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Up-regulation of *HOXB* cluster genes are epigenetically regulated in tamoxifen-resistant MCF7 breast cancer cells

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Tamoxifen (TAM) is commonly used to treat estrogen receptor (ER)-positive breast cancer. Despite the remarkable benefits, resistance to TAM presents a serious therapeutic challenge. Since several HOX transcription factors have been proposed as strong candidates in the development of resistance to TAM therapy in breast cancer, we generated an *in vitro* model of acquired TAM resistance using ER-positive MCF7 breast cancer cells (MCF7-TAMR), and analyzed the expression pattern and epigenetic states of *HOX* genes. *HOXB* cluster genes were uniquely up-regulated in MCF7-TAMR cells. Survival analysis of *in slico* data showed the correlation of high expression of *HOXB* genes with poor response to TAM in ER-positive breast cancer patients treated with TAM. Gain- and loss-of-function experiments showed that the overexpression of multi *HOXB* genes in MCF7 renders cancer cells more resistant to TAM, whereas the knockdown restores TAM sensitivity. Furthermore, activation of *HOXB* genes in MCF7-TAMR was associated with histone modifications, particularly the gain of H3K9ac. These findings imply that the activation of *HOXB* genes mediate the development of TAM resistance, and represent a target for development of new strategies to prevent or reverse TAM resistance. [BMB Reports 2018; 51(9): 450-455]

INTRODUCTION

Estrogen receptor (ER)-positive breast cancer constitutes almost 70% of the total number of breast cancer cases (1) and is likely to respond favorably to endocrine therapies such as tamoxifen (TAM) and aromatase inhibitors (AI). These drugs act by competitively binding to ER or by preventing the systemic conversion of testosterone to estrogen (2, 3). Even though endocrine therapy has been proven relatively safe and has significant therapeutic effects, a third of women treated with TAM for 5 years will have a relapse of the disease within 15 years (4). For decades, based on extensive studies investigating the molecular mechanisms of resistance to endocrine therapy, several important factors, such as *ER* gene (*ESR1*) mutations, epigenetic aberrations, or crosstalk between ER and growth factor signaling, have now come to our attention (5, 6). However, the investigation and discovery of novel biomarkers are still strongly required to predict responses to TAM resistance and develop personalized treatment strategies.

HOX are highly conserved transcription factors playing crucial roles in development, and several HOX genes are associated with cancer (7-9). Many previous studies have demonstrated abnormal HOX expression in breast cancer tissues and culture cells, and furthermore, their roles in tumorigenesis and metastasis of breast cancer (10-14). In addition, many HOX genes, such as HOXB5, HOXB7, HOXB13, HOXC10, HOXC11, and non-coding RNAs in HOX clusters are associated with endocrine resistance to breast cancer via repression of ER expression or activation of receptor tyrosine kinase pathways (15-19). However, the expression patterns and the functional characterization of the whole HOX cluster genes in TAM-resistant breast cancer cells have not been investigated.

Here, we generated an *in vitro* TAM resistance model using ER-positive MCF7 breast cancer cells (MCF7-TAMR), and analyzed the expression patterns of *HOX* genes as well as their epigenetic status. The correlation of *HOX* gene expression in breast cancer patients with survival has been further examined using publicly available datasets of human breast cancers. In addition, we investigated the functional impact of *HOX* gene expression on TAM sensitization and resistance by conducting gain-of-function and loss-of-function experiments.

RESULTS

HOXB genes are up-regulated in MCF7-TAMR cells

We generated an *in vitro* TAM-resistant MCF7 cell line (MCF7-TAMR) and confirmed the resistance to TAM in a concentration-dependent manner (Fig. 1A). MCF7 and MCF7-

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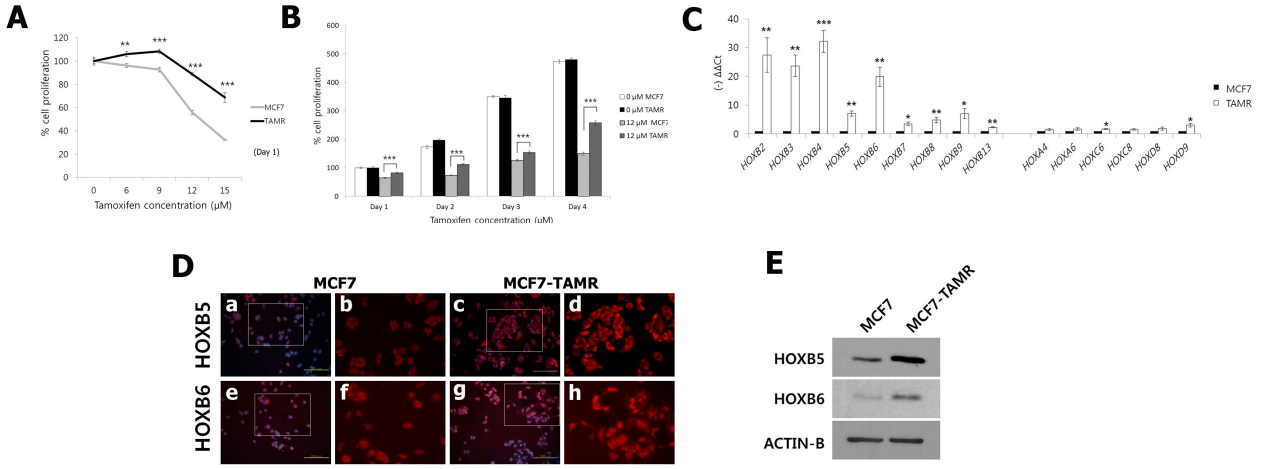

Fig. 1. Up-regulation of *HOXB* genes in MCF7-TAMR cells. (A) Cell viability test using MCF7 and MCF7-TAMR cells on day 1 after treatment with the indicated concentration of TAM. (B) Cell viability was measured from day 1 to day 4 after treatment with 0 and 12 μM TAM. (C) Real-time PCR analysis of whole *HOXB* genes, *HOXA4*, *HOXA6*, *HOXC6*, *HOXC8*, *HOXD8*, and *HOXD9* in MCF7 and MCF7-TAMR cells. (A-C) Data are presented as mean ± SEM from at least three independent assays; *P < 0.05; **P < 0.01; ***P < 0.001 vs. MCF7, by t-test. (D) Immunocytochemisry of HOXB5 (a-d) and HOXB6 (e-h) in MCF7 and MCF7-TAMR cells. The images in a, c, e, and g were overlaid with DAPI counterstain (×200), and boxed regions were magnified in b, d, f, and h, respectively (×400). (E) Western blots of HOXB5 and HOXB6 in MCF7 and MCF7-TAMR cells.

TAMR showed similar cell proliferation rates in normal medium, however, MCF7-TAMR cells showed increased time-dependent TAM resistance until day 4 (Fig. 1B). To investigate the altered *HOX* gene expression in TAM-resistant cells, we analyzed the levels of expression in 39 *HOX* genes in parental MCF7 and -TAMR cells. We found that the entire *HOXB* cluster genes (*HOXB2-B13*) were significantly up-regulated in MCF7-TAMR cells (Fig. 1C). However, only minor changes were detected in *HOXA*, *HOXC* and *HOXD* cluster genes (Fig. 1C and Supplementary Fig. 1). Representatively, the up-regulation of HOXB5 and HOXB6 expression in MCF7-TAMR cells was also confirmed at the protein level by immunocytochemistry and Western blotting analysis (Fig. 1D and E). To investigate whether the up-regulation of any *HOXB* gene is linked to the expression of other nearby *HOXB* genes, we analyzed publicly available datasets related to human cancer. We found that each *HOXB* gene is co-expressed with other *HOXB* genes in human breast cancer patients (Supplementary Table 1) suggesting that each *HOXB* gene expression is highly correlated with nearby *HOXB* genes.

Up-regulation of HOXB mid-cluster genes is associated with poor clinical outcome in ER-positive breast cancer

To assess the degree of survival of breast cancer patients depending on their *HOXB* expression levels, distant metastasis-free survival (DMFS) curves were plotted and compared using the Kaplan-Meier survival analysis and the log-rank test. There was no significant difference in the DMFS curves between the *HOXB*-high and -low groups in all patients (Fig. 2A). However, when analyzed only with ER-positive patients treated with TAM for therapy, DMFS was significantly lower in the *HOXB*-high groups (Fig. 2B). Among the *HOXB* genes, *HOXB5*, *B6* and *B7* genes, in particular, showed a significant difference in DMFS between high- and low expression groups (Fig. 2C-E). Due to the lack of dataset, the impact of *HOXB4* expression on breast cancer survival was not determined. Multigene prognostic tests also confirmed that the high expression of mid-cluster *HOXB* genes (*HOXB5-B7*) was associated with poor survival of patients with ER-positive breast cancer treated with TAM, compared with the anterior (*HOXB1-B3*) or posterior *HOXB* genes (*HOXB8-B13*) (Fig. 2F-H). In contrast, there were no significant differences in DMFS curves between the high- and -low expression groups of *HOXA* and *HOXC* cluster genes in all patients and ER-positive patients treated with TAM (Supplementary Fig. 2A-D). In case of *HOXD* cluster genes, the high expression was associated with poor prognosis; however, the expression levels were not elevated in MCF7-TAMR cells (Supplementary Fig. 1, and 2E and F).

Overexpression of mid-cluster HOXB gene induced TAM resistance in MCF7

Previous studies showed that several HOXB genes, such as HOXB5, HOXB7, and HOXB13, play a role in TAM resistance individually (16, 17, 19). However, there is no evidence to suggest whether the overexpression of multiple *HOXB* genes leads to additive or synergistic effects. To explore whether the combinatorial overexpression of multiple *HOXB* genes

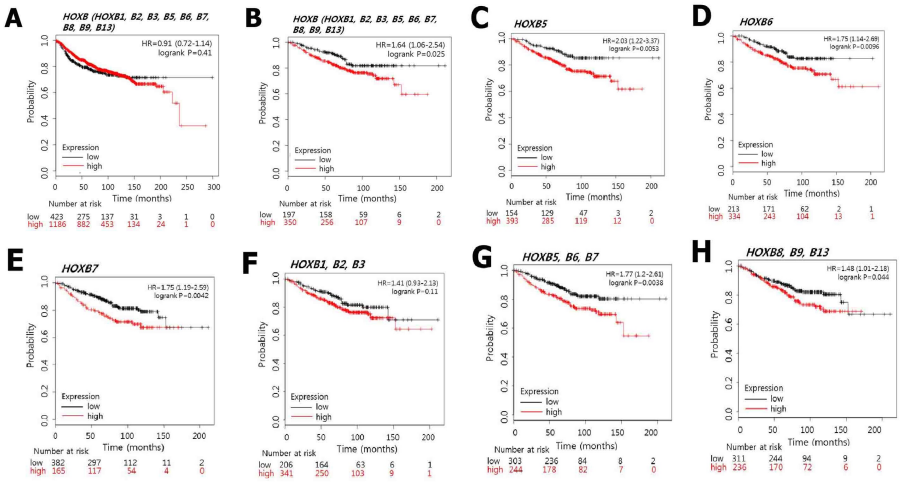

Fig. 2. Kaplan-Meier analysis of DMFS in breast cancer patients based on *HOXB* expression. (A, B) Survival rate was compared between groups of high and low *HOXB* expression in (A) all patients and in (B) ER-positive patients treated with TAM therapy. (C-E) DMFS of ER-positive patients who received TAM monotherapy were stratified by (C) *HOXB5*, (D) *HOXB6*, and (E) *HOXB7* expression level. (F-H) In multigene analysis, ER-positive breast cancer patients were divided into two groups according to the expression levels of (F) anterior *HOXB* (*HOXB1-B3*), (G) middle *HOXB* (*HOXB5-B7*), and (H) posterior *HOXB* (*HOXB8-B13*), and analyzed for DMFS.

induces higher TAM resistance compared with that of a single *HOXB* gene, we performed cell proliferation assay in the presence of TAM at 24 h post-transfection. Mid-cluster *HOXB* genes (*HOXB5*, *B6* and *B7*) were used in this experiment because of their potential roles in TAM resistance. The overexpression of each *HOXB* gene after transfection was confirmed by RT-PCR (Fig. 3A and C). The co-expression of *HOXB5*, *HOXB6* and *HOXB7* significantly increased the cell proliferation rate in the presence of TAM, compared with a single *HOXB* gene (Fig. 3B). The proliferation of MCF7 cells transfected with any combination of two *HOXB* genes (*B5*/*B6*, *B6*/*B7* and *B5*/*B7*) was slightly higher than the values of control cells. Furthermore, the overexpression of three mid-cluster *HOXB* genes (*HOXB5-B7*) led to the highest cell proliferation in the presence of TAM (Fig. 3D). Loss-of-function studies using *HOXB4*, instead of *HOXB7*, as siRNA target together with *HOXB5* and *B6* were performed because the up-regulation of *HOXB4* in TAMR cells (Fig. 1C) was considered much more relevant than the altered expression of *HOXB7*. In a series of knockdown experiments, the reduced mRNA expression of *HOXB4*, *B5*, and *B6* was confirmed (Fig. 3E, G, and I). Individual sets of MCF7-TAMR cells transfected with siRNA for a single gene showed slightly reduced cell proliferation in the presence of TAM, however, the effect was more pronounced when three *HOXB* genes were silenced simultaneously (Fig. 3F). The same patterns were observed when at least two genes (*HOXB4*/*B6* or *HOXB5*/*B6*) were silenced alone or combined (Fig. 3H and J).

Activation of *HOXB* gene expression in MCF7-TAMR cells is epigenetically regulated

The expression of *HOX* genes is tightly regulated by epigenetic mechanisms during normal development and cancer (20, 21). Since the *HOXB* cluster genes were generally upregulated as a whole in MCF7-TAMR cells, we expected that different epigenetic states can be generated in the *HOXB* locus during the transition to acquired TAM resistance. To test whether histone modifications at the *HOXB* locus serve as markers of differential gene expression in MCF7 and MCF7-TAMR cells, we performed ChIP analysis. Based on various sources of ChIP-Seq data in MCF7 cells deposited into the UCSC database, we determined the amplicon sites for the promoter region of each *HOXB* gene (Fig. 4A). ChIP-qPCR results revealed that increased H3K9ac at the proximal promoter region of each *HOXB* gene was associated with decreased H3K27me3 expression, as the transcript levels increased in MCF7-TAMR cells (Fig. 4B).

DISCUSSION

In this study, we showed that the *HOXB* cluster genes are activated as a whole in TAM-resistant MCF7 breast cancer cells. The results of survival analysis indicate that the elevated expression of *HOXB*, especially mid-cluster *HOXB*, is associated with poor survival in patients with ER-positive breast cancer who are treated with TAM therapy. Our functional studies via overexpression and knockdown experiments clearly confirm that the mid-cluster *HOXB* genes contribute to TAM resistance, and the activation of *HOXB* gene expression is mediated by epigenetic mechanisms.

HOX genes play a diverse role in adult tissues as well as during embryogenesis under endocrine control. Therefore, endocrine-HOX signaling has important clinical and molecular implications for human physiology and pathology (22). In human endometrium, HOX genes are dynamically expressed under the control of steroid hormones, and the decreased HOXA10 expression represents a possible mechanism of progesterone resistance in endometriosis (23). Evidence increasingly supports the contribution of HOX genes in endocrine therapy-resistant breast cancer (15). Although

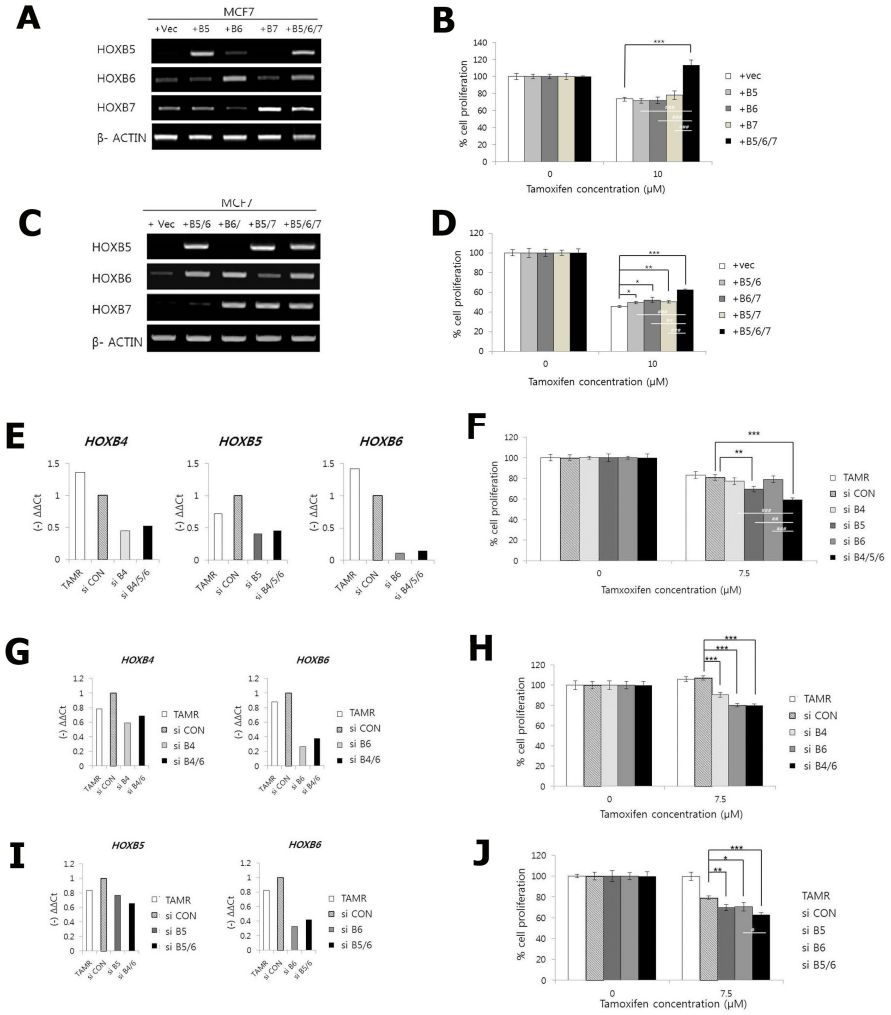

Fig. 3. Effects of mid-cluster *HOXB* genes expression in TAM sensitivity. (A, B) MCF7 cells were transfected with vector only (MCF7:Vec) and single plasmid DNA (MCF7:HOXB5, MCF7:HOXB6, MCF7:HOXB7), or co-transfected with multiple plasmid DNAs simultaneously (MCF7:HOXB5/6/7); (C, D) co-transfected with 2-3 plasmid DNAs for *HOXB5*, *B6*, and *B7* in different combinations (MCF7:HOXB5/6, MCF7:HOXB6/7, MCF7:HOXB5/7 and MCF7:HOXB5/6/7). (A and C): RT-PCR analysis of *HOXB5*, *B6*, and *B7* in each transfectant using *β-ACTIN* as an internal control. (B and D) All transfectants were treated with 10 μM of TAM and the cell proliferation rate was measured by CCK-8 assay on day 1. (E, F) MCF7-TAMR cells were transfected with siRNAs for control, *HOXB4*, *HOXB5*, *HOXB6* (TAMR-siCON, TAMR-siHOXB4, TAMR-siHOXB5, TAMR-siHOXB6), or co-transfected simultaneously (TamR-siHOXB4/5/6); (G, H) *HOXB4*, *HOXB6;* (I, J) *HOXB5*, *HOXB6*. (E, G and I): Q-PCR analysis for each HOXB expression. (F, H, and J): CCK-8 assay for cell proliferation rate on day 1. Data are presented as mean ± SEM from at least three independent assays. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs*. MCF7: Vec or TAMR-siCON, by t-test. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs*. MCF7:HOXB5/6/7 or TAMR-siHOXB4/5/6 (in F) or TAMR-siHOXB5/6 (in J), by t-test.

several HOX genes, such as HOXB7 and HOXB13, in TAM resistance have been well characterized (17, 19), cooperative and/or synergistic actions of clustered genes in TAM resistance have not been reported. Notably, the driving forces, which induce dysregulated gene expression in cancer, include gene copy number variations, epigenetic regulation, and coordinated actions of transcription factors. In this study, we reviewed The Cancer Genome Atlas (TCGA) breast cancer data to delineate the association between copy number amplification and *HOX* gene expression. We found a lack of positive correlation between the expression of HOXB mRNA and copy number in breast cancer patient samples (Supplementary Fig. 3), suggesting a rare functional relevance of *HOXB* amplification. In support, the copy number assay for each *HOXB5* and *HOXB6* gene locus in cell lines demonstrated a lack of *HOX* amplification in MCF7-TAMR cells compared with MCF7 (Supplementary Fig. 4). These data strongly exclude the possibility that the increase in copy number may have contributed to the increased expression. Thus, our proposition that the *HOXB* genes are up-regulated epigenetically in MCF7-TAMR cells seems more persuasive. Further, our findings support that consecutive *HOXB* genes mediate TAM resistance.

Nevertheless, additional studies are needed to explain the causal mechanism of action. Several HOX proteins sharing a high degree of homology are likely to share common molecular targets, probably via common signaling pathways. Further, non-coding RNAs such as miRNAs and long non-coding RNAs (lncRNAs) located in the HOX cluster regulate coordinated multi-gene expression during the development of TAM resistance. Several studies have shown that miRNAs are associated with drug resistance and

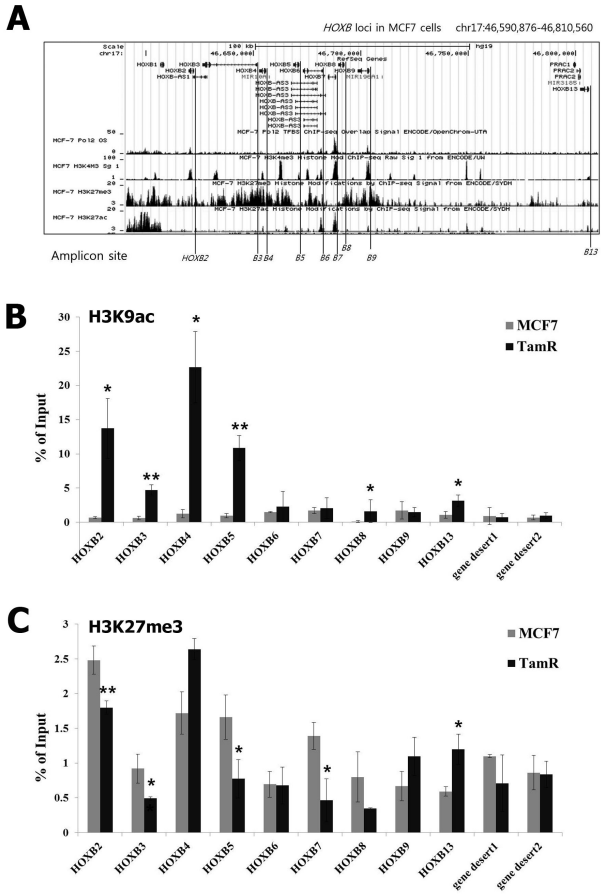

Fig. 4. Histone modifications in MCF7 and MCF7-TAMR cells. (A) Screenshot of the *HOXB* cluster in UCSC genome browser on Human (GRCh37/hg19) Assembly (http://genome.ucsc.edu/). Amplicons for each *HOXB* gene are marked. ChIP-seq data for Pol2 (GSM822295), H3K4me3 (GSM945269), H3K27me3 (GSM970218) and H3K27ac (GSM945854) were uploaded as custom tracks in the browser. (B) ChIP-qPCR analysis along the *HOXB* cluster. Immunoprecipitated and input DNAs were derived using anti-H3K9ac and anti-H3K27me3 antibodies. ChIPed DNAs for IgG were used as negative controls. Primers for gene desert regions were used as negative controls (gene desert #1: Chr 16: 62,732,615-62,732,729; gene desert #2: Chr 20: 56,403,369-56,403,521). Data are expressed as the percentage of input, after normalization with IgG; *P $<$ 0.05, **P $<$ 0.01.

prediction of outcome and therapeutic response in breast cancer (24, 25). MiR-196a and miR-10a, which are located in the *HOXB* loci, in particular, mediate endocrine resistance (15, 26). Although the potential *cis*- or *trans*-actions of these non-coding RNAs in multi-gene regulation, particularly in breast cancer cells resistant to TAM, remain to be characterized, this possibility must be taken into consideration.

Meanwhile, several studies have reported that epigenetic alterations are associated with drug resistance in breast cancer (27, 28). In this study, we showed the up-regulation of *HOXB* gene expression in TAM-resistant breast cancer cells, with accompanying changes in histone modification along the whole cluster. Consistent with these findings, a previous study of Barrett's esophagus (BE) showed that the activation of three consecutive mid-cluster *HOXB* genes (*HOXB5-B7*) is epigenetically correlated with H3K27me and AcH3 levels and chromatin compaction (29). Several human diseases including cancer are associated with altered high-order chromatin structure (30). Particularly, *HoxBlinc* (hoxb locus-associated long intergenic non-coding RNA) located between *hoxb4* and *-b5*, has been known to regulate *hoxb* gene transcription by modulating local chromatin alterations during murine embryonic stem cell differentiation (31). Considering the role of *HoxBlinc* RNA during embryogenesis in recruiting Set1/MLL complexes containing methyltransferase activity for H3K4 and its effect on 3D chromatin architecture, it is reasonable to expect that a large variety of lncRNAs modulate chromatin structure and gene expression in cancer cells.

In conclusion, we have shown the simultaneous activation of *HOXB* genes in TAM-resistant breast cancer cells and demonstrated the functional roles of mid-cluster *HOXB* genes in sensitizing and desensitizing TAM effect. These findings not only provide insight into the cumulative effect of *HOXB* gene expression in TAM resistance, but may also facilitate the development of new therapeutic approaches to re-sensitize resistant tumors by identifying factors that control the *HOXB* gene clusters.

MATERIALS AND METHODS

See Supplementary information.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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