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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 non-histone 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 performed immunohistochemistry (IHC) to assess expression levels of acetylated histone H4 (ac-H4), ac-H4K12, ac-tubulin, HDAC1, HDAC2 and HDAC6 in 58 breast specimens with synchronous normal epithelium (N), ductal carcinoma in situ (DCIS), and invasive ductal carcinoma (IDC) components. Differences in IHC scoring between groups were tested for significance using non-parametric tests.

Results—From N to DCIS, there was 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 HDACs 1, 2, and 6 were also significantly reduced but by a smaller magnitude. Greater reductions of H4 acetylation and HDAC1 levels were observed from N to DCIS in ER-negative compared to ER-positive, and in high grade tumors compared to non-high grade tumors.

Conclusion—Overall, there was a global pattern of hypoacetylation associated with progression from N to DCIS to IDC. Paradoxically, this was associated with reduction in HDAC expression levels, suggesting that this hypoacetylation pattern reflects a change in the balance between

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Statement of Translational Relevance

Biomarkers which are able to evaluate response to histone deacetylase (HDAC) inhibitors have not yet been identified. In this study, baseline levels of candidate markers for assessment of tumor response to HDAC inhibitor treatment are evaluated in invasive and *in situ* disease. 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 in early stages of breast cancer progression. Since ER-negative cases also showed hypoacetylation changes during histologic progression from normal epithelium to cancer, our data support that HDAC inhibitors might be a treatment option for ER-negative breast cancer patients as well.

HDAC and histone acetyltransferase (HAT) catalytic activities. These findings also suggest that the reversal of this hypoacetylation in DCIS and IDC could be an early measure of HDAC inhibitor activity.

Keywords

acetylation; histone deacetylase; ductal carcinoma in situ; immunohistochemistry; tumor progression

Introduction

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 (HDACs) 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 non-histone 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 FDA approved 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 and/or potentially useful for monitoring its antitumor activity.

Among class I HDACs (HDACs 1, 2, 3, 8), 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 appearing to be one of the more sensitive indicators of HDAC inhibition (2–6). Among class IIA (HDACs 4, 5, 7, 9), class IIB (HDACs 6, 10), and class IV (HDAC 11) HDACs, the best studied is HDAC 6 which is unique among all zinc-dependent HDACs because of its novel protein structure and primary cytoplasmic localization. Such localization functions to deacetylate substrates like the microtubule component α -tubulin and thereby regulate 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), while others have compared histone acetylation patterns with expression of individual cancer genes (23, 24). To date however, there has not been any description of how global patterns of histone acetylation or HDAC expression levels might differ between normal epithelium and either pre-neoplastic (e.g. in situ) or fully malignant epithelial lesions arising in the same organ. The present study was undertaken to immunohistochemically assess cellular levels of histone H4 and nonhistone (α -tubulin) protein acetylation, as well as nuclear and cytoplasmic HDAC (HDAC1, 2, 6) 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 IRB-approved research protocol, 61 formalin-fixed paraffin-embedded (FFPE) breast tissues with synchronous normal breast epithelium (N), in situ (DCIS) and invasive ductal cancer (IDC) were selected from pathology archives at the University of California, San Francisco. 3 cases were excluded because of inadequate quality of the samples, reducing the study number to 58 cases. All tumors were from female patients, and all cases contained N, DCIS, and IDC in the same section. Histologic grades were determined by Scarff-Bloom-Richardson (SBR) grade system for IDC, and by nuclear grade for DCIS. Two normal breast samples from reduction mammoplasties were used as positive controls.

Assay development of immunohistochemistry (IHC)

Three breast cancer cell lines, MCF7 (estrogen receptor alpha (ER) positive, progesterone receptor (PR) positive, human epidermal growth receptor type 2 (HER2) negative), SKBR3 (ER-negative, PR-negative, HER2-positive), and MDA231 (ER-negative, PR-negative, HER2-negative), were treated with 5 μ M SAHA for 24 hours. These cell lines were used for IHC assay development and for Western blotting to validate the IHC assay.

Whole cell lysates used for Western blotting were prepared by harvesting cells in a buffer containing 50 mM Tris (pH 7.5), 100 mM NaCl and 2% SDS followed by a brief sonication to shear chromatin. Equal protein aliquots were combined with 2X Laemmli buffer (20 mM Tris pH 6.8, 2% SDS, 20% glycerol and 40 mM beta mercaptoethanol), heated to 95 °C for 5 minutes, electrophoresed using 4–12% gradient gels (Invitrogen), transferred to nitrocellulose (Amersham Bioscience) and probed with specific antibodies at 1:1000 dilution in hybridization buffers of 20 mM Tris pH7.5, 130 mM NaCl, 0.05% Tween-20 with 5% non-fat milk. Horseradish peroxidase (HRP) coupled goat anti-mouse (BioRad, Hercules, CA) 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, Danvers, MA).

IHC assays to detect protein acetylation and HDAC expression levels were developed using cell buttons prepared from 3 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 3 cell lines to the same degree. Tubulin acetylation level was also increased with SAHA treatment of all 3 cell lines. Expression levels of all 3 HDACs did not show any change after SAHA treatment in any of 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. IHC results with cell lines were validated by Western blotting (Fig. 1).

IHC staining

Staining was performed on 5 μ m FFPE tissue sections using the streptavidin-biotin peroxidase method. Antibodies and dilutions used were determined as assay was developed: anti-acetyl-Histone H4 (Upstate 06-598, Lake Placid, NY, USA) at 1:100 dilution; Histone H4 (acetyl K12) (Abcam ab1761, Cambridge, MA, USA) at 1:75; Anti-Acetylated Tubulin (Sigma-Aldrich T7451, St Louis, MO, USA) at 1:400; Anti-HDAC1 (Upstate 06-720) at 1:50; HDAC2 (H-54) (Santa-Cruz Biotechnology sc-7899, Santa Cruz, CA, USA) 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 10mM 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-horse radish peroxidase (ABC-HRP). Staining was completed with incubation with DAB/H₂O₂, counterstained with hematoxylin, and mounted.

IHC scoring

IHC slides were evaluated blindly by two authors (JS and YYC) without clinical and pathological information, or treatment status for cell lines. For staining with all 6 antibodies, “H-score” was used to evaluate intensity and fraction of positive cells as previously described (25). Intensity was scored from 0 to 3, with 0 presenting no staining, 1 weak, 2 moderate, and 3 strong staining. The intensity and fraction of positive cells in each slide was 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 means absence of staining and 300 is the highest score. All scores are listed in Supplemental Table 1 along with clinical and pathological data.

ER/PR/HER2 status evaluation

ER and PR status were considered clinically positive when greater than or equal to 1% of tumor nuclei were stained regardless of intensity. ER and PR were also scored using H-score for normal epithelium, DCIS, and IDC components separately. ER- or PR-negative cases in which less than 1% of cells were stained could be scored greater than 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 intensity 2 were regarded indeterminate; and those with staining intensity 3 were considered positive. FISH analysis was performed on all cases showing score 2 by IHC. For FISH analysis, cases showing a ratio of HER2:centromere 17 copy number > 2.2 were considered positive for gene amplification, 1.8–2.2 were considered equivocal, and < 1.8 were considered negative.

Statistical analysis

Regression analysis and non-parametric Spearman rank correlation with significance at the 0.05 level was used to evaluate the correlation between antibodies. All data were stratified by ER status (positive vs. negative), PR status (positive vs. negative), HER2 status (positive vs. negative), SBR grades for IDC (1/2 vs. 3), nuclear grades for DCIS (low/intermediate vs. high), and age (< 50 vs. > 50), in each component for each antibody. A non-parametric test for non-normalized distribution of H-scores was used to compare changes in IHC markers: Mann-Whitney U test to test differences in H-scores between clinical subgroups, and Wilcoxon signed rank test to test between normal and DCIS and between DCIS and IDC for all paired samples. Significance was established at a p-value of < 0.05. All statistical analyses were performed by StatView version 5.0.1 (SAS Institute Inc).

Results

Clinical and pathological characteristics

Clinical and pathological 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 to 81). 21 cases were negative for ER, PR, 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. If nuclear grade alone was considered, invasive cancer and the DCIS component matched in 45 cases.

Acetylation levels

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 immunohistochemically. As expected, staining patterns for ac-H4 and ac-H4K12 were nuclear, while ac-tubulin was cytoplasmic. Overall, normal ductal epithelium showed stronger staining than either DCIS or IDC (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, Fig. 3, Fig. 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, 70% and 78% reduction, respectively). For tubulin, the changes associated with breast cancer progression were of much smaller magnitude (288 to 250 to 233, 14% and 19% reduction). These changes were all significant by Wilcoxon signed rank test.

Within individual cases, H-scores for ac-H4 and ac-H4K12 were correlated in normal epithelium, DCIS, and IDC ($R^2 = 0.129, 0.199, \text{ and } 0.359$, respectively; all $p < 0.01$ by Spearman rank correlation; see also Supplemental Table 2). Ac-tubulin staining showed no correlation with either ac-H4 or ac-H4K12.

HDAC levels

Antibodies specific for HDACs 1, 2, and 6 were used to assess total cellular expression levels. As expected, HDACs 1 and 2 showed nuclear staining, while 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, Fig. 3, Fig. 4). The magnitude of the reduction in HDAC expression was generally less than that for histone acetylation. HDAC2 and HDAC6 showed greater reductions from normal (median 17% and 14% for DCIS, 32% and 28% for invasive cancer) than HDAC1 (5% and 6%). In all three HDACs expression, there was a significant reduction from normal to DCIS and from DCIS to IDC.

Interestingly, there was no correlation between 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 ($R^2 = 0.045, 0.133, \text{ and } 0.216$ respectively, for normal epithelium, DCIS, and IDC; all $p < 0.005$).

Association with Phenotype

Other markers of tumor aggressiveness (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 their patterns of acetylation or HDAC expression compared to cases without these markers.

Overall, there was greater reduction from normal to DCIS/invasive cancer in the more aggressive phenotypes (ER negative, PR negative, high invasive grade, high DCIS grade) in acetylation levels (H4, H4K12, and α -tubulin) and HDAC1, but not in HDAC2 or HDAC6. For example, as described above, a distinct subset of cases showed marked reduction in HDAC1 expression from normal to DCIS (Supplemental Fig. 1); most of these cases were ER-negative, PR-negative, IDC grade 3, and DCIS high grade, and the differences between these positive vs. negative cases were all significant by nonparametric Mann-Whitney U test. Quantitation of ER and PR staining by H-score confirmed these observations. The rank

correlations between H-score of ER and ac-H4, ac-H4K12, and HDAC1 were all significantly correlated, similar to analyses using dichotomized hormone receptor status.

5 HER2-positive cases were analyzed; 3 cases were ER-positive and 2 cases were ER-negative. Changes in H-scores of all 6 markers from normal to DCIS and from DCIS to IDC showed no significant differences, with the exception of HDAC6, which showed significant difference from normal to DCIS. Similarly, magnitude of changes in H-scores with cancer progression did not differ by age group (≤ 50 vs. > 50) for all 6 markers.

Discussion

In recent years, there has been a 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 histone acetylation determined by the balance between the activities of histone deacetylases (HDACs) and histone acetyl transferases (HATs). 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 is rationale for studying the use of histone deacetylase inhibitors as novel anticancer agents. In breast cancer models, HDAC inhibitors can reactivate ER α transcription in the presence of a methylated and transcriptionally silenced ER gene (27). However, there are likely many estrogen independent pathways affected by HDAC inhibitors since ER-negative breast and other cancers respond clinically to HDAC inhibitors (28).

Our study was designed to determine whether the acetylation of both histone and non-histone substrates of HDACs, as well as HDAC expression, differed with breast cancer progression. The present study focused on three of the most representative HDACs (HDAC1, 2 and 6) and two of the most abundantly expressed substrates of these HDACs (ac-H4 and ac-tubulin). There has been very limited study of any of these biomarkers either in in vitro or in vivo studies. Further, there have been no prior evaluations of these biomarkers in the context of breast cancer progression and with regard to ER 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 N-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.

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 tumors of more advanced stage, whereas increased acetylation was associated with better prognosis (23). In prostate cancer histone H3K9 acetylation decreased from benign hyperplasia to intraepithelial neoplasia and

prostate adenocarcinoma (29). Higher acetylation levels of H3K9, H3K18, and H4K12 were similarly associated with better prognosis in this study (30). Other tumor types have shown changes in the opposite direction. Acetylation of histone H3K9 and histone H4K8 was higher in hepatocellular carcinoma than in normal or cirrhotic precursor lesions (24). In contrast to esophageal and prostate cancer, a better prognosis was seen in patients with decreased H3K9 acetylation in lung adenocarcinoma (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 organs.

Expression of all 3 HDACs 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 histone acetylase (HAT) and HDAC with breast cancer progression. HAT is an enzyme which 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, 34–42). In colorectal cancer, synchronous upregulation of HDAC1, p300, and CREB-binding protein has been demonstrated (12). Improved assays allowing direct measures of HDAC and HAT activities, rather than protein content, would enable better determination of the general affects of malignant progression on HAT and HDAC function, as well as correlation with acetylated protein changes.

We stratified the cohort into clinically meaningful subgroups to determine whether there existed an association between clinically validated breast cancer subtypes and the epigenetic markers studied. Interestingly, reduction in HDAC 1 expression associated with cancer progression from normal to DCIS and from DCIS to IDC was significantly larger in ER-negative compared to ER-positive, PR-negative compared to PR-positive, and high grade tumors to non-high grade tumors. HDAC6 expression was significantly reduced in high grade DCIS tumors compared to non-high grade DCIS. 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 HDAC 1 and 2 expression was observed at the polyp stage during the 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 androgen refractory aggressive tumors (15). However, in breast cancer, also it has been shown that HDAC6 expression is more prevalent in less advanced, less aggressive tumors (small, ER-positive, low tumor grade) and associated with better survival (8, 9), in concordance with the findings in our study.

Quantitation of IHC 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 fractions 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 IHC 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 intra- and inter-run

variability. In addition, inter-observer and intra-observer variability in scoring were found to be negligible.

In conclusion, we found that the IHC staining of ac-H4, ac-H4K12, ac-tubulin, HDAC1, HDAC2, and HDAC6 was highest in normal epithelium, with a significant reduction in IHC staining accompanying the progression to DCIS and IDC. Ac-H4 and ac-H4K12 showed greatest reduction from normal epithelium to DCIS. All 6 biomarkers evaluated in this study were reduced among all subgroups, which supports the possible efficacy of HDAC inhibitor therapy across all ER 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 ER-positive and ER-negative DCIS where 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 treatment of preinvasive cancers. Further, HDAC may be a novel target for future prevention studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Inhibition of intracellular histone H4 and α -tubulin 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 3 cell lines. Increased expression after SAHA treatment for ac-H4 in MCF7 is shown by immunohistochemistry (original magnification, $\times 200$).

Fig. 2.

Acetylation and HDAC expression in normal breast epithelium, DCIS, and IDC. The three different histologic components with the 6 antibodies are shown for one representative case (#38). Ac-H4, ac-H4K12, HDAC1, and HDAC2 show nuclear staining, while ac-tubulin and HDAC6 show cytoplasmic staining with substantial heterogeneity in background staining (original magnification, $\times 400$).

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.

Table 1

Clinical and pathological characteristics (n=58)

	No. of patients	(%)
Age (y)		
50	24	(41)
> 50	34	(59)
ER		
positive	35	(60)
negative	23	(40)
PR		
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		
T1 (≤ 2cm)	44	(76)
T2 (> 2, ≤ 5cm)	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

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		73		250		270		233		250	
IDC	150	0.0211	53	0.0003	233	0.0005	268	0.0046	190	0.0033	210	< 0.0001

[§] median H-score

* Wilcoxon signed rank test for difference between paired components