HDAC6 Expression Is Correlated with Better Survival in Breast Cancer

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ABSTRACT

Purpose: The structure and function of chromatin can be altered by modifications to histone. Histone acetylation in vivo is a dynamic reversible process governed by histone acetyltransferases (HATs) and histone deacetylases (HDACs). HDAC6 is a unique isoform among the HDACs, and a gene expression pattern study, with cDNA microarray technology, we have identified gene expression patterns in MCF-7 cells that indicate that a histone deacetylase (HDAC) gene (HDAC6) is a late responsive estrogen-induced up-regulated gene (2, 3). Therefore, we think that additional investigations into the levels of HDAC6 expression in breast cancer patients, either at the gene or protein level, are clearly warranted concerning the relationship between HDAC6 expression and response to endocrine therapy.

Experimental Design: In the present study, the level of HDAC6 mRNA expression was analyzed with quantitative real-time reverse transcription-PCR, in 135 female patients with invasive breast cancer. HDAC6 protein expression was also determined by immunohistochemistry. An association was sought between HDAC6 expression and various clinicopathologic factors.

Results: HDAC6 mRNA was expressed at significantly higher levels in breast cancer patients with small tumors measuring less than 2 cm, with low histologic grade, and in estrogen receptor α- and progesterone receptor-positive tumors. By contrast, no relationship was found between HDAC6 mRNA expression and any of the other clinicopathologic factors, namely, age, menopausal status, and axillary lymph node involvement. Patients expressing high levels of HDAC6 mRNA and protein had a better prognosis than those expressing low levels, in terms of disease-free survival. However, multivariate analysis failed to show that HDAC6 mRNA and protein are an independent prognostic factors for disease-free survival and overall survival. Furthermore, the patients with high levels of HDAC6 mRNA tended to be more responsive to endocrine treatment than those with low levels. Specific HDAC6 staining was found in the nucleus of some normal epithelial cells and in the cytoplasm of the majority of cancer cells. Although postmenopausal patients showed higher HDAC6 protein expression, there were no relationship between protein expression and any other clinicopathologic factors.

Conclusions: We conclude that the levels of HDAC6 mRNA expression may have potential both as a marker of endocrine responsiveness and also as a prognostic indicator in breast cancer. Additional investigations are warranted concerning the relationship between HDAC6 expression and response to endocrine therapy.

INTRODUCTION

It is well established that estrogens are important for the normal growth and development of mammary glands, as well as for the initiation and progression of estrogen-dependent breast cancer. The effect of estrogens on breast tissue tumorigenesis is believed to be mediated mainly through estrogen receptor α (ERα). Clinically, the ERα status of breast cancer patients is widely used both as an indicator for endocrine therapy responsiveness and also for prognosis. However, ERα is not a perfect predictor of endocrine therapy, because only about 70% of ERα positive patients respond to the therapy. Furthermore, 10% of patients who are ERα negative will also respond to endocrine therapy (1). Many studies have been performed that have sought to identify additional or alternative predictive factors. Expression profiling analysis with cDNA microarray technology could be an effective means of elucidating the system of estrogen signaling and thereby improve clinical decision-making. Using this microarray technology, we have identified gene expression patterns in MCF-7 cells that indicate that a histone deacetylase (HDAC) gene (HDAC6) is a late responsive estrogen-induced up-regulated gene (2, 3). Therefore, we think that additional investigations into the levels of HDAC6 expression in breast cancer patients, either at the gene or protein level, are clearly warranted to evaluate the clinical importance of this marker.

Within the nucleus of the cell, DNA and RNA are associated with a number of nuclear proteins known as chromatin. The structure and function of chromatin can be modified by many factors, of which the most extensively studied are histone acetylation and deacetylation. This dynamic reversible process is gov-
eral in vivo by histone acetyltransferases (HATs) and HDACs (reviewed in refs. 4, 5). In general, hypoacetylated chromatin is associated with silent genes, whereas hyperacetylation correlates with actively transcribed genes (6, 7). HDAC6 is a unique isoform among HDACs, because it contains two catalytic domains, compared with the one catalytic domain found in all other HDACs (8, 9). Furthermore, it is normally localized in the cytoplasm, and only a fraction of the protein relocalizes into the nucleus in response to stimuli for differentiation, suggesting that its natural substrates include non-histone acetylated protein (10).

Fluorescence in situ hybridization analysis localized the human HDAC6 gene to the sub-band border of chromosome Xp11.22–23, a region that is characterized by frequent gains and losses of chromosomal material in several types of cancer and neurologic disorders (11). In addition, HDAC6 has been shown to deacetylate α-tubulin in polymerized microtubules, thus potentially enhancing chemotactic cell motility (12). These findings have led us to hypothesize that altered HDAC6 expression or biological activity might be associated with malignant transformation. If a link could be established between levels of HDAC6 expression and the metastatic potential of breast cancer, it could prove useful as a prognostic indicator for these patients.

At present, the importance of HDAC6 expression levels to the clinicopathology of breast cancer remains unclear. Through the use of immunohistochemical staining and quantitative real-time reverse transcription-PCR, performed with LightCycler, we report here on a correlation between HDAC6 expression and the metastatic potential of breast cancer, it could prove useful as a prognostic indicator for these patients.

MATERIALS AND METHODS

Patients and Tumor Samples. Primary invasive breast carcinoma specimens were obtained, by surgical excision, from 135 female patients at the Department of Breast and Endocrine Surgery, Nagoya City University Medical School, Nagoya, Japan, between 1992 and 2000. Informed consent was obtained from all patients before surgery. The ethics committee of Nagoya City University Graduate School of Medicine, Nagoya, approved the study protocol. The median age of the patients was 53 years (range, 34–88 years). The patients’ tumors were classified with the International Union Against Cancer (UICC) staging system as follows: 32 cases were classified as stage I, 78 cases as stage II, 22 cases as stage III, and 3 cases as stage IV. As postoperative adjuvant treatment, tamoxifen was given to patients with ER+ and/or progesterone receptor (PgR)-positive tumors. Depending on tumor stage, the following chemotherapy regimens were administered: (a) oral 5-fluorouracil; (b) CFM [cyclophosphamide (100 mg orally on days 1–14), methotrexate (40 mg intravenously days 1 and 8), and 5-fluorouracil (500 mg intravenously days 1 and 8)]; or (c) CEF (cyclophosphamide 500 mg, epirubicin 60 mg, and 5-fluorouracil 500 mg, every 3 or 4 weeks). Since 1995, postoperative treatment has been performed with reference to the recommendation of Goldhirsch et al. (13). After recurrence, patients with ERo- and PgR- negative tumors were treated with CFM, CEF, and taxanes. Patients with hormone receptor-positive tumors and nonvisceral metastases were treated with endocrine therapy, such as antiestrogens, aromatase inhibitors, and medroxyprogesterone acetate.

Of the 135 patients, 10 cases who suffered recurrence were treated with tamoxifen but their disease progressed (4 cases) or had recurrence (6 cases) while on postoperative tamoxifen treatment. This group was defined as the nonresponders group. Eight cases who suffered recurrence were then treated with tamoxifen, and their disease improved (1 case of complete response, 5 cases of partial response, and 2 cases with stable disease over 6 months). This group was defined as the responders group. Patients were followed postoperatively, every three months. The median follow-up period was 61 months (range, 48–144 months).

Patients were graded histopathologically according to the modified Bloom and Richardson method proposed by Elston and Ellis (14). Samples were snap-frozen in liquid nitrogen and stored at −80°C until RNA extraction.

Isolation of Total RNA and Reverse Transcription. Total RNA from homogeneous breast cancer tissue, which was microscopically confirmed, was isolated from approximately 500 mg of frozen specimen. Total RNA was also isolated from one flask of the human hepatoma HepG2 cell line (kindly provided by Dr. N. Harada) for use as a positive control and to generate standard curves (15). mRNA was isolated with the TRIZOL reagent (Life Technologies, Inc., Tokyo, Japan) according to the manufacturer’s instructions. Reverse transcription reactions were performed as described previously (16). Briefly, each 20-μL cDNA synthesis mixture contained the following: 1 μg of total RNA, buffer [10 mmol/L Tris-HCl (pH 9.0), 50 mmol/L KCl, 1.5 mmol/L MgCl2], 1-mmol/L concentrations each deoxynucleotide triphosphate, 25 units of RNA-guard RNase inhibitor (Amersham Pharmacia Biotech Inc., Tokyo, Japan), 200 units of Superscript II reverse transcriptase (Life Technologies, Inc.), and 100 ng of pd(N)6 random hexamer (Amersham Pharmacia Biotech Inc.).

Primers and Probes. We conducted BLAST searches to confirm the specificity of the nucleotide sequences chosen for the primers and probes and to confirm the absence of DNA polymorphism. To avoid detection of contaminating genomic DNA, the primers were located at exon 28 and exon 29. The specific oligonucleotide primers were synthesized according to published information on the HDAC6 gene (GenBank accession no. NM-006044) as follows: sense primer, 5′-TCA GGT CAC TGG CAC TTT-3′ (3540–3559); and antisense primer, 5′-TCT CAC CAT GCT GAA GAG CC-3′ (3691–3672). The PCR product size is 152 bp. The donor probe 5′-ACA CCC GGC TGA CAT CAG GTG CAT GGA GAG CC-3′ has a fluorescein label at its 3′ end. The acceptor probe 5′-AGG ACC AGC GGG TGT CCA GAA TTT C-3′ has LC Red 640 at its 5′ end.

To ensure the fidelity of mRNA extraction and reverse transcription, all of the samples were subjected to PCR amplification with oligonucleotide primers and probes specific for the constitutively expressed gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and normalized. GAPDH primers were as follows: forward primer, 5′-AAA TCA GTA AGG GCG ATG CTG CTG-3′; and reverse primer, 5′-TTT TAA TTT TCA GAG ATG AGT ACC CCT TTG-3′. The sequences of the GAPDH probes used for real-time LightCycler PCR were 5′-AGG ACC AGC GGG CTC ATT TGC AGG G-3′ and 5′-GTC CAC TGG CGT CTT
CAC CAC CAT G-3'. All of the primers and probes were purchased from the Japanese Gene Institute (Saitama, Japan).

**Real-time Reverse Transcription-PCR.** PCR was performed with a LightCycler (Roche Molecular Biochemicals, Mannheim, Germany) as described previously (17). The PCR reaction was carried out in a final volume of 20 μL containing 2.4 μL of 25 mmol/L MgCl₂; 0.5 μL of 20 pmol/μL sense primer and antisense primer; 0.4 μL of 10 pmol/μL donor and acceptor probe; 2 μL of PCR master mix; 1.5 μL cDNA, and made up to 20 μL with water. After an initial denaturation step at 95°C for 60 seconds, temperature cycling was initiated. Each cycle consisted of denaturation at 95°C for 0 second, hybridization at 57°C for 5 seconds, and elongation at 72°C for 6 seconds. The fluorescence signal was acquired at the end of the hybridization step. A total of 55 cycles were performed. Cycling conditions for GAPDH were as follows: initial denaturation at 95°C for 30 seconds, followed by 50 cycles at 95°C for 0 seconds, 60°C for 5 seconds, and 72°C for 8 seconds.

**Standard Curves and Presentation of Results.** For each PCR run, a standard curve was constructed with serial dilutions of cDNA obtained from the HepG2 cell line. The level of expression of HDAC6 mRNA was given as relative copy numbers normalized against GAPDH mRNA and shown as mean ± SD. Relative HDAC6 mRNA expression was calculated by the formula: (HDAC6/GAPDH) x 1000.

A non-template negative control was included in each experiment. All of the non-template negative controls, the standard cDNA dilutions from the HepG2 cell line, and the tumor samples were assayed in duplicate. All of the patient samples with a coefficient of variation for gene mRNA copy number data 10% were retested with the method of Bieche et al. (18).

**Immunohistochemical Staining of HDAC6, Estrogen Receptor α, and Progesterone Receptor.** Immunostaining of HDAC6, ERα, and PgR was performed as described previously (19). Briefly, the slides were incubated at a dilution of 1:100, with either anti-HDAC6 rabbit polyclonal antibody (H-300, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-ERα primary antibody (ERID5, DAKO, Kobe, Japan) or anti-PgR primary antibody (PgR636, DAKO), with the streptavidin-biotin system (SAB-PO kit, Nichirei, Tokyo, Japan) according to the manufacturer’s instructions. The immunostaining of HDAC6, ERα, and PgR was subjectively assessed by two independent investigators (Z. Z. and H. I.), and discordant results were resolved by consultation with a third investigator (H. Y.). The expression of HDAC6 was scored by immunohistochemical score as described previously (20). The expression of ERα and PgR was scored by assigning a proportion score and an intensity score according to Allred’s procedure (21). In brief, the proportion of positive staining throughout the entire slide was assessed as 0 (negative), 1 (< 1%), 2 (1–10%), 3 (10–33%), 4 (33–67%), and 5 (> 67%), and the average staining intensity was lodged 0 (negative), 1 (weak), 2 (moderate), or 3 (strong) under light microscopy. The immunohistochemical score of each slide (0 or 2 to 8) was obtained as the sum of the proportion and intensity. ERα and PgR status by immunohistochemistry was then assessed as negative (scores 0 and 2) or positive (scores 3 to 8).

**Statistical Analysis.** The nonparametric Mann–Whitney U test and χ² test were used for the statistical analysis of the association between HDAC6 mRNA expression and clinicopathologic factors and the association between HDAC6 protein expression and clinicopathologic factors, respectively. Disease-free survival (DFS) and overall survival (OS) curves were generated by the Kaplan–Meier method and verified by the log-rank (Cox–Mantel) and Breslow–Gehan–Wilcoxon tests. Differences were considered significant when a P value < 0.05 was obtained.

**RESULTS**

**Patient Demographics and Tumor Characteristics.** Clinical characteristics are summarized in Table 1. Of the 135 patients examined with invasive carcinoma of the breast, 81 patients (60%) were postmenopausal and 54 (40%) were premenopausal; 53 patients (39.3%) had axillary lymph node metastases, and 82 (60.7%) had no metastases. In 33 patients (24.4%), the tumor measured <2 cm; in 102 (75.6%), it measured ≥2 cm. According to the immunohistochemical staining, the tumor was ERα-positive in 89 patients (65.9%), ERα-negative in 44 patients (32.6%), and of unknown ERα status in the remaining 2 patients (1.5%). By comparison, 72 patients (53.3%) had PgR-positive tumors, 56 (41.5%) had PgR-negative tumors, and in 7 patients (5.2%), the PgR status was unknown. Histologically, the tumor was classified as non-high-grade in 92 patients (68.1%), and high-grade in 43 patients (31.9%). The amount of HDAC6 mRNA expressed in the samples from the 135 patients ranged from 0 to 388 relative copy numbers (mean, 12).

The level of HDAC6 mRNA expression in patients with tumors measuring <2 cm (13 ± 39) was higher than in the group with tumors ≥2 cm (11 ± 7; P = 0.03). Additionally, it was found that the level of HDAC6 mRNA in the group of patients with non-high (i.e., grades 1 and 2) histologic tumor grade (15 ± 41) was higher than in the group with a high histologic grade (8 ± 6; P = 0.03). However, no significant correlation was found between the level of HDAC6 mRNA expression and any other clinicopathologic factors, such as age, menopausal status, or axillary lymph node metastasis (Table 1).

The level of HDAC6 mRNA correlated with tumor size and histologic grade. The level of HDAC6 mRNA expression in patients with tumors measuring <2 cm (13 ± 39) was higher than in the group with tumors ≥2 cm (11 ± 7; P = 0.03). Additionally, it was found that the level of HDAC6 mRNA in the group of patients with non-high (i.e., grades 1 and 2) histologic tumor grade (15 ± 41) was higher than in the group with a high histologic grade (8 ± 6; P = 0.03). However, no significant correlation was found between the level of HDAC6 mRNA expression and any other clinicopathologic factors, such as age, menopausal status, or axillary lymph node metastasis (Table 1).

The level of HDAC6 mRNA correlated highly significantly with ERα protein and PgR protein expression. A significantly higher level of HDAC6 mRNA expression was found in the ERα-positive group of patients (11 ± 12) compared with the ERα-negative group (7 ± 8; P = 0.0005). In addition, a significantly higher level of HDAC6 mRNA expression was found in the PgR-positive group of patients (16 ± 45) compared with the PgR-negative group (9 ± 16; P < 0.0001; Table 1).

**Patients Whose Tumor Expressed Higher Levels of HDAC6 mRNA Had Better Disease-Free Survival Rates and Tended to Have a Trend Toward Better Overall Survival Rates Than Those with Lower Expression Levels.** To identify a clinically meaningful cutoff point for a level of HDAC6 mRNA expression that could be used in the analysis of disease prognosis, various levels of HDAC6 mRNA expression were tested with the Kaplan–Meier method and verified by the log-rank (Cox–Mantel) and Breslow–Gehan–Wilcoxon tests. When analyzing disease-free survival rates,
we set the cutoff point for the level of HDAC6 mRNA expression at 3: patients with a high level of expression (14 ± 34; n = 111) tended to have a better prognosis than those with a low level (2 ± 1; n = 24); log-rank (Cox–Mantel) test, \( P = 0.0002\); Breslow–Gehan–Wilcoxon test, \( P = 0.0002\) (Fig. 1A). The same method was adopted for the analysis of overall survival rates. When the cutoff point for the level of HDAC6 mRNA expression was set at 3, patients with a high level of expression (14 ± 34, n = 111) tended to have a better prognosis than those with a low level [2 ± 1, n = 24]; log-rank (Cox–Mantel) test, \( P = 0.05\); Breslow–Gehan–Wilcoxon test, \( P = 0.07\); Fig. 1B).

**HDAC6 Protein Expression in Breast Cancer and Its Correlation with Clinicopathologic Factors.** Specific HDAC6 protein staining was found in the nucleus of some normal epithelial cells (Fig. 2A) and in the cytoplasm of the majority of breast cancer cells (Fig. 2B). Although postmenopausal patients showed higher HDAC6 protein expression, no relationship was found between protein expression and any other clinicopathologic factors (Table 1). Because of the high percentage (>80%) of breast cancer cells staining positive for HDAC6 protein in breast cancer tissue samples, we used the Kaplan–Meier method to analyze disease-free survival and overall survival rates in the different staining intensity groups and verified the results with the log-rank (Cox–Mantel) and Breslow–Gehan–Wilcoxon tests. The analysis showed that patients with moderate/strong staining intensity (\( n = 88 \)) had better disease-free survival rates than the weak-intensity group [\( n = 47 \); log-rank (Cox–Mantel) test, \( P = 0.03\); Breslow–Gehan–Wilcoxon test, \( P = 0.03\); Fig. 3A]. However, in terms of overall survival rates, there was a trend toward better prognosis for the moderate/strong-staining intensity group than for the weak-staining intensity group (Cox–Mantel test, \( P = 0.12\); Breslow–Gehan–Wilcoxon test, \( P = 0.05\); Fig. 3B).

**Univariate and Multivariate Prognostic Analysis of HDAC6 mRNA and Protein Expression in Breast Cancer for DFS and OS.** From the limited 135 cases of breast cancer patients, we failed to find that HDAC6 mRNA and protein expression are independent prognostic factors for disease-free survival (Table 2) and overall survival rates (Table 3).

**HDAC6 mRNA Expression and Responsiveness to Endocrine Therapy.** In this study, the number of recurrent or advanced patients receiving endocrine therapy was limited. Nevertheless, there was a tendency among the 8 patients who responded to endocrine therapy to express higher levels of HDAC6 mRNA than did the 10 nonresponders (Fig. 4). However, in this study, we did not find any correlation between HDAC6 mRNA and protein expression (data not shown).

**DISCUSSION**

Human HDACs isoforms are divided into three classes, based on sequence homology, intracellular localization, and association with the protein that forms the DNA-binding complex (22, 23). On the basis of its similarity to the yeast gene Hda1, HDAC6 (8, 9) was designated as class II, and is a unique deacetylase because it contains two functional catalytic domains and displays nucleocytoplasmic shuttling capabilities (24, 25). HDAC6 contains three nuclear export signals, the most NH2-terminal of the signals is responsible for the enzyme’s cytoplasmic localization in rapidly dividing cells (10); this raises the intriguing possibility that this deacetylase has unique cytoplasmic, nontranscriptionally related targets. In addition, HDAC6 has been shown to deacetylate \( \alpha \)-tubulin in polymerized micro-

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**Table 1  HDAC6 expression and clinicopathologic factors in invasive carcinoma cases**

<table>
<thead>
<tr>
<th>Age, y</th>
<th>No. of patients</th>
<th>HDAC6 mRNA (Mean ± SD)</th>
<th>Mann–Whitney U test, ( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;50</td>
<td>88</td>
<td>10 ± 13</td>
<td>NS</td>
</tr>
<tr>
<td>≤50</td>
<td>47</td>
<td>16 ± 53</td>
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</tr>
</tbody>
</table>

**Menopausal status**

| Post         | 81             | 14 ± 43                 | NS                            |
| Pre          | 54             | 10 ± 15                 |                               |

**Axillary lymph nodes**

| Negative     | 82             | 11 ± 13                 | NS                            |
| Positive     | 53             | 15 ± 53                 |                               |

**Tumor size**

| <2 cm        | 33             | 13 ± 39                 | 0.03*                         |
| ≥2 cm        | 102            | 11 ± 7                  |                               |

**Histological grade**

| 1,2          | 92             | 15 ± 41                 | 0.03*                         |
| 3            | 43             | 8 ± 6                   |                               |

**ERα**

| Positive     | 89             | 11 ± 12                 | 0.0005*                       |
| Negative     | 44             | 7 ± 8                   |                               |

**PgR**

| Positive     | 72             | 16 ± 45                 | <0.0001*                      |
| Negative     | 56             | 9 ± 16                  |                               |

**Abbreviation:** NS, not significant.

* \( P < 0.05 \).
tubules, thus potentially enhancing chemotactic cell motility (12). A study of gene expression patterns with cDNA microarray techniques in MCF-7 cells has shown that the HDAC6 gene is a late responsive, estrogen-induced, up-regulated gene (3). However, the importance of HDAC6 expression and its associated clinicopathologic function in breast cancer remains unclear. In the present study, it was found that HDAC6 mRNA expression was expressed at significantly higher levels in breast cancer patients with small tumors (<2 cm) of low histologic grade that were ERα- and PgR-positive. However, no relationship was found between HDAC6 mRNA expression and any of the other clinicopathologic factors under study, namely age, menopausal status, and axillary lymph node involvement.

Patients with high levels of expression of HDAC6 mRNA and protein expression had a better prognosis than those with low expression in terms of disease-free survival rates. Furthermore, the highly significant correlation found between HDAC6 mRNA expression and positive ERα (P = 0.0003) and PgR (P < 0.0001) status may suggest that, in breast cancer, HDAC6 levels might serve as a transcription activator of ERα and PgR gene expression. However, this needs to be confirmed by additional studies. Previous studies on the relationship between HDACs and ER have reported that in the ERα-negative breast cancer cell line MDA-MB-231, ER gene silencing was mediated by HDAC. Furthermore, this study showed that treatment with the HDAC inhibitor, trichostatin A, could lead to re-expression of ER mRNA without an apparent alteration in the methylation status of ER CpG island (26). However, the authors of the study did not identify whether one particular HDACs isoform was concerned with this finding. One recent study has shown that the HDAC inhibitor trichostatin A sensitized ERα-negative, anti-hormone-unresponsive breast cancer cell lines MDA-MB-231 and Hs578T, to tamoxifen treatment, possibly by up-regulating ERβ activity (27). Another study showed that overexpression of HDAC1 in stable transfected MCF-7 clones induced a loss of ERα and significantly increased cell proliferation and colony formation, as compared with the control MCF-7 cells. Whereas treatment of stable MCF-7 clones with the HDAC-specific

**Fig. 2** Representative microscopic views of HDAC6 staining with a polyclonal antibody H-300. A, HDAC6 staining was found in the nucleus of some normal epithelial cells. B, HDAC6 staining was also found in the cytoplasm of the majority of cancer cells. ×400.
inhibitor trichostatin A induced re-expression of ERα mRNA and protein, suggesting that HDAC1 affects breast cancer progression by promoting cellular proliferation in association with a reduction in both ERα protein expression and ERα transcriptional activity (28). Therefore, the highly positive correlation between HDAC6 mRNA and ERα and PgR, as shown in the present study, needs further investigation to clarify the functional significance of HDAC6 in estrogen signal transcription in breast cancer.

The significantly higher level of HDAC6 mRNA expression seen in ERα- and PgR-positive tumors compared with ERα- and PgR-negative tumors suggests that levels of this transcription-modulating factor might have potential as an indicator of responsiveness to endocrine therapy. Furthermore, HDAC6 mRNA expression may also have a value as a prognostic indicator for breast cancer progression. The reason for the significant correlation between increased HDAC6 mRNA and protein expression and a better prognosis in terms of DFS, seen in this study, remains unknown. Further research is needed to examine the HDAC6 gene and its function in non-histone protein transcription modulation, such as ER and PgR, and to clarify the details of its relationship with the clinicopathologic factors that were shown in the present study. Further investiga-

![Figure 3](image1.png)

**Fig. 3** Statistical analysis of HDAC6 protein expression intensity levels and disease-free survival and overall survival rates with the Kaplan-Meier method. A, the moderate/strong intensity group (n = 88) had better disease-free survival rates than the weak intensity group (n = 47; log-rank test, P = 0.03; Breslow–Gehan–Wilcoxon test, P = 0.03). B, for moderate/strong intensity groups there was a trend for better overall survival rates than that for weak intensity group (log-rank test, P = 0.12; Breslow–Gehan–Wilcoxon test, P = 0.05).

![Figure 4](image2.png)

**Fig. 4** The nonparametric Mann–Whitney U test was used for the statistical analysis of the association between HDAC6 mRNA expression and responsiveness to endocrine treatment by tamoxifen. The boxes represent the mean and the 70% confidence intervals; bars, SDs. The 8 endocrine treatment-responsive patients tended to have levels of HDAC6 mRNA that were higher than those of the 10 nonresponders (P = 0.13).

### Table 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Univariate P</th>
<th>Multivariate P</th>
<th>RR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymph nodes</td>
<td>0.0016</td>
<td>0.0063</td>
<td>0.364 (0.177–0.751)</td>
</tr>
<tr>
<td>ER</td>
<td>0.0003</td>
<td>0.0069</td>
<td>3.199 (1.377–7.435)</td>
</tr>
<tr>
<td>PgR</td>
<td>0.0078</td>
<td>0.2802</td>
<td>1.551 (0.699–3.441)</td>
</tr>
<tr>
<td>HDAC6 mRNA</td>
<td>0.0036</td>
<td>0.7254</td>
<td>1.164 (0.498–2.720)</td>
</tr>
<tr>
<td>HDAC6 intensity</td>
<td>0.0374</td>
<td>0.2307</td>
<td>1.534 (0.762–3.090)</td>
</tr>
</tbody>
</table>

Abbreviations: RR, relative risk; CI, confidence interval.

### Table 3

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Univariate P</th>
<th>Multivariate P</th>
<th>RR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymph nodes</td>
<td>0.0042</td>
<td>0.0110</td>
<td>0.324 (0.136–0.772)</td>
</tr>
<tr>
<td>ER</td>
<td>0.0134</td>
<td>0.0989</td>
<td>2.194 (0.863–5.582)</td>
</tr>
<tr>
<td>PgR</td>
<td>0.0020</td>
<td>0.0776</td>
<td>2.448 (0.906–6.617)</td>
</tr>
<tr>
<td>HDAC6 mRNA</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>HDAC6 intensity</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Abbreviations: RR, relative risk; CI, confidence interval; NS, not significant.
tion is also warranted concerning the relationship between HDAC6 expression and response to endocrine therapy. It is also of interest to notice the exporting of HDAC6 protein, as shown in the present study by immunohistochemistry, from the nucleus of normal epithelial cells to the cytoplasm of breast cancer cells. The modulating of HDAC6 protein shuttling between nucleus and cytoplasm compartment remains unclear (29). We postulate that in breast cancer the exporting of HDAC6 protein may be correlated with tumorigenesis. However, this also calls for additional studies.

In conclusion, in breast cancer, levels of HDAC6 mRNA may serve as a predictive indicator of responsiveness to endocrine treatment and also as a prognostic indicator for breast cancer progression.

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