HDAC6 inhibition enhances the anti-tumor effect of eribulin through tubulin acetylation in triple negative breast cancer cells

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Abstract

Background: Improved prognosis for triple-negative breast cancer (TNBC) has currently plateaued and the development of novel therapeutic strategies is required. This study aimed to explore the anti-tumor effect of combined eribulin and HDAC inhibitor (vorinostat: VOR, pan-HDAC inhibitor and ricolinostat: RICO, selective HDAC6 inhibitor) treatment for TNBC.

Methods: The effect of eribulin in combination with VOR or RICO was tested based on both concurrent and sequential administration to three TNBC cell lines (MDA-MB-231, Hs578T, and MDA-MB-157) and their eribulin-resistant derivatives. The expression of acetylated α-tubulin was analyzed by western blotting. Immunohistochemical analyses of clinical specimens obtained from breast cancer patients who underwent neoadjuvant chemotherapy with eribulin were also examined.

Results: The simultaneous administration of low concentrations of VOR (0.2 μM) or RICO (0.2 μM) enhanced the anti-tumor effect of eribulin in MDA-MB-231 and Hs578T cells but not in MDA-MB-157 cells. Meanwhile, pre-treatment with 5 μM of VOR or RICO enhanced sensitivity to eribulin in MDA-MB-231, Hs578T, and MDA-MB-157 cells. VOR or RICO increased acetylated α-tubulin expression in MDA-MB-231 and Hs578T cells in a dose-dependent manner (0.2 μM to 5 μM). In contrast, whereas 5 μM of VOR or RICO increased the expression of acetylated α-tubulin in MDA-MB-157 cells, low concentrations (0.2 μM or 0.5 μM) did not. Treatment with eribulin also increased the expression of acetylated α-tubulin in MDA-MB-231 and Hs578T cells but not in MDA-MB-157 cells. These phenomena were also observed in eribulin-resistant cells. Based on immunohistochemical analyses of clinical specimens, the expression of acetylated α-tubulin was increased after eribulin treatment in TNBC.

Conclusions: HDAC6 inhibition enhances the anti-tumor effect of eribulin through the acetylation of α-tubulin. This combination therapy could represent a novel therapeutic strategy for TNBC.

Background

Triple-negative breast cancer (TNBC) is a disease characterized by the lack of estrogen receptor (ER) and progesterone receptor expression, as well as the absence of human epidermal growth factor receptor 2 amplification, and accounts for 10–20% of all breast cancers. TNBC is the most aggressive subtype of breast cancer and thus is associated with poor clinical outcomes despite recent progress in the treatment for breast cancer [1, 2]. Although conventional cytotoxic chemotherapy is effective for a subset of patients with TNBC, some cases show a very aggressive clinical course, and fewer than 30% of patients with metastatic TNBC survive 5 years [3–6]. Therefore, there is an urgent need to develop novel therapeutic strategies for this disease subtype.

Eribulin mesylate (eribulin) is an inhibitor of microtubule dynamics and has been used worldwide for the treatment of metastatic breast cancer since 2011. This compound is a synthetic macrocyclic ketone analog of halichondrin B, which is naturally generated by marine sponges, and inhibits microtubule polymerization [7, 8]. When administered to patients with metastatic breast cancer who had previously

received both anthracycline and taxane, eribulin monotherapy significantly prolongs overall survival [9]. Consequently, this drug is currently used for patients with recurrent or metastatic breast cancer.

Eribulin has unique effects on epithelial–mesenchymal transition (EMT) that are distinct from those of other anti-tubulin agents [10]; specifically, it can induce mesenchymal–epithelial transition (MET) in TNBC cells [11], whereas paclitaxel can trigger EMT [12, 13]. We previously demonstrated that this opposing effect on the EMT–MET axis could induce a synergistic anti-tumor effect on TNBC when eribulin and paclitaxel were simultaneously administrated [14]. Previous reports revealed other favorable effects of eribulin on the tumor microenvironment (TME) such as vascular remodeling and improving the immunosuppressive TME [15, 16]. Therefore, when simultaneously administrated, eribulin might have the potential to enhance the anti-tumor effect of other anti-cancer drugs through its favorable influence on cancer cells and the TME although the precise mechanisms underlying these phenomena remain unclear.

Microtubules, which are the target molecule of eribulin, are complex polymers that repeatedly undergo rapid and stochastic transitions between growth and contraction, thus enabling localized changes for specific physiologic purposes [17]. This instability is tightly regulated by the acetylation of α-tubulin [18, 19], and higher levels of acetylated α-tubulin expression are correlated with sensitivity to anti-tubulin agents including paclitaxel and other taxane anti-cancer agents [20]. Among regulators of α-tubulin modification, histone deacetylase (HDAC) 6 is known as the major deacetylase of this protein [21]. The inhibition of HDAC was found to result in anti-tumor effects on various malignancies, and this approach is considered promising for the treatment of cancers [22, 23]. HDACs are classified into 11 families, and a considerable number of HDAC inhibitors have been developed. Many HDAC inhibitor monotherapies or combination therapies with other anti-cancer agents are being investigated in clinical trials for the treatment of various cancers [24]. Among HDAC inhibitors, vorinostat (VOR), which is a pan-HDAC inhibitor, was approved for the treatment of cutaneous T cell lymphoma for the first time by the FDA [25]. In addition, ricolinostat (RICO), which is a selective HDAC6 inhibitor, has shown anti-tumor effects on hematologic malignancies and melanoma [26–28] and is thus being tested in several clinical trials (NCT02189343, NCT01997840).

To date, there has been one report that demonstrated a synergistic anti-tumor effect of eribulin and HDAC inhibitor combination therapy on TNBC [29]; however, the mechanisms underlying this synergistic effect have not been fully elucidated. We hypothesized that the inhibition of HDAC6 sensitizes TNBC cells to eribulin through the acetylation of α-tubulin. In this study, we aimed to test this notion and demonstrated that the inhibition of HDAC6 by pan- or selective- inhibitors enhanced the anti-tumor effect of eribulin on TNBC cells through the acetylation of α-tubulin.

Methods

Cell culture and reagents

Three TNBC cell lines (MDA-MB-231, Hs578T, and MDA-MB-157) and MCF7, which is an ER–positive breast cancer cell line, were purchased from the American Type Cell Collection (Manassas, VA) in 2017

and passaged in our laboratory. All cell lines were tested monthly for mycoplasma contamination using the MycoAlert mycoplasma detection kit (Lonza Walkersville, Inc, Walkersville, MD) and were cultured for no more than 20 passages from the validated stocks. All cell lines were cultured in RPMI with 10% FBS at 37.0 °C with 5% CO_2. Eribulin-resistant TNBC cells were previously established in our laboratory [30]. Eribulin was purchased from Eisai Co., Ltd. (Tokyo, Japan). Vorinostat was purchased from Sigma-Aldrich (Saint Louis, MO) and ricolinostat was purchased from Sellek Chemicals (Houston, TX).

WST Assays

The growth inhibitory effects of eribulin and HDAC inhibitors were quantitated using a tetrazolium salt-based proliferation assay (WST assay; Wako Chemicals, Osaka, Japan) according to the manufacturer's instructions. Briefly, 4×10^3 cells were cultured in 96-well plates, in triplicate, with 100 µl of growth medium with a graded concentration of eribulin or HDAC inhibitors for 72 h. Subsequently, 10 µl of WST-8 solution was added to each well, and the plates were incubated at 37 °C for another 3 h. Absorbance was measured at 450 and 640 nm using the SoftMax Pro (Molecular Devices, Tokyo, Japan), and cell viability was determined. Each experiment was independently performed and repeated at least three times. To evaluate the synergistic effect of HDAC inhibitors and eribulin, an isobologram was plotted based on data from the WST assays [31]. In an isobologram, a diagonal line represents an additive effect. Experimental data points, represented by dots located below, on, or above the line, indicate synergistic, additive, or antagonistic effects, respectively.

Western Blotting

Proteins were isolated from cells, as previously described, and were then used for western blot analyses (10 µg/lane) [32]. For experiments on drug exposure, the proteins were isolated from cells treated with drugs for 48 h. The membrane was probed with the following antibodies: anti-Bcl-2 (1:1000; Abcam, Cambridge, UK), anti-acetylated α-tubulin (1:200; Santa Cruz Biotechnology, Heidelberg, CA), anti-ZEB1 (1:1000; Cell Signaling Technology, Danvers, MA), anti-E cadherin (1:50000, Gene Tax, Irvine, CA), anti-Slug (1:1000; Cell Signaling Technology, Danvers, MA), and anti-vimentin (1:1000; Cell Signaling Technology, Danvers, MA). β-Actin (1:5000; Sigma-Aldrich, Saint Louis, MO) or α-tubulin (1:200, Santa Cruz Biotechnology, Heidelberg, CA) was used as a loading control. Each experiment was repeated independently at least three times, and one representative blot was chosen for the figures.

Cell Growth Assay

The growth of parental and eribulin-resistant MDA-MB-231, Hs578T, and MDA-MB-157 cells pre-treated with DMSO and VOR (5 µM) or RICO (5 µM) for 48 h was measured by performing a WST assay (Wako Chemicals, Osaka, Japan). Briefly, 1×10^5 cells/well were seeded in 6-well plate and cultured for 24 h. Thereafter, a medium change was performed with DMSO, VOR, or RICO. After incubation for another 48 h, 4×10^3 cells/well were seeded in 96-well tissue culture plates in 100 µl of medium without VOR or RICO. After each indicated period, the absorbance was measured after adding WST solution. Each experiment was independently performed and repeated at least three times.

Small Interfering RNA (siRNA) Transfection

ON-TARGETplus siRNA targeting ZEB1 (M-006564) and the negative control (D-001810) were purchased from GE Healthcare (Buckinghamshire, UK). Transfection of each siRNA (10 nM) was performed using Lipofectamine RNAi-MAX (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's instructions. Twenty-four hours after transfection, the proteins were extracted and 4×10^3 cells/well were cultured in 96-well tissue culture plates and incubated for 72 h after adding stepwise-diluted VOR or RICO. Finally, the absorbance was measured after adding WST solution, as described previously.

Apoptosis Analysis

Cells were plated in six-well plates at a density of 5×10^4 cells/well. After 24 h, cells were treated with eribulin (1 nM for the parental cells, 3 nM for eribulin-resistant MDA-MB-231 cells, 70 nM for eribulin-resistant Hs578T cells) and/or 0.5 μM of VOR or RICO and were cultured for 48 h. To detect apoptotic cell death, DNA fragmentation was detected using a Cell Death Detection ELISAplus (Roche Applied Science, Tokyo, Japan) following the manufacturer's instruction.

Immunohistochemistry

Tissue sections were obtained from 26 breast cancer patients who enrolled in a randomized controlled trial for peripheral neuropathy comparing weekly paclitaxel (80 mg/m^2) for 12 cycles with eribulin mesylate (1.4 mg/m^2) on day 1 and 8 (1 cycle; 21 days) for four cycles followed by tri-weekly FEC (500 mg/m^2 fluorouracil, 100 mg/m^2 epirubicin, and 500 mg/m^2 cyclophosphamide) as neoadjuvant chemotherapy (JONIE-3 study: UMIN000012817) between 2014 and 2016. This study was conducted according to the ethical guidelines of the Declaration of Helsinki, and specific approval was obtained from the Ethics Committee of Shinshu University School of Medicine. The tissue sections were obtained by core needle biopsy before treatment and after eribulin treatment for each patient. Immunohistochemical staining for acetylated α-tubulin (anti-acetylated α-tubulin, 1:500; Santa Cruz Biotechnology, Heidelberg, CA) was performed as previously described [32]. The H-score was used to evaluate the intensity and the fraction of positive cells. Intensity was scored from 0 to 3, with 0 representing no staining, 1 weak, 2 moderate, and 3 strong staining. H-score was calculated as a sum of the intensity of staining multiplied by the percentage of stained cells for each intensity, where 0 indicated the complete absence of staining and 300, the highest score, showing the highest intensity of staining in all cells. All immunohistochemical specimens were evaluated by two observers who were blind to the conditions of the patients.

Statistical analysis

Data were tested for significance by performing a Mann-Whitney U-test or paired two-tailed t-test; a p-value < 0.05 was considered statistically significant (StatFlex ver.6, Artech Co., Ltd., Osaka, Japan).

Results

Sensitivity to HDAC inhibitors in parental and eribulin-resistant TNBC cells

To evaluate potential growth-inhibitory effects by HDAC inhibitors (VOR and RICO) on TNBC cells in vitro, MDA-MB-231, Hs578T, and MDA-MB-157 cells were treated with VOR or RICO for 72 h, and cell viability was measured by performing WST assays (Additional file 1; Table S1). The IC_{50} of VOR for MDA-MB-231, Hs578T, and MDA-MB-157 cells was 1.8 ± 0.4 μM, 1.3 ± 0.5 μM, and 1.6 μM ± 0.3 μM, respectively. On the other hand, the IC_{50} of RICO for these three cell lines was 2.0 ± 0.5 μM, 1.8 ± 0.3 μM, and 2.4 ± 0.4 μM, respectively. There were no significant differences in the IC_{50} of VOR or RICO among these three cell lines (Additional file 2; Fig. S1).

Next, we investigated the growth-inhibitory effects of HDAC inhibitors on eribulin-resistant TNBC cells (MDA-MB-231/E, Hs578T/E, MDA-MB-157/E), which were established previously in our laboratory [30]. The IC_{50} of VOR for MDA-MB-231/E, Hs578T/E, and MDA-MB-157/E cells was 2.0 ± 0.5 μM, 1.8 ± 0.3 μM, 1.8 ± 0.4 μM, respectively. Meanwhile, the IC_{50} of RICO for these three eribulin-resistant TNBC cell lines was 1.8 ± 0.5 μM, 2.2 ± 0.3 μM, and 2.2 ± 0.6 μM, respectively. Thus, no significant differences in the IC_{50} of VOR or RICO were observed among the three eribulin-resistant TNBC cell lines (Additional file 2; Fig. S1). Moreover, no cross-resistance to eribulin and VOR or RICO was observed in both between these parental cells and eribulin-resistant cells.

Low concentrations of VOR or RICO enhance the anti-tumor effect of eribulin in MDA-MB-231 and Hs578T cells

Next, we analyzed whether the co-administration of low concentrations of VOR or RICO could enhance the anti-tumor effect of eribulin on TNBC cells. The concentrations of co-administrated VOR or RICO were determined to be 0.2 μM and 0.5 μM because we confirmed that these concentrations do not affect cell growth as a single agent before this experiment (Additional file 2; Fig. S1). The growth-inhibitory effect of eribulin was enhanced when low concentrations (0.2 or 0.5 μM) of VOR or RICO were simultaneously added to MDA-MB-231 and Hs578T cells. However, in MDA-MB-157 cells, low-dose VOR or RICO did not enhance sensitivity to eribulin (Fig. 1a). Isobologram analysis of VOR and eribulin results demonstrated that each experimental data point was located below the diagonal line for MDA-MB-231 cells and Hs578T cells, indicating that VOR and eribulin acted synergistically (Additional file 3; Fig. S2). In contrast, when we examined the growth-inhibitory effect of combined eribulin and HDAC inhibitors on MCF7 cells, RICO was not found to enhance eribulin sensitivity (Additional file 4; Fig. S3).

Next, we examined the induction of apoptosis after single treatment with eribulin, VOR, or RICO, as well as a combination of eribulin with VOR or RICO, in MDA-MB-231 and Hs578T cells. Whereas the administration of 0.5 μM VOR or RICO for 48 h did not induce apoptosis in MDA-MB-231 and Hs578T cells, 1 nM of eribulin induced apoptosis significantly compared to that in cells treated with DMSO alone. Notably, the addition of 0.5 μM VOR or RICO to 1 nM eribulin significantly enhanced the induction of apoptosis compared to that induced by monotherapy comprising 1 nM of eribulin (Fig. 1b).

Next, to gain further insight into apoptosis induction by eribulin and HDAC inhibitors, we analyzed alterations in the levels of Bcl-2, which is known as an anti-apoptotic protein, in TNBC cell lines (Fig. 1c). Whereas the administration of VOR or RICO did not change the expression of Bcl-2, treatment with eribulin (1 nM) decreased the expression of Bcl-2. Furthermore, the addition of VOR or RICO to eribulin enhanced this decrease in Bcl-2 expression compared to that induced by eribulin monotherapy, which was concordant with the results of apoptosis assays mentioned previously herein. These results indicate that low concentrations of VOR or RICO enhance the anti-tumor effect of eribulin by augmenting eribulin-mediated induction of apoptosis in MDA-MB-231 and Hs578T cells.

Low concentrations of VOR or RICO restore eribulin-resistance in eribulin-resistant MDA-MB-231 and Hs578T cells

As we found that low concentrations of HDAC inhibitors could enhance sensitivity to eribulin in the parental MDA-MB-231 and Hs578T cells, we next examined whether HDAC inhibitors could restore eribulin sensitivity in three eribulin-resistant TNBC cell lines (MDA-MB-231/E, Hs578T/E, MDA-MB-157/E). The co-administration of a low concentration (0.2 μM or 0.5 μM) of VOR or RICO partially restored eribulin sensitivity in MDA-MB-231/E and Hs578T/E cells, though this did not reach the level of eribulin sensitivity in parental cells. However, as in parental cells, a low concentration of VOR or RICO did not alter the sensitivity of eribulin in MDA-MB-157/E cells (Fig. 2a).

Next, we examined whether VOR or RICO could enhance the induction of apoptosis in eribulin-resistant MDA-MB231 and Hs578T cells, as was observed for their parental cells. Eribulin monotherapy for 48 h (3 nM for MDA-MB-231/E cells, 70 nM for Hs578T/E cells) induced apoptosis significantly in both eribulin-resistant cell lines. Whereas the addition of 0.5 μM of VOR or RICO for 48 h did not induce apoptosis in the eribulin-resistant MDA-MB-231 and Hs578T cells, the combination of eribulin with the same dose of VOR or RICO significantly enhanced the induction of apoptosis in these cell lines (Fig. 2b). These results indicate that a low concentration of VOR or RICO can enhance apoptosis induced by eribulin and restore sensitivity to eribulin in the eribulin-resistant MDA-MB-231 and Hs578T cells, as well as in their parental cells.

EMT is not involved in enhanced eribulin sensitivity induced by HDAC inhibitors

As we previously reported that EMT induction enhances eribulin sensitivity in a subset of TNBC cell lines (MDA-MB-231 and Hs578T) [14], we also examined whether VOR or RICO treatment would alter EMT markers in these cell lines. ZEB1, vimentin, and Slug were studied as mesenchymal markers, whereas E-cadherin was studied as an epithelial marker. Western blotting demonstrated that the administration of 0.5 μM VOR or RICO did not alter the expression of these mesenchymal markers in MDA-MB-231 and Hs578T cells. E-cadherin expression was not detected in both cell lines treated with DMSO alone and 0.5 μM of VOR or RICO (Fig. 3a).

We previously reported that the siRNA-mediated knockdown of ZEB1, which serves as a transcriptional activator of mesenchymal differentiation, confers eribulin resistance to MDA-MB-231 and Hs578T cells,

indicating that the EMT–MET axis is involved in eribulin sensitivity [14]. However, the involvement of the EMT–MET axis in sensitivity to HDAC inhibitors has not been elucidated. Hence, we tested whether ZEB1 inhibition by siRNA could alter VOR or RICO sensitivity in three TNBC cell lines. The inhibition of ZEB1 expression by siRNA was confirmed by western blotting (Fig. 3b). In all three cell lines, no difference in the growth-inhibitory effect of VOR or RICO was observed between cells treated with control siRNA and those treated with siRNA targeting ZEB1 (Fig. 3c). These results indicate that the administration of HDAC inhibitors does not alter the EMT–MET phenotype in TNBC cells; moreover, the EMT–MET axis is less likely to be associated with TNBC cell sensitivity to HDAC inhibitors.

Low concentrations of HDAC inhibitors and eribulin increase the expression of acetylated α-tubulin in MDA-MB-231 and Hs578T cells, but not in MDA-MB-157 cells

Next, to further investigate the mechanism underlying the increase in eribulin sensitivity induced by VOR and RICO, we examined differences in acetylated α-tubulin expression caused by VOR or RICO treatment for 48 h among these cell lines. Western blotting demonstrated that VOR or RICO addition increased the expression of acetylated α-tubulin in a dose-dependent manner in MDA-MB-231 and Hs578T cells. In contrast, although 0.2 and 0.5 μM VOR or RICO did not alter the expression of acetylated α-tubulin, 5 μM VOR or RICO drastically increased the expression of acetylated α-tubulin in MDA-MB-157 cells. A similar change in the expression of acetylated α-tubulin observed in parental TNBC cell lines was induced in their eribulin-resistant sublines (Fig. 4a).

Then, we examined alterations in the expression of acetylated α-tubulin induced by eribulin in TNBC cell lines because paclitaxel, which is another anti-tubulin agent, has been reported to increase the expression of this marker [33]. The expression of acetylated α-tubulin was increased in MDA-MB-231 and Hs578T cells in a dose-dependent manner after the addition of 0.5, 1, and 2 nM eribulin. However, eribulin at these concentrations did not alter the expression of acetylated α-tubulin in MDA-MB-157 cells (Fig. 4b).

Next, we analyzed the alteration of acetylated α-tubulin expression when eribulin was administered in combination with VOR or RICO to the parental TNBC cell lines. Combination therapy comprising 0.5 nM eribulin with 0.5 μM VOR or RICO additively upregulated the expression of acetylated α-tubulin in MDA-MB-231 and Hs578T cells, whereas no effect on the expression of acetylated α-tubulin was observed in MDA-MB-157 cells (Fig. 4c). These results indicate that both eribulin and HDAC inhibitors might increase the acetylation of α-tubulin in MDA-MB-231 and Hs578T cells but not in MDA-MB-157 cells.

Increasing the expression of acetylated α-tubulin through VOR or RICO pre-treatment enhances eribulin sensitivity in TNBC cells

Next, we examined whether the upregulation of acetylated α-tubulin induced by VOR or RICO pre-treatment could alter the sensitivity to eribulin. Results showed that 5 μM VOR or RICO could increase the expression of acetylated α-tubulin in MDA-MB-231, Hs578T, and MDA-MB-157 cells. However, all three cell lines treated with 5 μM VOR or RICO for 72 h did not survive, as demonstrated in Fig. S1, whereas the same dose of VOR or RICO for 48 h did not inhibit cell proliferation, and the cells grew similarly to those

without treatment after passage (Additional file 5; Fig. S4). Therefore, 5 μM VOR or RICO was used for this experiment. After pre-treating MDA-MB-231, Hs578T, and MDA-MB-157 cells with 5 μM VOR or RICO for 48 h, the pre-treated cells were seeded in a 96-well plate and tested for sensitivity to eribulin (Fig. 5a). After incubation for another 72 h, cell viability was measured. As a result, pre-treatment with VOR or RICO for 48 h enhanced sensitivity to eribulin in all three cell lines (Fig. 5b).

Furthermore, the effect HDAC inhibitor pre-treatment on sensitivity to eribulin was tested in eribulin-resistant TNBC cells. As shown in Fig. 5c, pre-treatment with VOR or RICO enhanced eribulin sensitivity in the three eribulin-resistant TNBC cell lines (MDA-MB-231/E, Hs578T/E, and MDA-MB-157/E cells), as observed in their parental cell lines (Fig. 5c). These results indicate that the increase in the expression of acetylated α-tubulin mediated by VOR or RICO pre-treatment enhances TNBC cell sensitivity to eribulin.

Increased expression of acetylated α-tubulin induced by eribulin treatment in clinical TNBC specimens

As our in vitro results suggested the possibility that α-tubulin acetylation might be increased by eribulin treatment in a subset of TNBC cells (Fig. 4b), we next analyzed whether eribulin would increase the acetylation of α-tubulin in clinical TNBC specimens. The expression of acetylated α-tubulin was evaluated by immunohistochemical staining in clinical specimens obtained from breast cancer patients who underwent neoadjuvant treatment with eribulin. The tissue sections were obtained by core needle biopsy before the initiation of treatment and after four courses of treatment with eribulin from 26 breast cancer patients who enrolled in the JONIE-3 study. Regarding ER expression, 16 cases were ER-positive and eight were ER-negative. Of the eight cases of ER-negative breast cancer, six were TNBC. ER-negative cases tended to exhibit higher baseline expression of acetylated α-tubulin compared to that in ER-positive cases though there was no statistical significance (Fig. 6a). We then analyzed the change in acetylated α-tubulin expression with eribulin treatment in five TNBC cases because one case presented with a clinically complete response to neoadjuvant treatment with eribulin and thus a post-treatment specimen could not be obtained. In two cases that showed a high H-score before eribulin treatment (275 and 300), high expression of acetylated α-tubulin was maintained throughout treatment with eribulin. In the other three cases, H-scores were increased by eribulin treatment (Additional file 6; Table S2). The H-scores of six cases at each point, the rate of H-score change, and responses to eribulin treatment are shown in Table S2, and the representative findings of immunohistochemical analyses are shown in Fig. 6b–e.

Next, to investigate whether altered α-tubulin acetylation, induced by eribulin, was associated with ER status, we analyzed eribulin-mediated changes in acetylated α-tubulin expression in ER-positive and ER-negative patients. Although there was no significant change in acetylated α-tubulin expression in ER-positive breast cancer specimens ($p = 0.994$), the expression of acetylated α-tubulin significantly increased in ER-negative breast cancer specimens after treatment with eribulin ($p = 0.012$; Fig. 6f). Notably, no cases of ER-negative breast cancer were associated with decreases in the expression of acetylated α-tubulin expression, whereas this decreased in 37.5% of ER-positive breast cancers (Fig. 6g). These results indicate that the increased acetylation of α-tubulin induced by eribulin is correlated with ER signaling.

Furthermore, we examined whether altered acetylated α-tubulin expression induced by eribulin treatment is associated with the response to eribulin in TNBC because increased acetylated α-tubulin expression resulted in higher sensitivity to eribulin in vitro. Two of three TNBC patients who showed a partial response (PR) exhibited > 2-fold positive conversion of acetylated α-tubulin expression. Another patient with a PR had a high level of acetylated α-tubulin expression (H-score: 300) before treatment, and thus acetylated α-tubulin could not be upregulated. In contrast, two patients who did not show a response to eribulin (stable disease) maintained acetylated α-tubulin expression during eribulin treatment (Additional file 6; Table S2). These results suggest that an increase in acetylated α-tubulin expression was associated with a favorable response to eribulin treatment in TNBC patients.

Discussion

In the present study, we demonstrated that HDAC6 inhibition, by both a pan-HDAC inhibitor (VOR) and selective HDAC6 inhibitor (RICO), enhances the anti-tumor effect of eribulin on TNBC cells in vitro. The administration of low doses of VOR or RICO, which alone exerted little growth-inhibitory effects, enhanced sensitivity to eribulin. Moreover, pretreatment with VOR or RICO increased acetylated α-tubulin expression and enhanced the anti-tumor effect of eribulin in both TNBC cells and their eribulin-resistant derivatives. To the best of our knowledge, this is the first report demonstrating potential enhancement of the anti-tumor effect of eribulin with HDAC6 inhibition for TNBC.

HDAC inhibitors cause changes in the acetylation status of chromatin and other non-histone proteins, resulting in changes in gene expression, the induction of apoptosis, and the inhibition of metastasis in cancer [34]. The HDAC family is divided into four classes and 11 isoforms (HDAC1–11). To date, HDAC inhibitors have been shown to exert an anti-tumor effect on various malignancies. Vorinostat, which has broad-substrate specificity for HDACs (pan-HDAC inhibitor), has been approved for the treatment of cutaneous T-cell lymphoma [35]. Not only HDAC inhibitor monotherapy but also combination therapy comprising HDAC inhibitors and other anti-cancer agents is expected to represent a promising therapeutic strategy because synergistic anti-tumor effects have been found with such combinations [26–28, 33, 36–40]. Indeed, many clinical trials based on other HDAC inhibitors with or without anti-cancer agents are ongoing. Moreover, HDAC inhibitors have the potential to overcome resistance to paclitaxel and tyrosine kinase inhibitors for epithelial growth factor receptor (EGFR) [41, 42]. Regarding breast cancer, Ono et al. recently reported that an HDAC inhibitor (OBP-801) and eribulin can synergistically inhibit the growth of TNBC cells by suppressing the survivin, Bcl-xL, and MAPK pathway [43]. However, the precise mechanism underlying the synergistic anti-tumor effect of HDAC inhibitors and eribulin had not been not fully elucidated.

Among HDACs, HDAC6 is a unique class IIb HDAC that contains two homologous catalytic domains, as compared to one catalytic domain in other HDACs [44]. HDAC6 is known as a deacetylase of α-tubulin [21], and the hyperacetylation of α-tubulin due to HDAC6 inhibition has been shown to reduce microtubule dynamic instability resulting in cell apoptosis [18]. In the present study, we demonstrated that low concentrations (0.2 μM and 0.5 μM) of VOR or RICO could enhance the anti-tumor effect of eribulin in

MDA-MB-231 and Hs578T cells by increasing the induction of apoptosis. In these cells, a low concentration of VOR or RICO upregulated the expression of acetylated α-tubulin. In contrast, a low concentration of VOR or RICO neither altered the expression of acetylated α-tubulin nor improved eribulin sensitivity in MDA-MB-157 cells. Similar to that with HDAC inhibitors, the upregulation of acetylated α-tubulin by eribulin was only observed in MDA-MB-231 and Hs578T cells, and not in MDA-MB-157 cells. However, the induction of α-tubulin acetylation by 5 μM VOR or RICO was found to enhance sensitivity to eribulin in all three TNBC cell lines. These results indicate that the required concentration of HDAC inhibitors to increase the expression of acetylated α-tubulin and the mechanisms underlying tubulin acetylation by eribulin are different among cell lines. However, the induction of α-tubulin acetylation by high-dose HDAC inhibitors was found to render TNBC cell lines sensitive to eribulin. Furthermore, an analysis of immunohistochemical staining of clinical breast cancer specimens revealed that eribulin treatment increased the expression of acetylated α-tubulin, particularly in ER-negative breast cancer. Moreover, consistent with in vitro results of this study, patients with increased expression of acetylated α-tubulin respond favorably to eribulin treatment. These results were consistent with previous studies that demonstrated that paclitaxel increases the acetylation of α-tubulin and that a combination of paclitaxel and HDAC inhibitors can enhance the acetylation of α-tubulin compared to that with each monotherapy [33, 40]. However, our study provides additional evidence regarding the combination of HDAC6 inhibitor and anti-tubulin agent treatment, suggesting that not only paclitaxel but also eribulin can target the same axis to induce the upregulation of acetylated α-tubulin in TNBC, and thus their combination exerts a synergistic anti-tumor effect.

Notably, increased expression of acetylated α-tubulin was likely to occur in ER-negative breast cancer but not in ER-positive cancer, indicating the correlation between ER signaling and tubulin acetylation. Indeed, a previous report showed that membrane-localized ER associates with HDAC6 and causes the rapid deacetylation of tubulin in breast cancer cells [45]. Thus, ER signaling might act on the opposite axis targeted by eribulin treatment in regard with tubulin acetylation; specifically, we suggest that ER signaling induces deacetylation, whereas eribulin treatment induces acetylation of α-tubulin. Although our study could not demonstrate statistical significance in the expression level of acetylated α-tubulin between ER-positive and ER-negative cases, possibly due to the small number of included patients, in a larger patient cohort study comprised of 392 patients, TNBC showed significantly higher levels of acetylated α-tubulin than ER-positive breast cancer [46]. Therefore, an increase in acetylated α-tubulin might occur preferentially in ER-negative breast cancer, likely due to the absence of ER–HDAC6 interactions. Our in vitro study demonstrating that ER-positive breast cancer cells (MCF7) do not show enhanced eribulin sensitivity when treated with an HDAC6 inhibitor further support this ER–tubulin acetylation association.

Breast cancer usually develops resistance to anti-cancer drugs despite showing a response in the early phase of treatment. Further, the mechanisms underlying drug resistance are varied. ATP-binding cassette transporters comprise one of the primary mechanisms involved in drug resistance through the efflux of agents from cancer cells [47]. We previously reported that two transporters (ABCB1, ABCC11) confer eribulin resistance [30]. To overcome resistance to anti-cancer drugs, although inhibitors of ATP-binding cassette transporters have been developed [48–50], a strategy to block ATP-binding cassette transporters

has not been successful. Thus, other strategies to overcome drug resistance are needed. In the present study, we demonstrated that low concentrations of VOR or RICO upregulate the expression of acetylated α-tubulin, enhancing sensitivity to eribulin and inducing apoptosis in eribulin-resistant MDA-MB-231 and Hs578T cells but not MDA-MB-157 cells. In contrast, the upregulation of acetylated α-tubulin after VOR or RICO pre-treatment restored eribulin resistance in all three TNBC cells. These results are similar to those obtained with their parental cells and indicate the correlation between the increased expression of acetylated α-tubulin and eribulin sensitivity, even in eribulin-resistant cells, and suggest that combination treatment with HDAC inhibitors and eribulin could be a novel promising strategy for TNBC after acquired resistance to eribulin.

EMT plays a crucial role in the development of invasive and metastatic properties during cancer progression [10, 51, 52]. Cytotoxic agents such as paclitaxel and 5-FU have been reported to induce EMT directly in breast cancer cells [53, 54]. However, eribulin exerts the opposite effect on the EMT–MET axis as other cytotoxic drugs, inducing MET in TNBC cells [11]. Furthermore, Dezzo et al. [55] reported that the expression of EMT-related genes is positively associated with eribulin sensitivity in breast cancer. Currently, whether HDAC inhibitors induce or suppress EMT in cancer cells is controversial. Kong et al. reported that HDAC inhibitors induce EMT in prostate cancer cells [56], whereas Sakamoto et al. reported that they suppress EMT in biliary tract cells [57]. In the present study, we determined if treatment with HDAC inhibitors would alter the expression of EMT-related proteins and whether sensitivity to HDAC inhibitors would be affected by phenotypic changes through EMT. As a result, treatment with HDAC inhibitors did not change the expression of EMT-related proteins (ZEB1, vimentin, slug, E-cadherin) and forced transition to an epithelial phenotype via ZEB1 inhibition did not alter HDAC inhibitor sensitivity. These results indicate that alterations to the MET–EMT axis are not involved in the enhanced anti-tumor effect induced by the combination of HDAC inhibitors and eribulin in TNBC cells.

Several limitations of this study also need to be considered. First, though we focused on the acetylation of α-tubulin, HDAC6 inhibition could alter a variety of other gene expression patterns and the acetylation status of other proteins. Therefore, the possibility that other mechanisms contribute to enhanced eribulin sensitivity should be considered. Second, the number of clinical specimens was small in our study. This was because neoadjuvant therapy with eribulin has not been approved yet, and thus, the opportunity to obtain clinical specimens before and after eribulin treatment is limited to clinical trials. Thus, further investigations and a large number of clinical specimens are required to validate our results and elucidate the other mechanisms underlying enhanced eribulin sensitivity in TNBC caused by HDAC inhibitors.

Conclusions

The findings of the present study demonstrate that increased expression of acetylated α-tubulin, induced by HDAC6 inhibition, enhances the anti-tumor effect of eribulin in parental and eribulin-resistant TNBC cells. A combination of eribulin and HDAC inhibitor could be a potent and novel therapeutic strategy for TNBC patients.

List Of Abbreviations

EMT, epithelial–mesenchymal transition; ER, estrogen receptor; FEC, 5FU, epirubicin and cyclophosphamide; HDAC, histone deacetylase; MET, mesenchymal–epithelial transition; PR, patient response; RICO, ricolinostat; siRNA, small interfering RNA; TME, tumor microenvironment; TNBC, triple-negative breast cancer; VOR, vorinostat

Declarations

- Ethics approval and consent to participate: This study was approved by the Medical Ethics Committee on Clinical Investigation of Shinshu University (No. 3819). Written informed consent was obtained from all patients.
- Consent for publication: Not applicable
- Availability of data and material: The data the support the findings of this work are available from the authors upon reasonable request.
- Competing interests: The authors declare that they have no competing interests.
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- Authors' contributions: TO and KI contributed to the conception and design of the experiments, data analysis, interpretation, and drafted the manuscript TO, MO, and YH performed the experiments and contributed to the acquisition of the data. MH and TU evaluated the IHC of acetylated α-tubulin. All authors read and approved the final manuscript.
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Additional Files

Additional file 1: Table S1 (.docx)

IC_{50} for eribulin, vorinostat, and ricolinostat in the parental and eribulin-resistant TNBC cell lines

Additional file 2: Figure S1 (.pdf)

Sensitivity to vorinostat and ricolinostat in eribulin-resistant breast cancer cells and their parental cells.

Sensitivity to vorinostat (VOR) (a) and ricolinostat (Rico) (b) was assayed by using the WST assay. Each cell line with"/E"indicates an established eribulin-resistant cell line. Closed circles (●) indicate parental cells, whereas closed triangles (▲) indicate eribulin-resistant cells. The error bars represent the standard deviations of the values obtained; experiments were performed in triplicate.

Additional file 3: Figure S2 (.pdf)

Combinatory effect of eribulin and vorinostat on growth inhibition of triple-negative breast cancer (TNBC) cells *in vitro*.

The combinatory effect of eribulin (ERI) and vorinostat (VOR) on MDA-MB-231 and Hs578T cells was tested using WST assays. The experimental data were plotted on an isobologram. The dots located below, on, or above the diagonal line indicate synergistic, additive, and antagonistic effects, respectively.

Additional file 4: Figure S3 (.pdf)

Combined effect of eribulin and ricolinostat on MCF7 cells.

The combined effect of eribulin (ERI) and ricolinostat (RICO) on MCF7 cells *in vitro* was tested using WST assays. Sensitivity to ERI in the presence or absence of low doses of RICO was tested. Closed circles (●) indicate control, closed triangles (▲) indicate 0.2 μM RICO, closed squares (■) indicate 0.5 μM RICO. The error bars represent the standard deviations of the values obtained; experiments were performed in triplicate.

Additional file 5: Figure S4 (.pdf)

Cell growth after pre-treatment of vorinostat or ricolinostat.

Results of cell growth assays performed using MDA-MB-231 and Hs578T cells pre-treated with DMSO (control), vorinostat (VOR), or ricolinostat (RICO). The error bars represent the standard deviations of the values obtained; experiments were performed in triplicate.

Additional file 6: Table S2 (.docx)

H-score of acetylated α-tubulin before and after treatment of eribulin

Figures

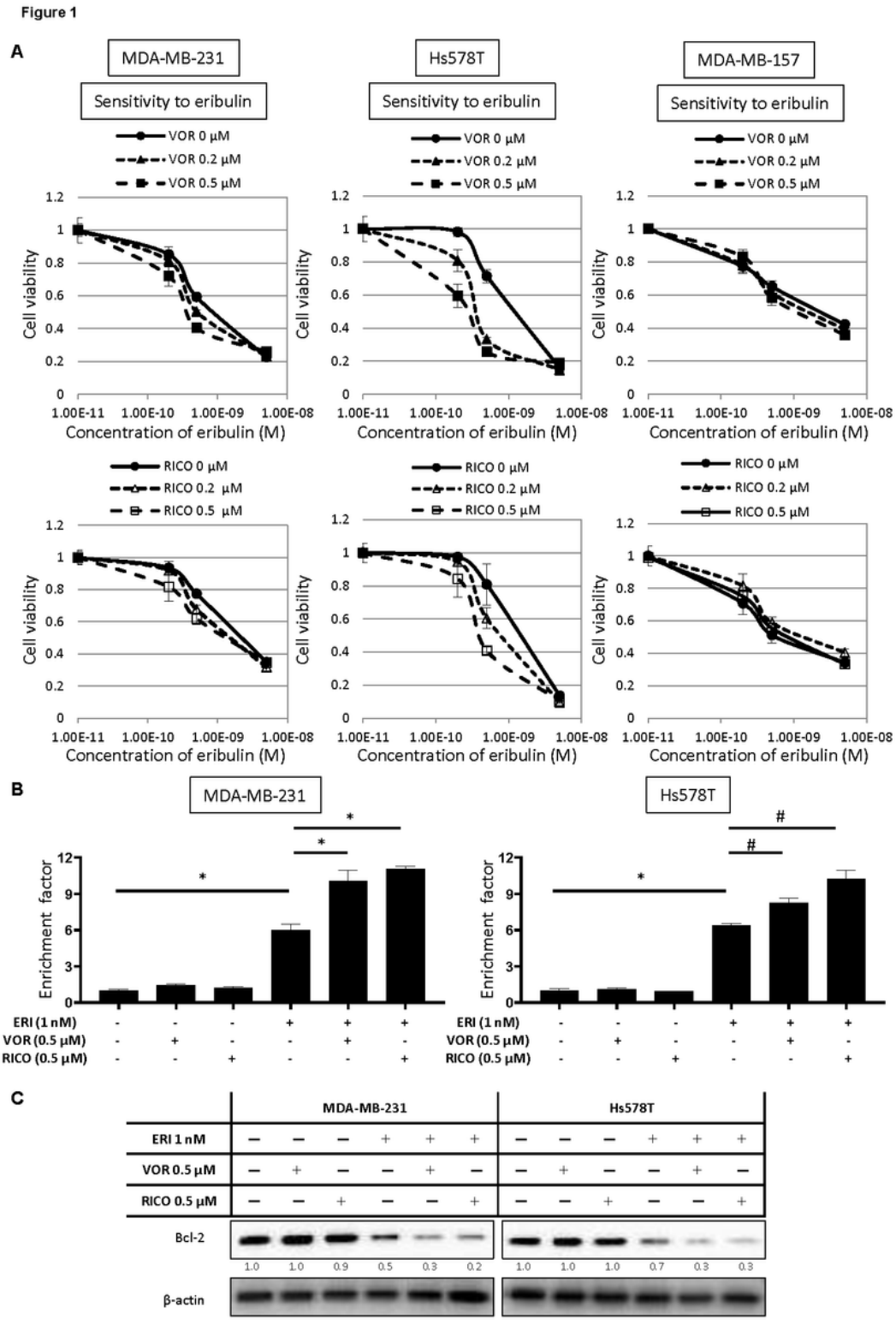

Figure 1

Combined effect of eribulin and vorinostat or ricolinostat on triple negative breast cancer growth inhibition. (a) The combined effect of eribulin (ERI) and vorinostat (VOR) or ricolinostat (RICO) on MDA-MB-231, Hs578T, and MDA-MB-157 cells in vitro was tested using WST assays. Sensitivity to ERI in the presence or absence of low doses of VOR (upper panels for each cell line) and RICO was tested (lower panels for each cell line). Closed circles (●) indicate control, closed triangles (▲) indicate 0.2 μM VOR,

closed squares (■) indicate 0.5 μM VOR, open triangles (△) indicate 0.2 μM RICO, and open squares (□) indicate 0.5 μM RICO. The error bars represent the standard deviations of the values obtained; experiments were performed in triplicate. (b) Effect of ERI, VOR, RICO, and a combination of ERI and VOR or RICO on apoptosis in MDA-MB-231 and Hs578T cells. The Cell Death Detection ELISA plus kit was used to quantify apoptosis in MDA-MB-231 and Hs578T cells in the presence of either VOR (0.5 μM), RICO (0.5 μM), ERI (1 nM), or a combination of ERI (1 nM) and VOR (0.5 μM) or RICO (0.5 μM) for 48 h. The error bars represent the standard deviations of the values obtained; experiments were performed in triplicate. (c) Expression of Bcl-2 in MDA-MB-231 and Hs578T cells treated with VOR (0.5 μM), RICO (0.5 μM), ERI (1 nM), or a combination of ERI (1 nM) and VOR (0.5 μM) or RICO (0.5 μM) for 48 h. β-actin was used as a loading control. The experiments were repeated independently at least three times, and one representative blot is provided in the figures.

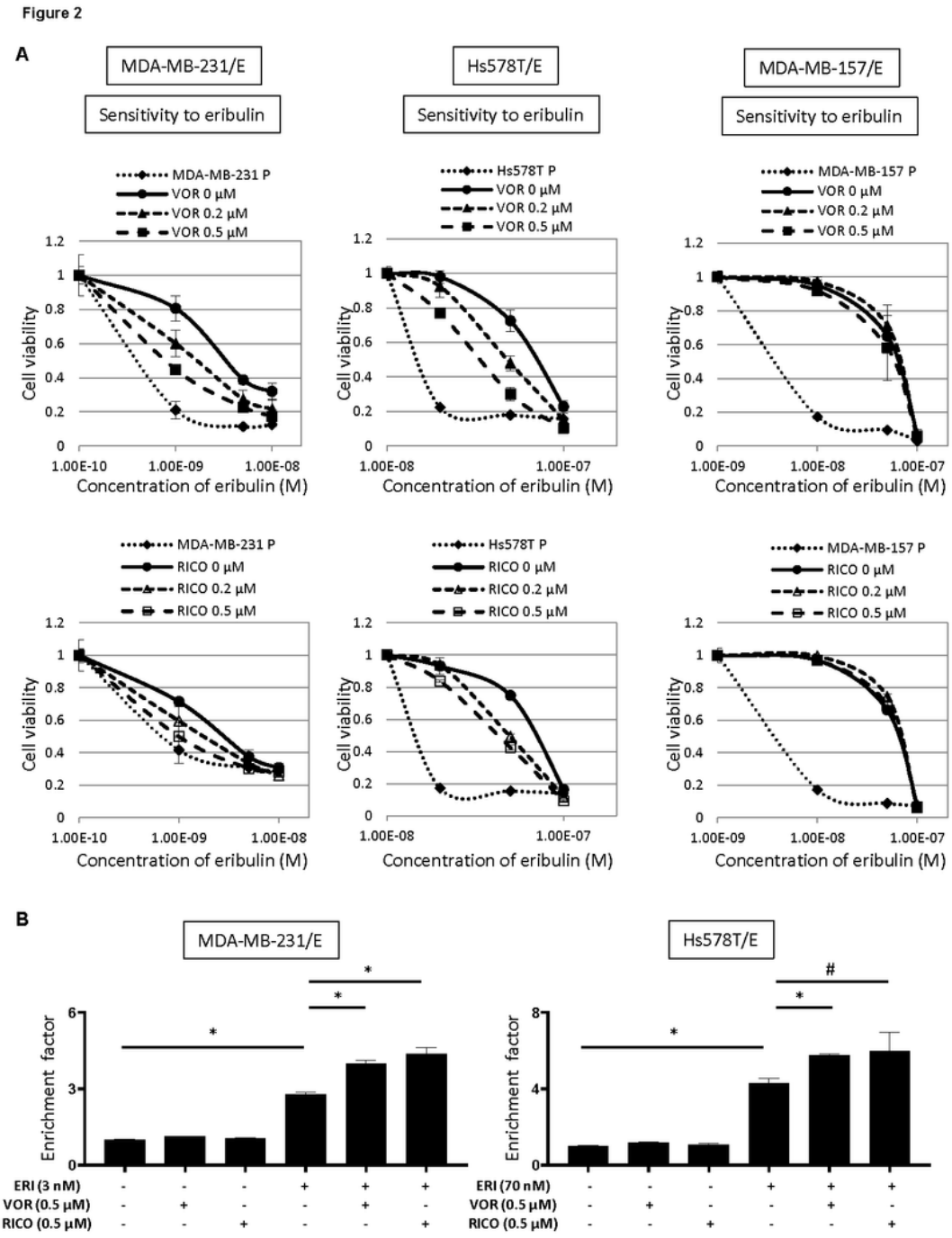

Figure 2

Combined effect of eribulin and vorinostat or ricolinostat on eribulin-resistant triple-negative breast cancer cell growth. The combined effect of eribulin (ERI) and vorinostat (VOR) or ricolinostat (RICO) on eribulin-resistant MDA-MB-231, Hs578T, and MDA-MB-157 cells (MDA-MB-231/E, Hs578T/E, MDA-MB-157/E) was tested in vitro using WST assays. (a) Sensitivity to ERI in the presence or absence of low doses of VOR (upper panels for each cell line) and sensitivity to ERI in the presence or absence of low

doses of RICO (lower panels for each cell line). Closed diamonds (◆) indicate parental cells. Closed circles (●) indicate control, closed triangles (▲) indicate 0.2 μM VOR, closed squares (■) indicate 0.5 μM VOR, open triangles (△) indicate 0.2 μM RICO, and open squares (□) indicate 0.5 μM of RICO. The error bars represent the standard deviations of the values obtained; experiments were performed in triplicate. (b) Effect of ERI, VOR, RICO, and a combination of ERI and VOR or RICO on apoptosis in MDA-MB-231/E and Hs578T/E cells. The Cell Death Detection ELISA plus kit was used to quantify the apoptosis of MDA-MB-231/E and Hs578T/E cells in the presence of either VOR (0.5 μM), RICO (0.5 μM), ERI (3 nM for MDA-MB-231/E, 70 nM for Hs578T/E), or a combination of ERI (3 nM for MDA-MB-231/E, 70 nM for Hs578T/E) and VOR (0.5 μM) or RICO (0.5 μM) for 48 h. The error bars represent the standard deviations of the values obtained; experiments were performed in triplicate.

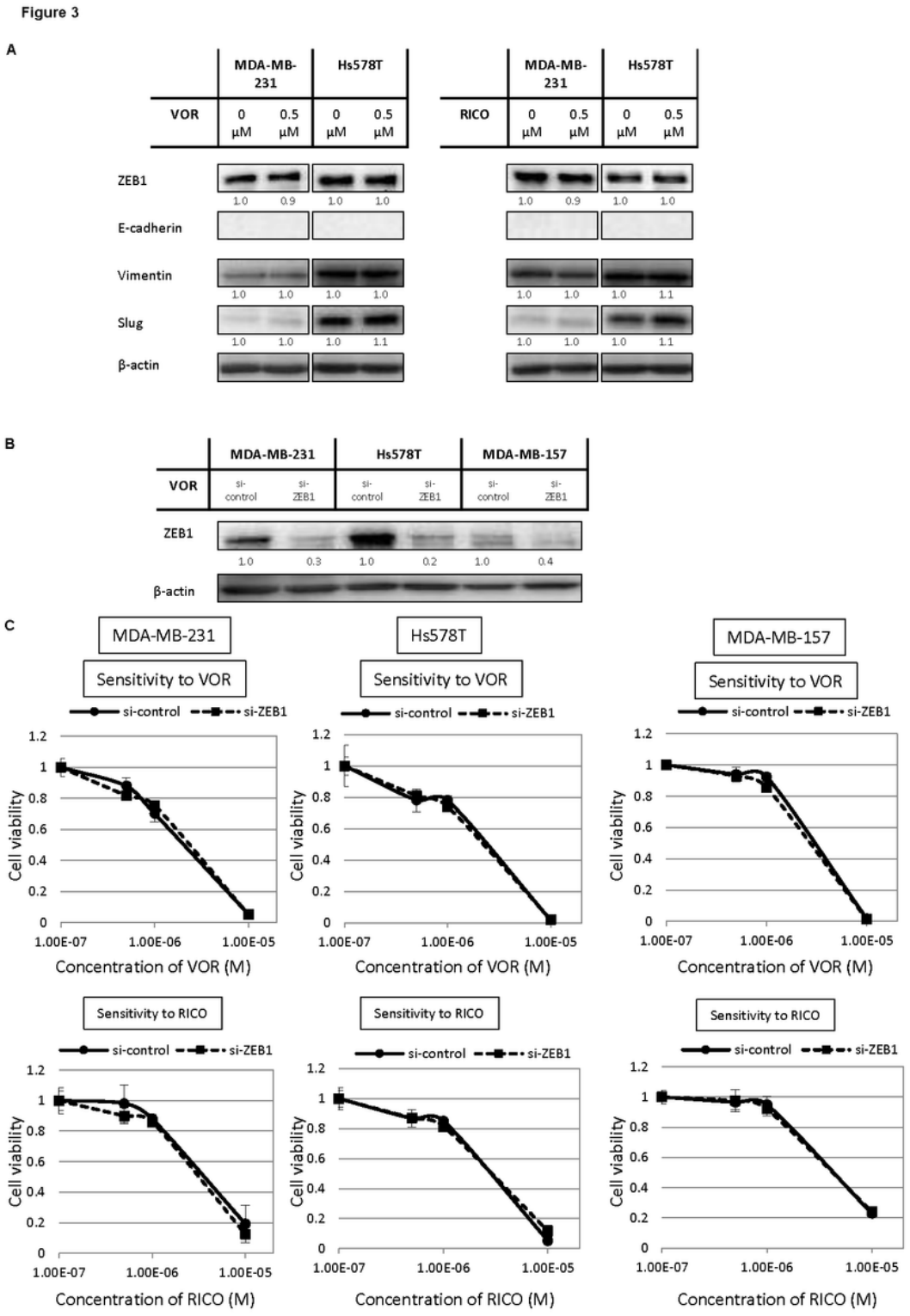

Figure 3

Effects of HDAC inhibitors on the expression of epithelial/mesenchymal markers in triple-negative breast cancer cells. (a) MDA-MB-231, Hs578T, and MDA-MB-157 cells were treated with 0.5 μM vorinostat (VOR) or 0.5 μM ricolinostat (RICO) for 48 h. The expression of epithelial/mesenchymal markers was examined by western blotting. Representative results of western blot analyses are shown. β-actin was used as a loading control. The expression of ZEB1 was inhibited by siRNA and sensitivity to VOR or RICO was

tested using WST assays. The cells were transfected with siRNA targeting ZEB1 (si-ZEB1) or control siRNA (si-control). Twenty-four hours after transfection, protein was extracted and 4 × 103 cells/well were cultured in 96-well tissue culture plates and incubated for 72 h after adding stepwise dilutions of VOR or RICO. (b) The expression of ZEB1 in the cells was analyzed by western blotting. (c) Sensitivity to ERI was measured using WST assays. Closed circles (●) indicate cells transfected with control siRNA, and closed squares (■) indicate cells transfected with si-ZEB1. The error bars represent the standard deviations of the values obtained; experiments were performed in triplicate.

Figure 4

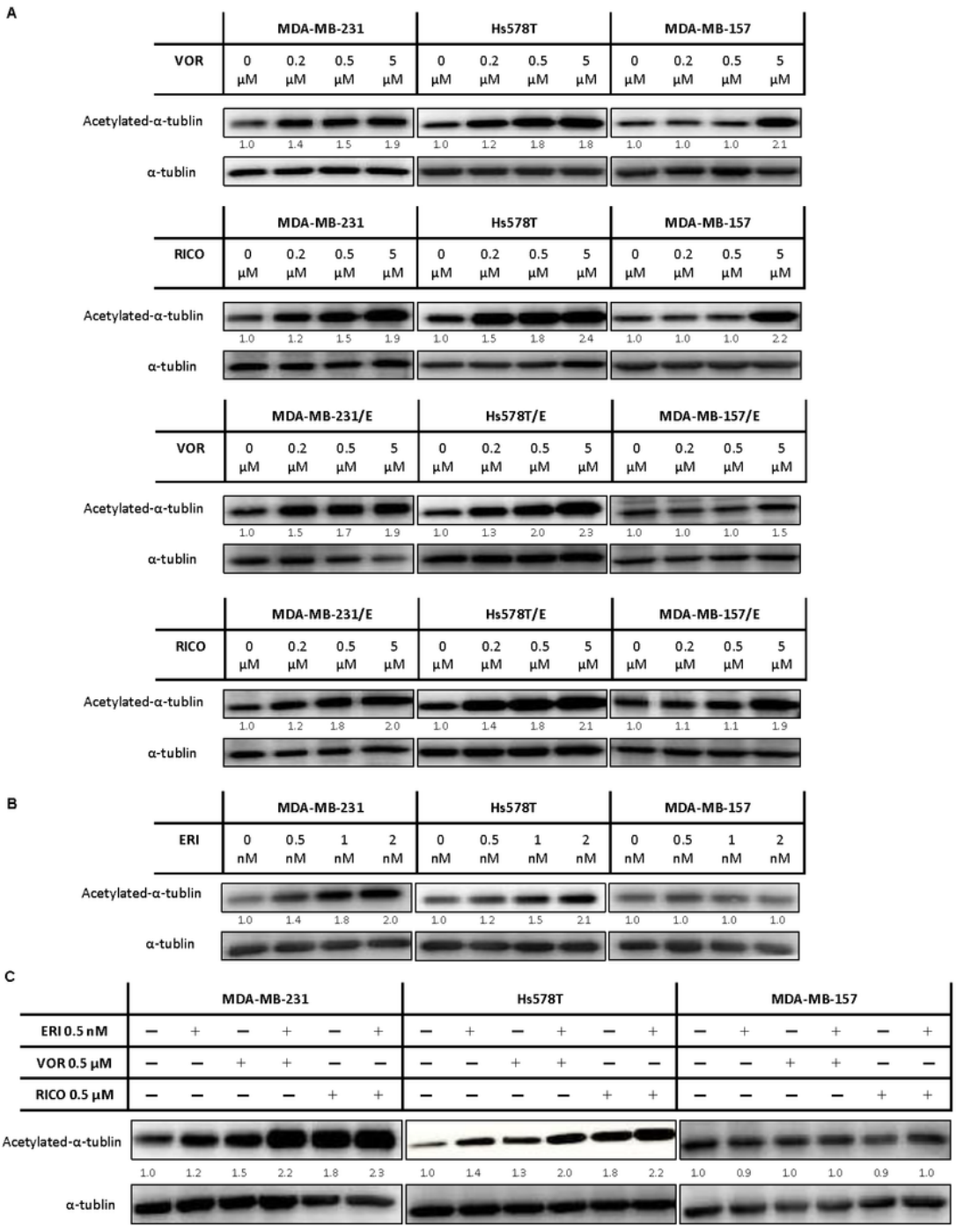

Figure 4

Expression of acetylated α-tubulin in triple-negative breast cancer cell lines. The expression of acetylated α-tubulin was examined by western blotting. Representative results of western blot analyses are shown. α-tubulin was used as a loading control. The experiments were repeated independently at least three times, and one representative blot is provided in the figures. (a) Expression of acetylated α-tubulin in MDA-MB-231, Hs578T, MDA-MB-157, MDA-MB-231/E, Hs578T/E, and MDA-MB-157/E cells treated with vorinostat (VOR; 0.2, 0.5, and 5 μM) or ricolinostat (RICO; 0.2, 0.5, and 5 μM) for 48 h. (b) Expression of acetylated α-tubulin in MDA-MB-231, Hs578T, and MDA-MB-157 cells treated with eribulin (ERI; 0.5, 1, and 2 nM) for 48 h. (c) Expression of acetylated α-tubulin in MDA-MB-231, Hs578T, and MDA-MB-157 cells treated with eribulin (ERI; 0.5 nM), vorinostat (VOR; 0.5 μM), ricolinostat (RICO; 0.5 μM), and a combination of these (ERI 0.5 nM + VOR 0.5 μM, and ERI 0.5 nM + RICO 0.5 μM) for 48 h.

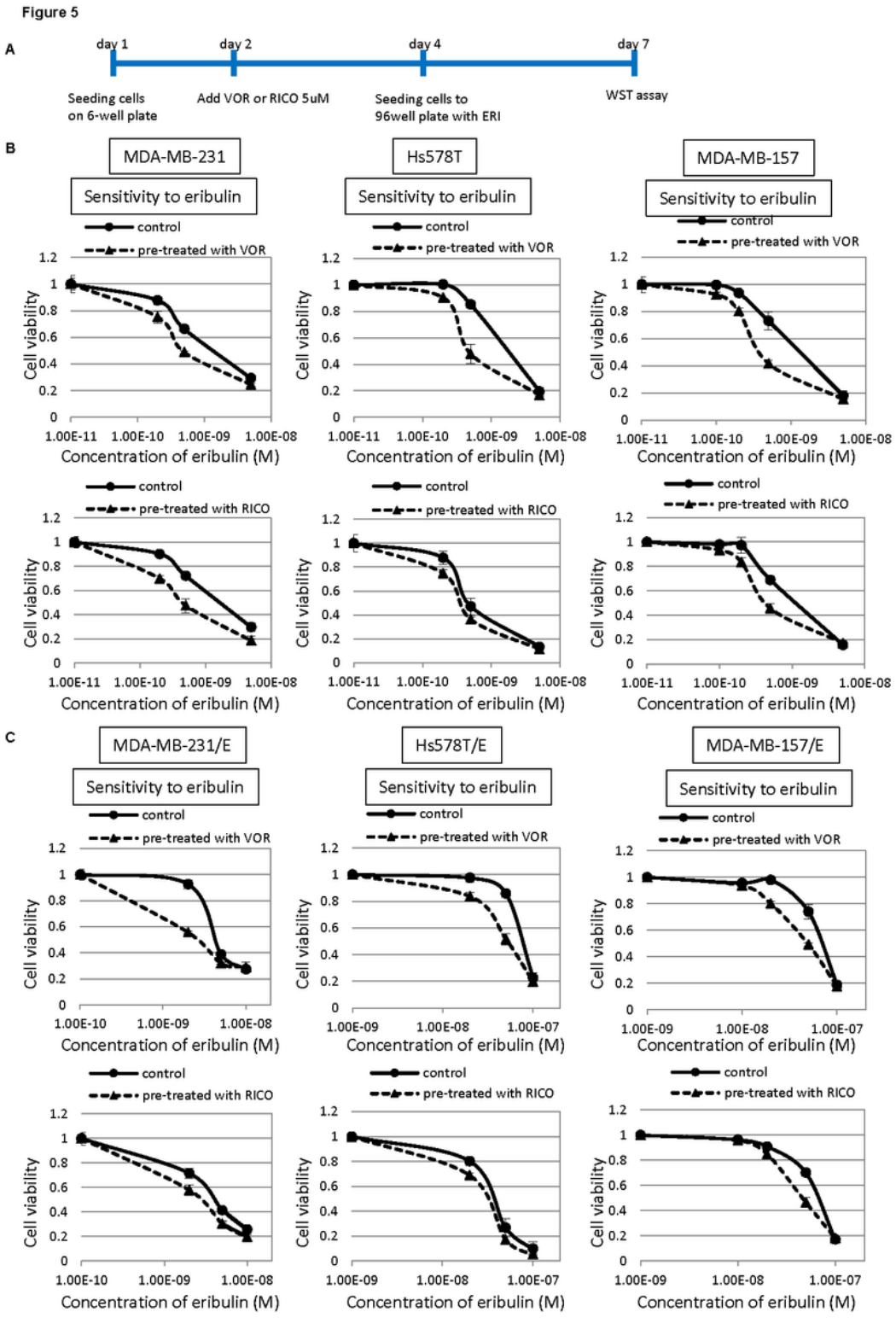

Figure 5

Vorinostat or ricolinostat effects on eribulin sensitivity in triple-negative breast cancer cells and eribulin-resistant derivatives. Sensitivity to eribulin (ERI) was tested in cells pre-treated with vorinostat (VOR) or ricolinostat (RICO) using MDA-MB-231, Hs578T, MDA-MB-157, MDA-MB-231/E, Hs578T/E, and MDA-MB-157/E cells. (a) Schematic representation of the experiment. (b) Sensitivity to ERI in MDA-MB-231, Hs578T, MDA-MB-157, MDA-MB-231/E, Hs578T/E, and MDA-MB-157/E cells was measured in cells pre-

treated with VOR (5 μM) or RICO (5 μM) for 48 h. Cells pre-treated with DMSO alone were used as a control. The error bars represent the standard deviations of the values obtained; experiments were performed in triplicate.

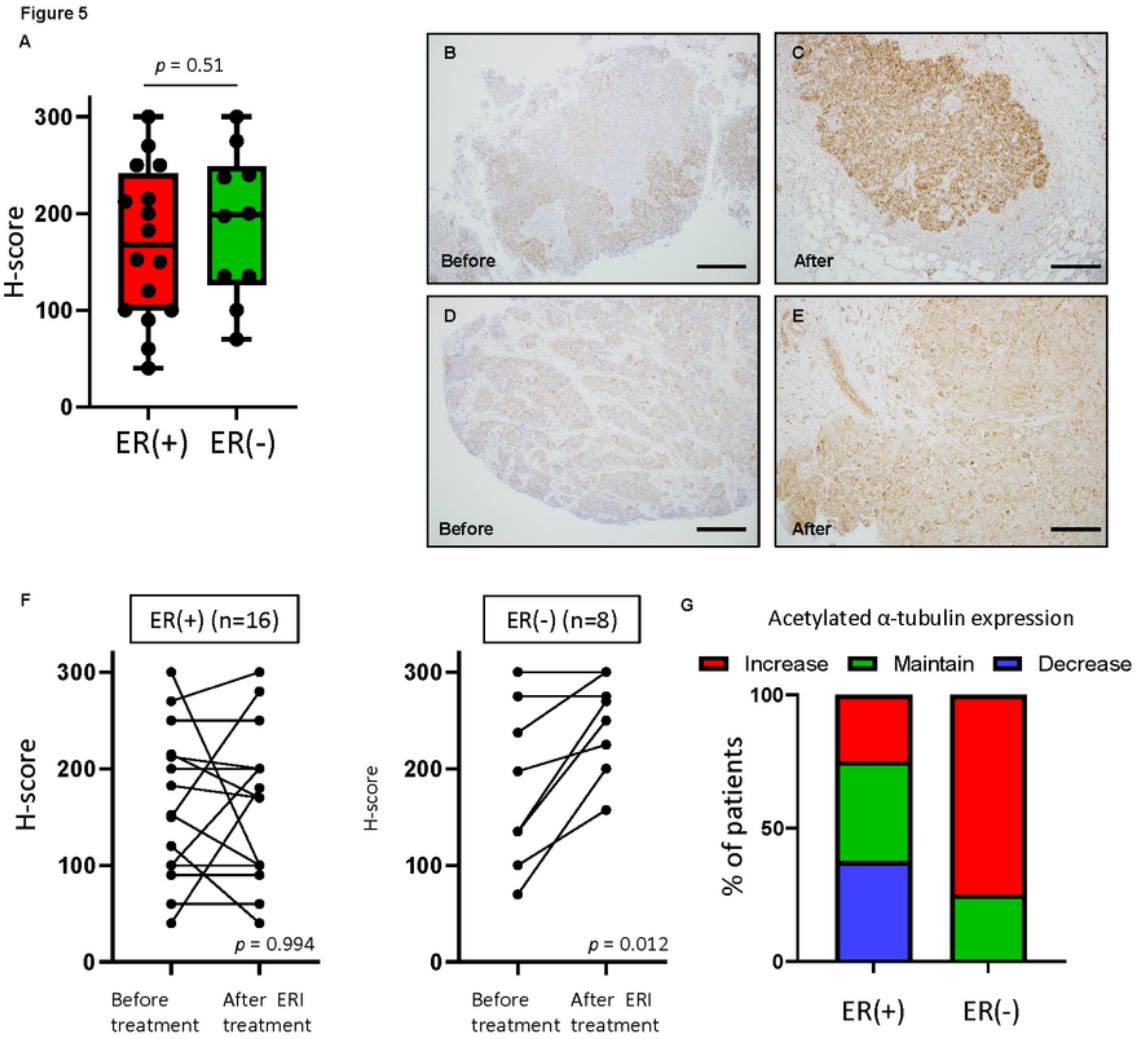

Figure 6

Expression of acetylated α-tubulin in clinical specimens. The expression of acetylated α-tubulin was examined by immunohistochemical staining for acetylated α-tubulin using clinical specimens from

breast cancer patients who underwent neoadjuvant chemotherapy comprising eribulin. (a) Box-and-whisker plot with individual H-score values of acetylated α-tubulin in estrogen receptor (ER)-negative and ER-positive breast cancer. p = 0.51 by Mann-Whitney U-test. (b–f) Representative photographs of two cases based on immunohistochemistry (×100) for acetylated α-tubulin. Scale bars = 50 μm. Shown are before the treatment of case 2 (b) and 3 (c) and after the eribulin treatment of case 2 (d) and case 3 (e) described in Supplementary Table 2; (f) Altered acetylated α-tubulin expression in ER-negative and ER-positive breast cancer. p = 0.994 for ER-positive and p = 0.012 for ER-negative by paired two-tailed t-test. (g) Distribution of patients who showed decrease, maintain, and increase of acetylated α-tubulin expression in ER-negative and ER-positive breast cancer.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- ObaetalSupplementaryTable2.docx
- ObaetalSupplementaryFigures.pdf
- ObaetalSupplementaryTable1.docx