and IDC to normal epithelium from reduction mammoplasties, or pure normal cells was used as a further test to assess for biomarker changes that occur during progression from normal cells to DCIS and eventually to invasive cancer. As in the comparisons of neoplastic tissue to synchronous normal epithelium, all of the acetylation markers showed a similar high level of staining in the pure normal samples, which were significantly higher than levels of acetylation seen in DCIS. Thus, it seems that epigenomic changes have not yet occurred in pathologically normal epithelium, even when located adjacent to DCIS or invasive cancer.

Changes in histone H3 and H4 acetylation levels with tumor progression have been reported in other tumor types, although these reports are inconsistent. In squamous cell carcinoma of the esophagus, hypoacetylation of histone H4 was seen in

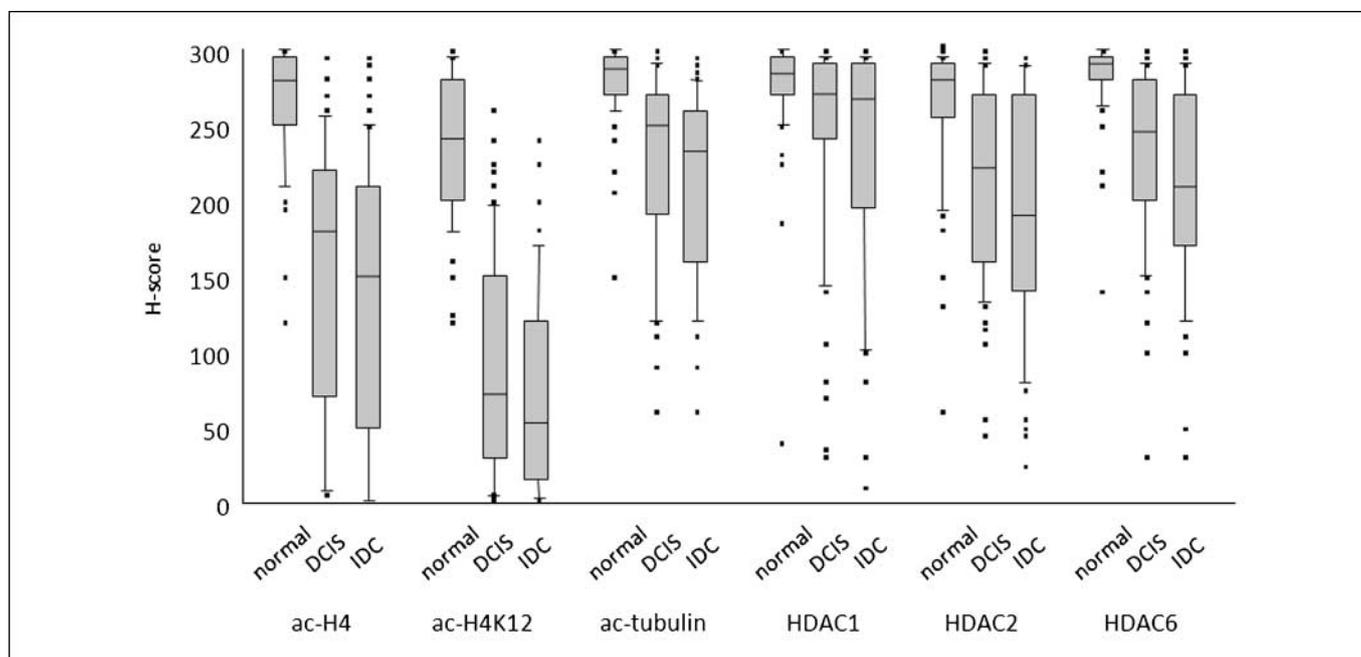


Fig. 3. Distribution of staining by H-score. Box plot distributions are shown for normal epithelium, DCIS, and IDC. Plots show median (horizontal line), 25%-75% range (boxes), 10%-90% range (vertical lines), and outlier cases (individual points).

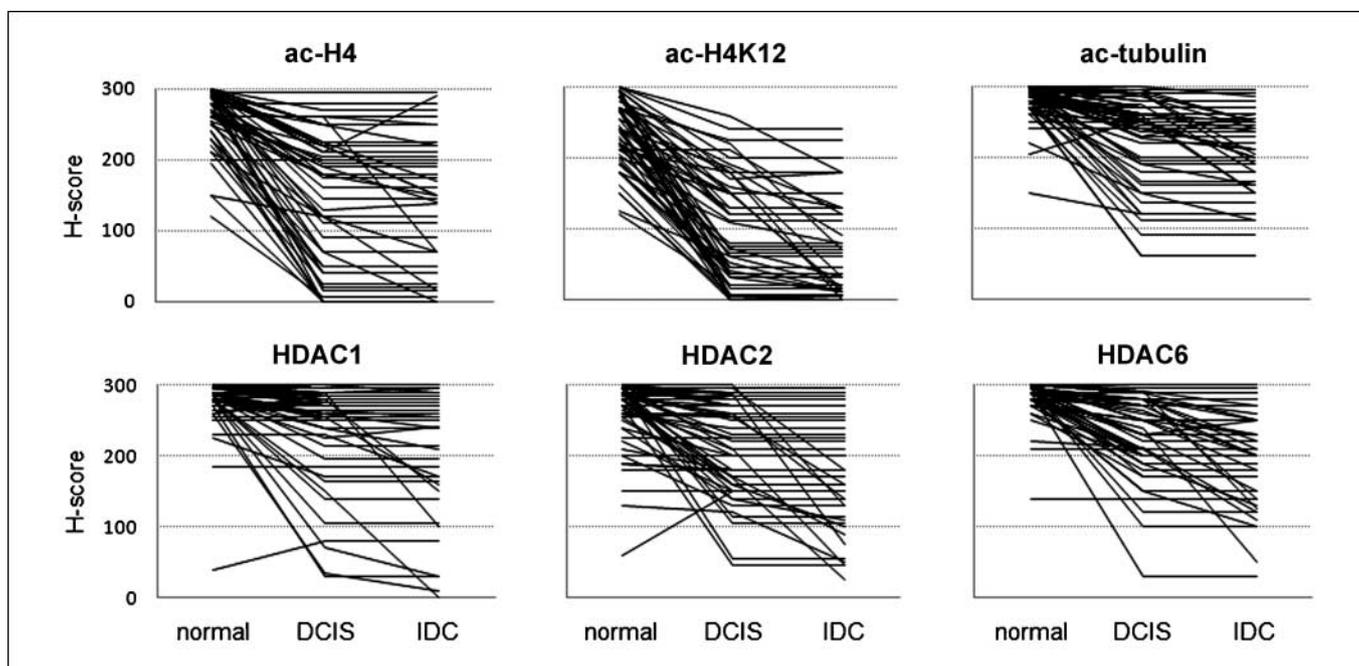


Fig. 4. Patterns of biomarker expression during tumor progression. Each line represents the H-score for an individual case for normal epithelium, DCIS, and invasive cancer.

tumors of more advanced stage, whereas increased acetylation was associated with better prognosis (23). Similarly, in prostate cancer, histone H3K9 acetylation decreased from benign hyperplasia to intraepithelial neoplasia and prostate adenocarcinoma (29), and higher acetylation levels of H3K9, H3K18, and H4K12 were associated with better prognosis (30). Conversely in the liver, acetylation of histone H3K9 and histone H4K8 was higher in hepatocellular carcinoma than in normal or cirrhotic precursor lesions (24). In adenocarcinoma of the lung as well, better prognosis was seen in patients with decreased H3K9 acetylation (31). Such heterogeneous results may reflect differing mechanisms of carcinogenesis in different tumor types and may be due to either specificity of acetylation targets or a range of baseline acetylation levels among different organs according to changes present in nonneoplastic tissue.

In addition to substrate acetylation, HDAC expression was also analyzed. Expression of HDAC1, HDAC2, and HDAC6 decreased with tumor progression while acetylation also decreased, which was unexpected. One possible explanation for this observation is an alteration in the relative activities of both HAT and HDAC with breast cancer progression. HAT is an enzyme that modifies chromatin structure and transcriptional activities by acetylating histone. Thus, substrate acetylation levels reflect the combined activity levels of HDAC and HAT. Several proteins and genes, such as p300, BRCA2, hMOF, AIB1, and CREB-binding protein, have been reported to have intrinsic HAT activity and altered levels of their expression have been reported in variety of cancers including breast cancer (12, 32–40). In colorectal cancer, synchronous up-regulation of HDAC1, p300, and CREB-binding protein has been shown (12). As future advances in direct assays of HDAC and HAT activity are made, better determination of the general effects of malignant progression on HAT and HDAC function, as well as improved correlation with protein acetylation are anticipated.

We stratified the cohort into clinical subgroups to determine whether there existed an association between breast cancer phenotypes and HDAC expression. Interestingly, in estrogen receptor–negative compared with estrogen receptor–positive; progesterone receptor–negative compared with progesterone receptor–positive; and high-grade tumors with non–high-grade tumors, HDAC1 expression was significantly reduced with cancer progression. In contrast, published studies of HDAC expression in other solid tumors have shown opposite findings (11–18, 24). The association between tumor progression and HDAC expression levels has been studied in colorectal cancer, where up-regulation of HDAC1 and HDAC2 expression was observed at the polyp stage during colorectal cancer progression (13). In prostate cancer, HDAC1 levels were low in normal and in benign hyperplasia whereas higher levels were observed even in intraepithelial neoplasia and the highest expression in invasive cancer, especially in androgen refractory aggressive tumors (15). In breast cancer, however, it has been shown that HDAC6 expression is more prevalent in less advanced, less aggressive tumors (small, estrogen receptor positive, low tumor grade) and associated with better survival (8, 9), in concordance with the findings in our study. In aggregate, these studies suggest the likelihood of organ specificity of HDAC expression in solid tumors.

Quantitation of immunohistochemistry staining is dependent on the scoring method used, and may in part account for the variation among studies. Many biomarkers show heterogeneity of staining within a sample for both intensity and fraction of stained cells. An H-score was used for this study, as we felt that it was important to account for changes in both intensity and prevalence of staining. This method detects a broad range of expression, and is useful for development of immunohistochemistry assays in which predefined thresholds are not available. Later studies may use biologically derived cut points, or may apply quantitative digital image analysis

(29). In the current study, not only was the intensity decreased from normal epithelium to DCIS/IDC, but also the fraction of positive cells decreased, so the reduction in H-score was more reliable than scoring of intensities alone. The reproducibility of the H-scores was validated in this study by staining duplicate samples to rule out intrarun and interrater variability. In addition, interobserver and intraobserver variability in scoring were found to be negligible.

Finally, several considerations regarding study design merit discussion. We believe that an important strength of the study is the use of concurrent samples, which is an effective approach to evaluate cancer progression within a single case. Many previous studies including work from our own group have shown considerable pathologic, biological, and genomic heterogeneity between individual DCIS lesions, such that DCIS and synchronous IDC are more closely related to each other than two DCIS from different patients. We sought to minimize this between-tumor variability by using synchronous normal, DCIS, and IDC; all cases met a strict criterion of having all three components present within the same block. The plan of analysis incorporated the advantages inherent in this paired design. Overall, our analysis with concurrent normal, DCIS, and IDC components was able to show the changes of expression levels during carcinogenesis for each case, with greater validity than an alternate study design using unpaired samples.

DCIS is a nonobligate precursor to invasive cancer. Thus, DCIS without invasive cancer at diagnosis probably represents two broad categories of lesions: those with high potential to progress to invasive cancer and those with limited potential. Although outside the scope of this study, it would clearly be of enormous interest and importance to determine whether epigenetic differences could differentiate these two groups in a cohort of DCIS patients with long-term outcome data. Such studies could validate whether the biomarkers in this study could have prognostic, as well as predictive utility.

In conclusion, we found that the immunohistochemistry staining of ac-H4, ac-H4K12, ac-tubulin, HDAC1, HDAC2, and HDAC6 was highest in normal epithelium, with a significant

reduction in immunohistochemistry staining accompanying the progression to DCIS and IDC. Ac-H4 and ac-H4K12 showed greatest reduction in the transition from normal epithelium to DCIS. Importantly, all six biomarkers evaluated in this study were reduced among all subgroups, which supports the possible efficacy of HDAC inhibitor therapy across all estrogen receptor and HER2 subsets. Clinical trials are critical to determine how effectively HDAC inhibitors could reverse the hypoacetylation observed in tumor, particularly if the proper response biomarkers are applied. Acetylation levels of histone and tubulin could both predict response and assess degree of response to HDAC-targeted drug therapies. Furthermore, some epigenetic markers may be clinically relevant prognostic factors as well. We have initiated a window trial of SAHA in both estrogen receptor-positive and estrogen receptor-negative DCIS in which histone acetylation levels of both H4 and H4K12 will be measured at baseline and following therapy. The primary outcome of interest will be to determine if a short course of HDAC inhibitors can reduce proliferation (Ki67) in DCIS, and whether degree of hypoacetylation is associated with magnitude of change in Ki67. Such clinical trials will provide important insight into how HDAC inhibitors could reverse the hypoacetylation observed in carcinogenesis, and which groups of patients might benefit most from this treatment. Our findings suggest that HDAC-targeted therapy may have a role in the treatment of preinvasive cancers. Further, HDAC may be a novel target for future prevention studies.

Disclosure of Potential Conflicts of Interest

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