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 situ 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, 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) Immunocytochemistry 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
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 ampiclon 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
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
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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.

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.

ACKNOWLEDGEMENTS

We thank Clara Yuri Kim for editing the manuscript. This research was supported by the National Research Foundation (NRF) funded by the Korean Government (MSIP, NRF-2014R1A1A2056986, NRF-2016R1D1A1B03930822, and NRF-2016R1A2B1057486).

CONFLICTS OF INTEREST

The authors have no conflicting interests.

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