The role of HOX genes in tamoxifen-resistant MCF7 breast cancer cells

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The role of HOX genes in tamoxifen-resistant MCF7 breast cancer cells

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ABSTRACT

The role of HOX genes in tamoxifen-resistant MCF7 breast cancer cells

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Endocrine therapy, such as tamoxifen and aromatase inhibitors, has been used to treat both early and advanced estrogen receptor α (ER)-positive breast cancer. Despite improvements in treatment, resistance to the current therapeutics can occur in up to one quarter of all cases and thus presents a serious therapeutic challenge. Multiple mechanisms responsible for endocrine resistance have been proposed, however, the molecular events underlying resistance to therapeutic agents are not clearly understood. Therefore, a better understanding of gene expression alterations associated with the resistance would suggest alternative regimens that overcome endocrine resistance. HOX transcription factors have

recently been implicated as strong candidates to control cancer progression and metastasis. Previously, a number of strong pieces of evidence suggest that HOX genes, such as HOXB7 and HOXB13, play a critical role in the development of resistance against endocrine therapy in breast cancer. To identify other HOX genes involved in tamoxifen resistance, here we have generated an *in vitro* model of acquired tamoxifen resistance using MCF breast cancer cells (MCF7-TamR) and analyzed the expression patterns of HOX genes. MCF7-TamR cells were more resistant to tamoxifen than MCF7 cells and exhibited up-regulation of HOXB genes. Meanwhile, Kaplan-Meier analysis of the distant metastasis free survival (DMFS) for ERα-positive patients with breast cancer on adjuvant tamoxifen monotherapy showed the correlation of high HOXB expression with a poor response to tamoxifen therapy. In this report, we provide evidence that multi HOXB genes overexpression renders MCF7 cells resistant to tamoxifen. In contrast, midcluster HOXB genes knockdown in MCF7-TamR cell confer TAM sensitivity. In MCF7-TamR cells, the activation of HOXB genes was associated with histone modification with the gain of H3K9ac and loss of H3K27me3, compared to MCF7 cell. These results suggest a functional role of epigenetically regulated HOXB in the development of acquired tamoxifen resistance in breast cancer.

Key Words: Breast cancer, tamoxifen resistance, HOX genes

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Ⅰ. INTRODUCTION

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females worldwide.[1] Approximately 70% of breast cancer is classified as estrogen receptor α (ER) positive in that their growth is dependent on the continued presence of estrogen.[2] ER-positive patients are able to benefit from endocrine therapies such as Tamoxifen (TAM) and aromatase inhibitors. Despite the relative safety and significant therapeutic effects of endocrine therapy, one-third of women treated with tamoxifen for 5 years will have recurrent disease with 15 years.[3] For decades, extensive studies have identified multiple mechanisms including activation of ERα, receptor tyrosine kinase signaling (EGFR, HER2) and cell cycle regulators by which breast cancers mediate endocrine resistance.[4-5] However, which critical pathway should be targeted in order to overcome endocrine resistance in breast cancer is not clear.

HOX genes are a subset of homeobox genes and encode transcription factors which play important roles in cell proliferation, differentiation, and the determination of cell identity.[6] In addition, many HOX genes have been shown to be associated with cancer.[7-8] Previous studies showed that HOX genes were already altered expression in breast cancer cell compared with normal breast cell [9] and some of dysregulated HOX gene, such as HOXA5[10], HOXA10[11], HOXB5[12], HOXB7[13], and HOXB13[14], were involved in tumorigenesis and metastasis. Similarly, altered expression of HOX genes in cancer cells were known to be involved in acquired endocrine resistance. A number of strong pieces of evidence suggest that HOX genes and associated microRNAs play a critical role in the development of resistance against therapy in cancer.[15] Several HOX genes, such as HOXB5, HOXB7 and HOXB13, have already been reported to be significant players in endocrine resistance in breast cancer. MCF7 breast cancer cells overexpressing HOXB5 were more resistant to tamoxifen.[12] HOXB7 overexpression renders breast cancer cells resistant to tamoxifen through activation of several receptor tyrosine kinase pathways, including the EGFR pathway.[16] HOXB13 confers tamoxifen resistance via repression of ER expression, as well as induction of IL6 expression.[17] However, the other HOX genes and their roles in tamoxifen resistance have not been investigated.

To find out other HOX genes involved in tamoxifen resistance, here we have generated an *in vitro* model of acquired tamoxifen resistance using MCF breast cancer cells (MCF7-TamR) and analyzed the expression patterns of 39 HOX genes. Then, we investigated the role of dysregulated HOX gene in the MCF7-TamR cell line.

Ⅱ. MATERIALS AND METHODS

1. Cell culture and establishment of MCF7-TamR cells

MCF7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; WelGENE Inc., Deagu, Korea) supplemented with 10% fetal bovine serum (FBS, WelGENE Inc.) and 1x antibiotic antimycotic solution (WelGENE Inc.). To generate an *in vitro* model of acquired tamoxifen resistance, MCF7 breast cancer cells were grown in the presence of 0.1 μM 4-OH-tamoxifen (Sigma-Aldrich).

2. Cell proliferation assay

Cell proliferation assay was carried out using the Cell Counting Kit-8 (CCK-8) according to the manufacturer's instruction (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) Briefly, cells were trypsinized, counted, and plated in 96-well plates at a density of $9x10^3$ cells per well. On designated days, the cells were stained with 5 μl of WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] for 3.5 hr at 37°C. The absorbance was measured with an ELISA reader (Softmax Pro) at 450 nm. All experiments were performed in triplicates.

3. Total RNA isolation and RT-PCR

Total RNA was isolated from the cultured cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was conducted with 1 μg of total RNA using ImProm-ll TM Reverse Transcriptase (Promega, Madison, WI, USA). PCR was performed using Taq polymerase (Bioneer, Seongnam, Korea). For the quantitative PCR, SYBR Green PCR Master Mix (Applied Biosystems,

Calrlsbad, CA, USA) was used and then subjected to real time PCR quantification using the ABI7300 (Applied Biosystems). All reactions were done in triplicates, and the relative amounts of all mRNAs were calculated by using the comparative CT method. β-actin mRNA was used as the invariant control. All primer sequences were provided in Table 1.

4. Immunocytochemistry

For the immunocytochemistry, the cells were fixed with 4% PFA and incubated in blocking buffer (0.1% Triton X-100 containing 1% goat serum) for 30 min. Then cells were incubated overnight with primary antibodies to HOXB5 (Abcam, Cambrige, UK) and HOXB6 (Sigma Cat HPA042063). The cells were then washed three times in 500 μl of blocking solution. After wash, the cells were incubated with secondary antibodies for 1.5 ~ 3 hr at room temperature.

5. *In silico* analysis

A. Gene Expression Omnibus (GEO)

To search HOX gene expression in another tamoxifen resistant breast cancer cell line, the bioinformatics approach was applied: using GEO (http://www.ncbi.nlm.nih.gov/geo/). GSE5840 datasets showed differential gene expression pattern between MCF7 cell and MCF7-TamR cell treated with DMSO or Estrogen.[18] Among retrieved gene profile images, datasets with different values and ranks were chosen, and then, chromosome neighbors were examined because HOX genes are clustered on four different chromosomes. Data were median-centered and normalized before hierarchical cluster analysis by GeneCluster 2.0. TreeView software was used for visualization of the data.

B. Analysis of co-expression genes

To search HOX genes co-expressed in breast cancer patients, cBioPortal (http://www.cbioportal.org/) dataset was utilized. cBioPortal for Cancer Genomics provides visualization, analysis and download of large-scale cancer genomics data sets. Gene expression was analyzed using Breast Invasive Carcinoma (TCGA, Cell 2015) data set; 1,105 samples from 1,098 patients.

C. Survival analysis - Kaplan-Meier plotter

The Kaplan Meier plotter is capable of assessing the effects of 54,675 genes on survival using 4,142 breast cancer samples. Gene expression data and relapse free and overall survival information were downloaded from GEO (Affymetrix microarrays only), EGA and TCGA. To analyze the prognostic value of HOX genes, the patient samples were split into low- and high-expression groups using the median as the auto select best cutoff. Distant metastasis-free survival (DMFS) time was measured from the date of operation to the date of the first distant metastasis.

6. Plasmids, si-RNA and reagents

For the overexpression studies, a full-length cDNA of the HOXB5, -B6 and -B7 gene was cloned into the pcDNA3-HA-tagged expression vector. pcDNA3-HA-tagged expression vector was transiently transfected into MCF-7 cells by use of Attractene Transfection Reagent (Qiagen, Hilden, Germany). For the knockdown studies, the MCF7-TamR cells were transfected with 15 nM siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. siRNAs were purchased from GE Dharmacon

(Waltham, MA, USA) ; ON-TARGETplus Non-targeting Pool (Cat. # D-001810-10-05 5 nmol), SMARTpool: ON-TARGETplus HOXB5 siRNA (cat. L-017532-02-0005 5 nmol) and Santa Cruz (CA, USA) ; HOXB4 siRNA(h) (sc-38692), HOXB6 siRNA(h) (sc-38694).

7. Chromatin Immunoprecipitation (ChIP) analysis

For the ChIP assay, antibodies for histone modifications were purchased from Abcam (Cambrige, UK); anti-H3K9ac antibody (Cat. No. ab12179) and anti-H3K27me3 antibody (Cat. No. ab1791). IgG controls for mouse (Cat. No. sc-2025) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). ChIP assays were performed following the protocol outlined by the manufacturer (Abcam) with minor modifications. Briefly, cells were cross-linked with 1% formaldehyde for 15 min at room temperature. The cross-linking was stopped by adding glycine (0.125 M final concentration), and cells were incubated for 10 min. After washing twice with cold PBS, the cells were resuspended in cell lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-Cl [pH 8]), containing protease inhibitor (Roche), and kept on ice for 10 min. The chromatin was sonicated 48 times for 10 sec on ice to generate DNA fragments with an average length of 500-1000 bp, followed by centrifugation at 8,000 x g for 30 sec. The supernatants were collected and diluted 10-folds in RIPA buffer (0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, 2 mM EDTA [pH8], 150 mM NaCl, 50 mM Tris-Cl [pH 8]) with protease inhibitor mixture. One percent of the supernatant fraction was saved to quantitate the amount of input DNA. The rest was precleared with Protein A/G PLUS-Agrose (Santa Cruz Biotechnology, Inc.) for 1 hour at 4℃ and immunoprecipitated with antibodies overnight. The immunoprecipitates were colleted with protein A/G agarose beads and washed sequentially for 30 min at 4℃. The DNA was eluted in 120 μl of elution buffer (1% SDS, 100 mM $NaHCO_3$) for 30 min at 30℃, and

incubated with RNaseA overnight at 65℃. Finally, DNA was purified using PCR purification kit (COSMO Genetech Co., Ltd, Seoul, Korea). After reversal of cross-linking, nucleic acids were eluted and analyzed by ChIP-PCR. All primer sequences are provided in Table 2.

8. Statistical analysis

Survival curves based on the Kaplan–Meier method were compared using a log-rank test. All statistical tests were two-sided and *p*-values of less than 0.05 were considered statistically significant. Results from the *in vitro* assay are expressed as mean ± SEM and were analyzed using the paired t-test. Differences were considered significant at * $p < 0.05$ **p<0.01 and ***p<0.001.

Table 1. Primers used for RT-PCR and realtime RT-PCR

	Forward primer (5'-3')	Reverse primer (5'-3')
HOXA1	TCATATGGACAGGAGCACCA	TGACCCAGGTAGCCGTACTC
HOXA2	CCTTTTGAGCAGACCATTCC	AGGGATTCTTTGTGGCTGAG
HOXA3	CTGCTCAACTCACCCACAGT	GCTTTCGCCTGAGCTGGA
HOXA4	CCCACCTTCCTTACCTCCTC	CCCAGAAGGGGACAACAGTA
HOXA5	ACCCACATCAGCAGCAGAGA	GGCCGCCTATGTTGTCAT
HOXA6	TTTTCTCCCGAGCAGCAGTA	ATGGCTCCCATACACAGCAC
HOXA7	ATGTACGCCCTGATGTTTCC	ACAGGAGATGAAGGGCATTG
HOXA9	CCACGCTTGACACTCACACT	AGTTGGCTGCTGGGTTATTG
HOXA10	GGGGACTTCTCTTCCAGTTTC	GGGAGAATTGTGGTGTGCTT
HOXA11	CTCAGTGTCTGGCTGCAGAG	CCTCACTCTCAGGCTCTTGG
HOXA13	CACGAACCCTTGGGTCTTC	CATCCGAGGGATGGGAGA
HOXB1	CTTATGGGAACGAGCAGACC	CTGACACCTTCGCTGTCTTG
HOXB2	TTCACCAGTACGCTCTGTGC	AAAGATAACCGAGTGCCCAAT
HOXB3	CAGAAGTCCAGCATTGCTCA	CATGACAGGAAACACAATGTCC
HOXB4	CATTCACTGAGGGCCAGAAT	CCAGCTCCCAGAACTCAACT
HOXB5	CCAATTTCACCGAAATAGACG	CGGTCATATCATGGCTGATG
HOXB6	TCTACCGCGAGAAAGAGTCG	GGAGGAACTGTTGCACGAAT
HOXB7	CGAGTTCCTTCAACATGCAC	GTTTGCGGTCAGTTCCTGAG
HOXB8	CGTGGATCTCCTTCCCTTCT	GAATTACGGCGTGAATAGGC
HOXB9	GGGAGCTGCTCAAACAGG	GGAGGGGTTGGTTTGATCC
HOXB13	GGAAAAGGCCAAAGAGTGTG	GGAAGGCAGAAAGTGACCTG
HOXC4	CACCCCGAGAAATCACAGTC	TTCACCGTGCTAACGTGAAT
HOXC5	CTCTAACCTCGCCCTCTCCT	GGGGAAGAGGTTGTGGAGAT
HOXC6	CATGCTCTCAAACTGCAGACA	ACCCCACTGTGCGAATTCAT
HOXC8	ATGCGGTTTTGTTGTCGTTT	TTCTTTCGAGCACATTGCAT
HOXC9	GGGAGGGTTCAGTGTTGAGA	GGGATGACCTGGACCAAATA
HOXC10	AACATCTGGAATCGCCTCAG	GTCAGCCAATTTCCTGTGGT
HOXC11	TTAGGGGGCAGCTAAATGTG	GAAACCTCAATGCACCGATT
HOXC12	CAGTCGCTGGAATCCGACT	GAGAGCGGCTGTTGATCG
HOXC13	GGCTACCTGGACGTGTCG	ACGTCTGGGAAGGGAGACTT

	Forward primer (5'-3')	Reverse primer (5'-3')
HOXD1	ACCCTGGTGCTTTCCAGAC	GCTAGCGGCCCCATACTC
HOXD3	CATCTTCACCCACCAATCCT	CTCTTGTCCTCGCAGCTCTC
HOXD4	GCTCCAGGAGAGCCTTGC	GTAGTTGGGGTTCACCGAAT
HOXD8	CCGCGAAGTTTTACGGATAC	GGAGCTGCTTGTGGTCTCAT
HOXD9	TTCTCGTGCAACTCGTTCCT	TTGTTTGGGTCAAGTTGCTG
HOXD10	CCACCAAAGTCTCCCAGGT	CAGTGAGCCAATTGCTGGT
HOXD11	GCAGTCCCTGCACCAAGG	TTCGCGGATCTGGTACTTG
HOXD12	AGCGGCCAAGTATGACTACG	AGTGAAGGTCGCAGGCAAG
HOXD13	GGGCTATACGAGCCCTTACC	CGGCTGATTTAGAGCCACAT

Table 2. Primers used for ChIP-PCR

	Forward primer (5'-3')	Reverse primer (5'-3')
HOXB2	CCTGGTGGAAAACAGAGAGC	CTGCTGGCCACGTAAAGAAG
HOXB3	GGACCTCAGAGACAGAAAGCTA	GTATCGCTTTCCTCTGAGGTG
HOXB4	CTCGGAGGATCACGTGGGCG	TC GACCCCTGACTCGTTTTCCT
HOXB5	CAAAGCCAACCTTCTCTCTGTT	CGTAAATTCTCGCTGATGACC
HOXB6	GTCTCACTCACTGTTGCACG	CACTTCCTCCTATTACCCGC
HOXB7	GTCCCTGCCTACAAATCATCC	CATGTTGAAGGAACTCGGCTC
HOXB8	CACCACTTAAAGAGGTCCTCG	CTCGCTCTGCGTTCTGTC
HOXB9	GATTTATGTCGGAGCTGACGC	GCTAAGCGTCCCAGAAATGG
HOXB13	GGACGTGTAAATGAGACTCTGC	CCTAAAGGCAGAAACTGCG
gene desert	TGGTGGTCTGCCTTCTGCCAGT	TCACGTGGGAGGAAGAAGTAGGGC

Ⅲ. RESULT

1. Generation of tamoxifen resistant MCF7 breast cancer cell and analysis of HOX gene expression

A. Generation of *In vitro* tamoxifen resistant MCF7 (MCF7-TamR) cell model

To generate in vitro acquired tamoxifen resistant breast cancer model, we derived MCF7 ER-positive breast cancer cells and exposed to 0.1 μM 4-OH-tamoxifen for at least six months. Tamoxifen resistant MCF7 cells (MCF7-TamR) exhibited significant resistance to TAM treatment as determined by cell proliferation assay (Fig. 1B, C). MCF7-TamR cells were prominently resistant to tamoxifen than MCF7 cells at both low and high TAM concentrations. Also, low TAM sensitivity of MCF-TamR cells sustained over time, up to two days (1, 2 days). Meanwhile, MCF7 and MCF7-TamR showed same cell proliferation rates in normal media condition (no tamoxifen treatment) (Fig. 1A). Overall, MCF7-TamR cell is more resistant to tamoxifen than MCF7 cell.

B. Analysis of HOX gene expression in MCF7-TamR cell line

Some HOX genes, such as HOXB7 and HOXB13, were dysregulated in breast cancer and involved in tumorigenesis and metastasis. Also these altered expression HOX genes have been reported to promote endocrine resistance in breast cancer. To investigate dysregulated HOX genes in our in vitro tamoxifen resistant model (MCF7-TamR), we analyzed 39 HOX gene mRNA expression levels in the parent MCF7 and MCF7-TamR (Fig. 2A, B). Interestingly, in the RT-PCR and Real-time

RT-PCR results, HOXB cluster genes (HOXB2, 3, 4, 5, 6, 7, 8, 9, 13) were significantly up-regulated in MCF7-TamR cells. On the contrary, only minor changes were seen in HOXA, HOXC and HOXD clusters. Also, higher expressions of HOXB5 and HOXB6 in MCF7-TamR were observed compared with MCF7 at the protein level (Fig. 3).

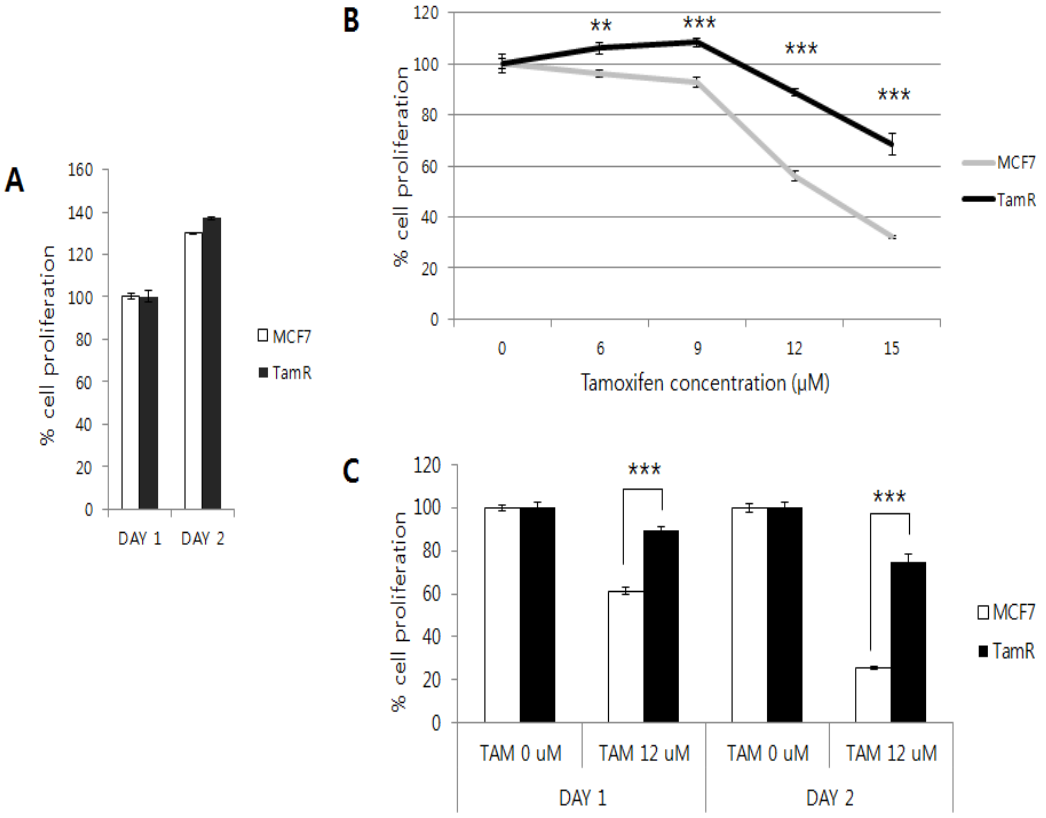

Figure 1. Tamoxifen resistance of MCF7-TamR cell. (A) MCF7 and MCF7-TamR cell growth rates were measured by CCK-8 cell proliferation assay at Day 1 and Day 2. (B) MCF7 and MCF7-TamR cell viability day after treatment with 0, 6, 9, 12 and 15 μM 4-OH TAM. (C) Cell viability was measured at Day 1 and Day 2 after treatment with only 12 μM 4-OH TAM. **p<0.01; ***p<0.001 vs. MCF7, by t-test. Data are presented as mean ± SEM.

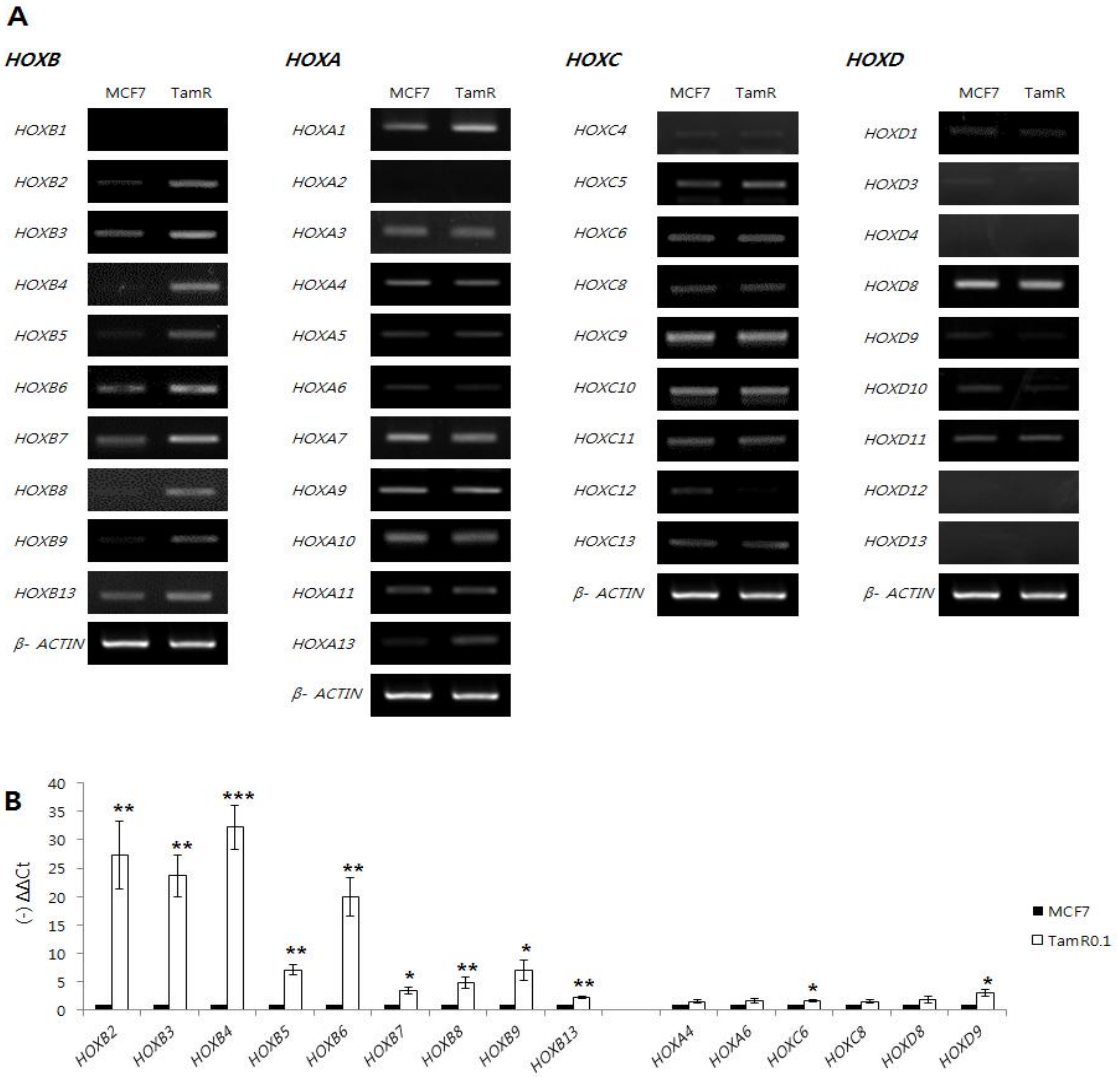

Figure 2. HOXB up-regulated in MCF7-TamR cells. (A) RT-PCR analysis of HOXA, HOXB, HOXC and HOXD genes in MCF7 and MCF7-TamR. (B) Real-time RT-PCR analysis of HOXB genes (HOXB2, 3, 4, 5, 6, 7, 8, 9, 13), HOXA4, HOXA6, HOXC6, HOXC8, HOXD8 and HOXD9 in MCF7 and MCF7-TamR. The bar represents the ratio of increased expression in MCF7-TamR compared with MCF7 level. Relative values ± S.D. were obtained from at least three independent assays.*p<0.05; **p<0.01; ***p<0.001 vs. MCF7, by t-test. Data are presented as mean ± SEM.

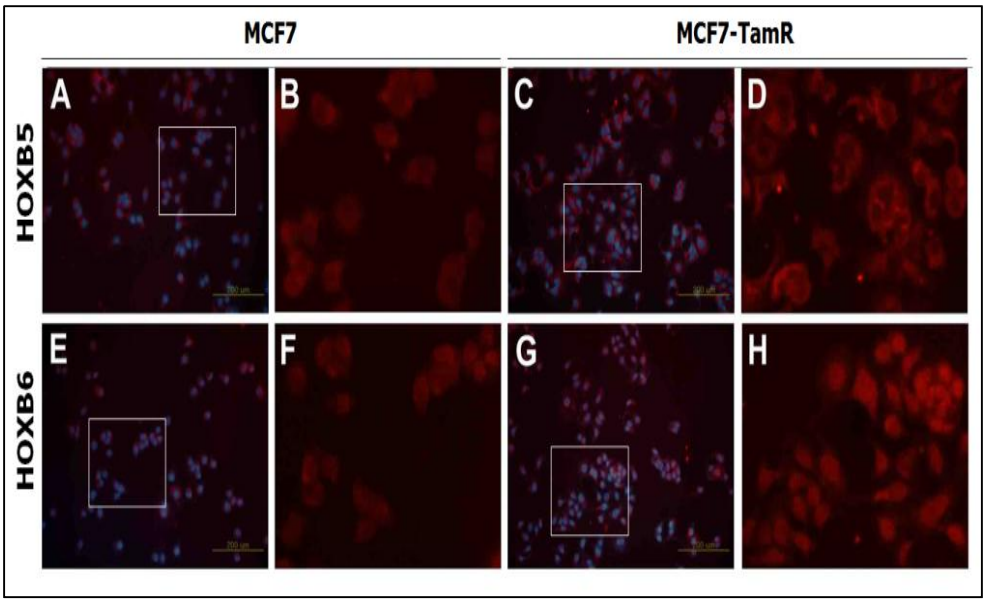

Figure 3. Up-regulation of HOXB5 and HOXB6 in MCF7-TamR cells. Detection of HOXB5 (A-D) and HOXB6 (E-H) protein expression in MCF7 and MCF7-TamR cell lines. The images were overlaid with DAPI counterstain (A, C, E, and G; x200). Boxed regions in A, C, E and G were magnified in B, D, F, and H (x600).

C. Analysis of *HOX* gene expression in MCF7-TamR using *In silico* data set

Since HOXB cluster genes were up-regulated in our *in vitro* tamoxifen resistant MCF7 cell line compared with the parent MCF7 cell line, we analyzed whether the HOXB cluster genes were up-regulated in other tamoxifen resistant breast cancer cell system, using GEO data set. The Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) is an international public repository for high-throughput microarray and next-generation sequence functional genomic data sets submitted by the research community.[19] GEO dataset offers microarray-based experiments measuring the abundance of mRNA. We re-analyzed publicly available data set profiling gene expression in wild-type MCF7 cells as well as its TamR derivatives treated with ethanol or 17β-estradiol (E2) for 4 hrs (GSE5840). Heat map shows that most HOXB (HOXB2, 3, 4, 5, 7, 9) and HOXC (HOXC4, 5, 6, 8, 10, 11) genes were up-regulated in MCF7-TamR cells treated with ethanol or 17β-estradiol (E2) compared with MCF7 cells treated with ethanol (Fig. 4B, C). On the other hand, most HOXA and HOXD gene expressions showed minor changes in MCF7-TamR cells (Fig. 4A, D). These results revealed that the HOXB cluster genes expression level was consistently higher in two different MCF7-TamR cell lines.

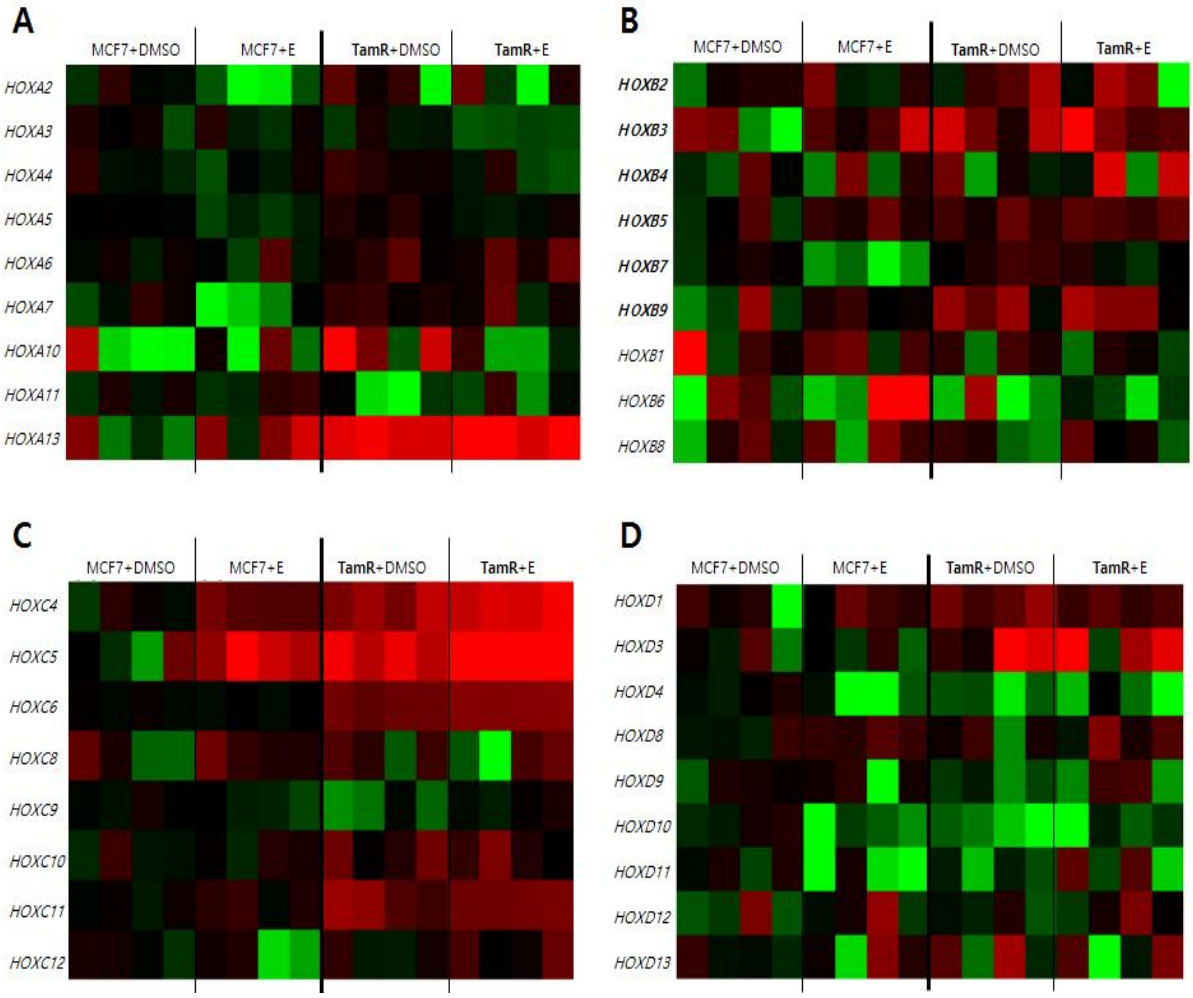

Figure 4. Hierarchical clustering analysis of the GSE5840 dataset.(A, B, C, D) Microarray data were downloaded from GEO with GES5840 and re-analyzed for HOXA, HOXB, HOXC and HOXD expression. Gene expression values were standardized against the control (MCF7+DMSO) average value, then log-transformed. The level of gene expression is color-coded. Red, higher mRNA expression; green, lower mRNA expression; black, no difference.

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2. Up-regulation of HOXB cluster genes in breast cancer patients

A. Co-expression of HOXB genes in breast cancer patients

Since the expression of HOXB cluster genes increased altogether in the MCF7-TamR cell line, the up-regulation of one HOXB gene might have influenced the other HOXB gene expression level or other HOXB genes were expressed together. To investigate whether the up-regulation of one HOXB gene is linked to another nearby HOXB gene expression, we analyzed Breast Invasive Carcinoma (TCGA, Cell 2015)[20] dataset; 1,105 samples from 1,098 patients using cBioPortal. It showed that each HOXB gene expression is correlated with nearby HOXB genes. Expression correlation is listed up in Table 3. A perfect correlation of +1 (positive) or −1 (negative) occurs when each of the variables is a perfect monotone function of the other. We selected only gene pairs with values > 0.3 in both Pearson's and Pearson's measure. Strong correlations were written in bold (value > 0.7). Above all, HOXB5 and HOXB6 mRNA expression levels were most highly correlated among the HOXB cluster (Pearson: 0.79, Spearman: 0.89) (Fig. 5A). HOXB4 and HOXB5 genes showed moderate correlation (Pearson: 0.61, Spearman: 0.71) (Fig. 5B). Furthermore, high correlations of gene expression were shown at HOXB3, HOXB4, HOXB5, HOXB6 and HOXB7. Taken together, co-expression of HOXB genes were detected in both breast cancer patient samples and *in vitro* MCF7-TamR cell line.

Table 3. The list of genes with the highest expression correlation with the *HOXB* genes

HOXB2		
Correlated Gene	Pearson's Correlation	Spearman's Correlation
HOXB3	0.69	**0.86**
HOXB4	0.55	0.66
HOXB5	0.39	0.6
HOXB6	0.35	0.6
HOXB1	0.31	0.55
HOXB3		
Correlated Gene	Pearson's Correlation	Spearman's Correlation
HOXB4	**0.73**	**0.76**
HOXB2	0.69	**0.86**
HOXB5	0.62	**0.7**
HOXB6	0.6	**0.71**
HOXB8	0.34	0.42
HOXB7	0.33	0.51
HOXB1	0.33	0.54
SKAP1	0.3	0.47
HOXB4		
Correlated Gene	Pearson's Correlation	Spearman's Correlation
HOXB3	**0.73**	**0.76**
HOXB5	0.61	**0.71**
HOXB2	0.55	0.66
HOXB6	0.52	**0.71**
HOXB7	0.33	0.52
HOXB5		
Correlated Gene	Pearson's Correlation	Spearman's Correlation
HOXB6	**0.79**	**0.89**
HOXB3	0.62	**0.7**
HOXB4	0.61	**0.71**
HOXB8	0.49	0.62
FGF14	0.43	0.34
HOXB2	0.39	0.6

HOXB7	0.37	0.63
SKAP1	0.32	0.33
HOXB6		
Correlated Gene	Pearson's Correlation	Spearman's Correlation
HOXB5	**0.79**	**0.89**
HOXB7	0.6	**0.75**
HOXB3	0.6	**0.71**
HOXB8	0.57	0.63
HOXB4	0.52	**0.71**
HOXB9	0.37	0.5
HOXB2	0.35	0.6
HOXB7		
Correlated Gene	Pearson's Correlation	Spearman's Correlation
HOXB6	0.6	**0.75**
HOXB8	0.42	0.6
PRAC2	0.39	0.33
HOXB9	0.38	0.55
HOXB5	0.37	0.63
HOXB4	0.33	0.52
HOXB3	0.33	0.51
HOXB8		
Correlated Gene	Pearson's Correlation	Spearman's Correlation
HOXB6	0.57	0.63
HOXB5	0.49	0.62
HOXB9	0.48	0.59
HOXB7	0.42	0.6
HOXB3	0.34	0.42
HOXB9		
Correlated Gene	Pearson's Correlation	Spearman's Correlation
HOXB8	0.48	0.59
HOXB7	0.38	0.55
HOXB6	0.37	0.5

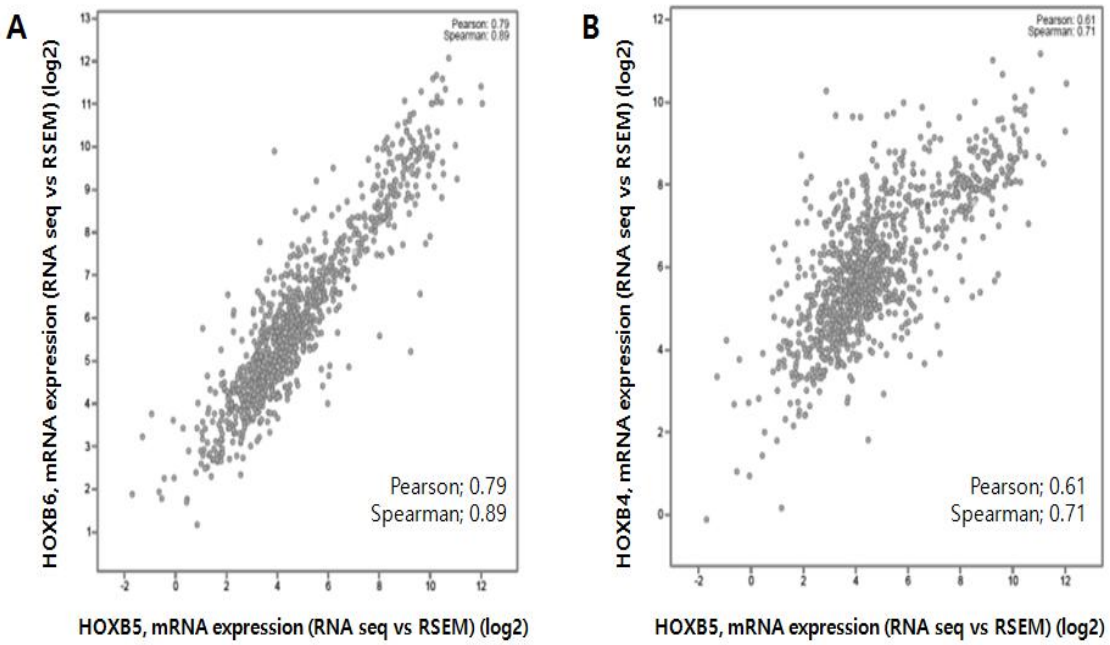

Figure 5. Correlation of mRNA expression (RNA Seq vs RSEM) of two HOXB genes. Co-expression of two genes was represented by the Pearson's Correlation and Spearman's Correlation value. (A) Co-expression of HOXB5 vs. HOXB6; Pearson: 0.79, Spearman: 0.89 (B) Co-expression of HOXB4 vs. HOXB5; Pearson: 0.61, Spearman: 0.71. A positive correlation coefficient corresponds to an increasing monotonic trend between X and Y. A perfect correlation of +1 (positive) or −1 (negative) occurs when each of the variables is a perfect monotone function of the other.

B. Kaplan-Meier Survival analysis

To assess the survival of breast cancer patients who treated tamoxifen therapy in the context of HOXB expression level, DMFS curves were drawn and compared using the Kaplan-Meier method and the log-rank test. We found no significant difference between the HOXB-high and -low groups with all patients in the DMFS curves (Fig. 6A). However, when analyzed with ER positive patients treated with tamoxifen for therapy, DMFS was significantly different between the HOXB-high and -low groups (Fig. 6B). Survival analysis of breast cancer patients showed a correlation between high expressions of HOXB with a poor response to endocrine therapy. When analyzed, in the context of DMFS of tamoxifen treated ER positive patients, out of the HOXB gene cluster (HOXB1, 2, 3, 5, 6, 7, 8, 9, 13), only HOXB5, -6 and -7 expression levels showed a significant difference in high- and low expression groups (Fig. 6C, D, E). In these results, the midcluster HOXB gene (HOXB5, 6, 7) expression was especially related with a poor response to tamoxifen. To confirm whether midcluster HOXB were key element of tamoxifen resistance, we separated HOXB genes into anterior (HOXB1, 2, 3), middle (HOXB5, 6, 7) and posterior (HOXB8, 9, 13) parts, and analyzed using DMFS with ER positive patient treated with tamoxifen (Fig. 6F, G, H). HOXB4 was excluded from the HOXB midcluster genes due to the lack of survival analysis dataset. We found that the high expression of HOXB, especially the midcluster genes (HOXB5, 6, 7) was largely influenced on poor response to tamoxifen in ER positive breast cancer patients.

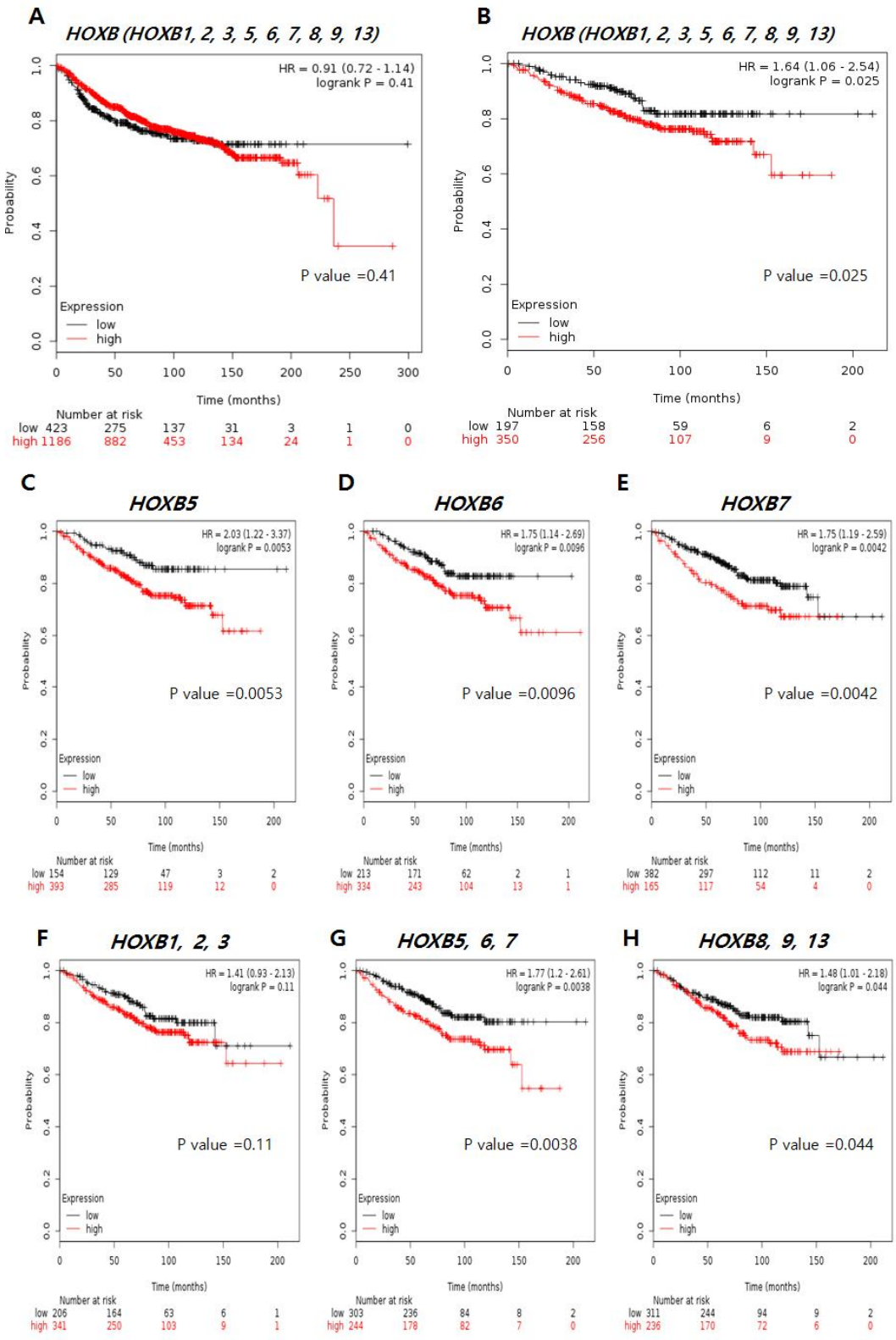

Figure 6. Kaplan-Meier analysis of distant metastasis-free survival (DMFS) based on HOXB expression in the online dataset. Survival was compared between the HOXB-high and –low expression groups (A) with all patients and (B) with ER positive patients who received tamoxifen therapy. DMFS of ER positive patients who received TAM monotherapy were stratified by (C) HOXB5 or (D) HOXB6 or (E) HOXB7 expression level. Multi-gene expression of HOXB (F) anterior (HOXB1, 2, 3), (G) middle (HOXB5, 6, 7) and (H) posterior (HOXB8, 9, 13) were separated expression-high and –low group and analyzed DMFS from ER positive breast cancer patients.

3. The role of midcluster HOXB genes in tamoxifen resistance in breast cancer cell

A. Gain of function study

From the survival analysis data, we present evidence that HOXB midcluster overexpression in ER positive breast cancer cells were related to TAM resistance. In addition, there were studies already established, suggesting that overexpression of HOXB5 or HOXB7 in breast cancer cell line induce tamoxifen resistance. [12, 16] To explore whether the midcluster HOXB overexpression is more significantly correlated with tamoxifen resistance than single HOXB (HOXB5 or HOXB7) overexpression in MCF7 cells, we transiently multi–overexpressed midcluster HOXB (HOXB5, 6, 7) in wild type MCF7 cells using pcDNA3-HA expression vector. One day after HOXB gene transfection, cells were treated with 10 μM 4-hydorxytamoxifen (4-OH TAM, the active metabolite of TAM) *in vitro*, for 24 hrs. MCF7 cells with multi overexpression HOXB midcluster gene had higher survival than with single gene overexpression at 10 μM 4-hydorxytamoxifen concentration (Fig. 7A). To evaluate the transfection efficiency, the mRNA level of each HOXB genes were analyzed by RT-PCR (Fig. 7B). This finding indicated that multi HOXB gene expression, especially micluster gene overexpression rendered tamoxifen resistance in ER positive breast cancer cells. Next, we tested that multi-overexpression of not only three HOXB genes, but also two HOXB genes in MCF7 cell induced tamoxifen resistance. We made 5 different MCF7 cell lines; transfected vector control, two genes of midcluster HOXB (B5 and B6, B6 and B7, B5 and B7) and three midcluster genes (HOXB5, 6, 7) in MCF7 cells. Then we treated each transfected MCF7 cells with 10 μM 4-OH TAM for 24 hrs and measured cell proliferation. HOXB expression in each transfected MCF7 cells was detected by RT-PCR (Fig. 7D). Overexpression of two HOXB genes resulted in a slight increase in TAM resistance, but overexpression of three midcluster HOXB

genes significantly induced TAM resistance in MCF7 cells (Fig. 7C). These results supported that midcluster HOXB expression and TAM resistance are closely linked.

B. Loss of function study

In contrast, to evaluate the effect of midcluster HOXB genes on TAM resistance, we transfected HOXB siRNAs in MCF7-TamR cells. We chose HOXB4, B5 and B6 siRNA for knockdown study instead of HOXB5, B6, and B7 siRNA, because previous results showed that HOXB4 was more up-regulated than HOXB7 in MCF7-TamR cells (Fig 2A, 2B). Efficiently decreased HOXB 4, 5, 6 mRNA expression in multi HOXB siRNA transiently transfected TamR cells was observed by Realtime PCR. (Fig. 8A). A day after we treated the cells with 7.5 μM 4-OH TAM, TamR-siHOXB4, 5, 6, cell proliferation significantly decreased compared with TamR-siCon cells (Fig. 8B). Next, we also investigated that not only three HOXB genes multi-knockdown of, but also two HOXB genes multi-knockdown in MCF-TamR cells could promote re-sensitivity to tamoxifen. Then, we treated each transiently transfected MCF7-TamR cells with 7.5 μM 4-OH TAM for 24 hrs and measured cell proliferation. To see transfection efficiency, HOXB4, B5 and B6 expression of each transfected MCF7-TamR cells was analyzed by Realtime PCR (Fig. 8C, 8E). From the cell proliferation assay results, we found that the knockdown of at least two mid-cluster HOXB (HOXB4 and B6 or HOXB5 and B6) renders TAM sensitivity in MCF7-TamR cells.

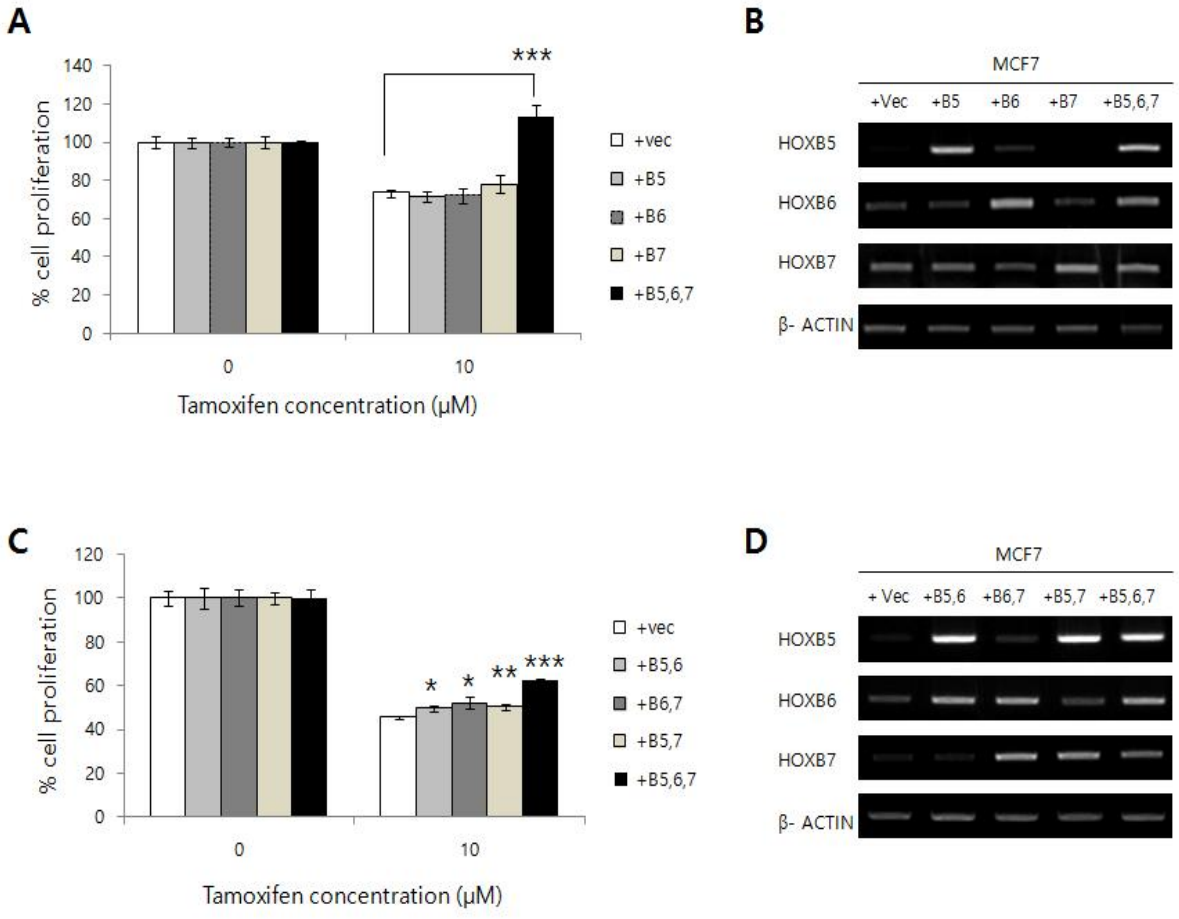

Figure 7. Overexpression of midcluster HOXB in MCF7 cells. (A) Cell proliferation was measured by CCK-8 assay on Day 1 with treatment of 10 μM tamoxifen in TamR cells with high HOXB5, HOXB6, HOXB7, HOXB midcluster genes (HOXB5, B6 and B7) and control MCF7-vec cells (C) Control vector and gene combination with HOXB5 and B6; HOXB6 and B7; HOXB5 and B7; HOXB5, B6 and B7 were overexpressed in MCF7, then treated with 7.5 μM tamoxifen and measured for cell proliferation on Day 1. RT-PCR analysis of HOXB5, B6, B7 expression in (B) MCF7-vec, MCF7-B5, MCF7-B6, MCF7-B7, MCF7-B5, 6, 7, (D) MCF7-vec, MCF7-B5, 6 MCF7-B6, 7 MCF7-B5, 7 and MCF7-B5, 6, 7 cell. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. MCF7-vec, by t-test. Data are presented as mean ± SEM.

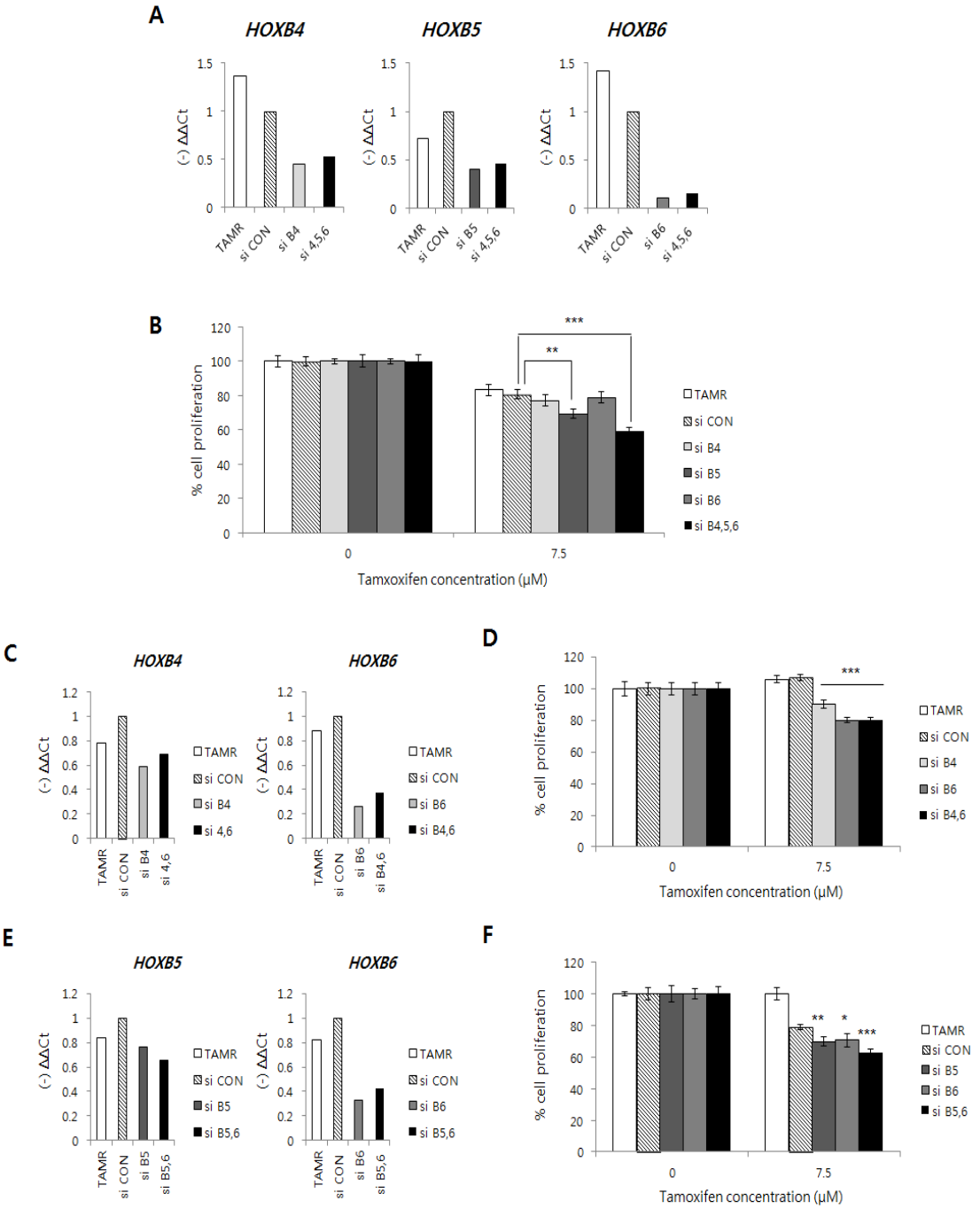

Figure 8. Knockdown of midcluster HOXB in MCF7-TamR cells. Realtime PCR analysis of HOXB4, B5 and B6 expression fold change in (A) TamR, TamR-siB4, TamR-siB5, TamR-siB6, TamR-siB4, 5, 6, (C) TamR, TamR-siB4, TamR-siB6, TamR-siB4, 6, (E) TamR, TamR-siB5, TamR-siB6 and TamR-siB5, 6 cells compared with TamR-siCon. (B, D, F) Cell proliferation was measured by CCK-8 assay on Day 1 with treatment of 7.5 μM tamoxifen in the si-RNA (HOXB4, HOXB5 and HOXB6) transfected MCF7-TamR cells and TamR-siCon cells *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. TamR-siCon, by t-test. Data are presented as mean ± SEM.

4. The correlation with epigenetic modification and HOXB activation in MCF7-TamR cells

HOX genes are regulated by epigenetic mechanisms involving the histone code during ES cell differentiation.[21] Also, in cancer, change of HOX gene expression has been found to be correlated with both DNA methylation and histone modification.[22] Histone modifications ultimately affect the degree of chromatin compaction and the accessibility to transcriptional machinery.[22-23] To test whether these epigenetic modifications occur at HOXB gene promoters in MCF7-TamR cells, we performed ChIP assay. First, we designed the amplicon site in each HOXB promoter region (Fig. 9A). Second, we performed ChIP assay using the H3K9ac antibody as an activation marker and H3K27me3 as a repressive marker at the promoter region. Finally, we analyzed ChIP-PCR, and found a gain of H3K9ac and a loss of H3K27me in MCF7-TamR compared with MCF7 at the promoter region of HOXB2, 3, 4, 5, 6, 7, 8, 9, 13 (Fig. 9B). Hence, we concluded that the activation of HOXB genes in MCF7-TamR correlated with levels of H3K9ac and H3K27me3 at gene regulatory regions.

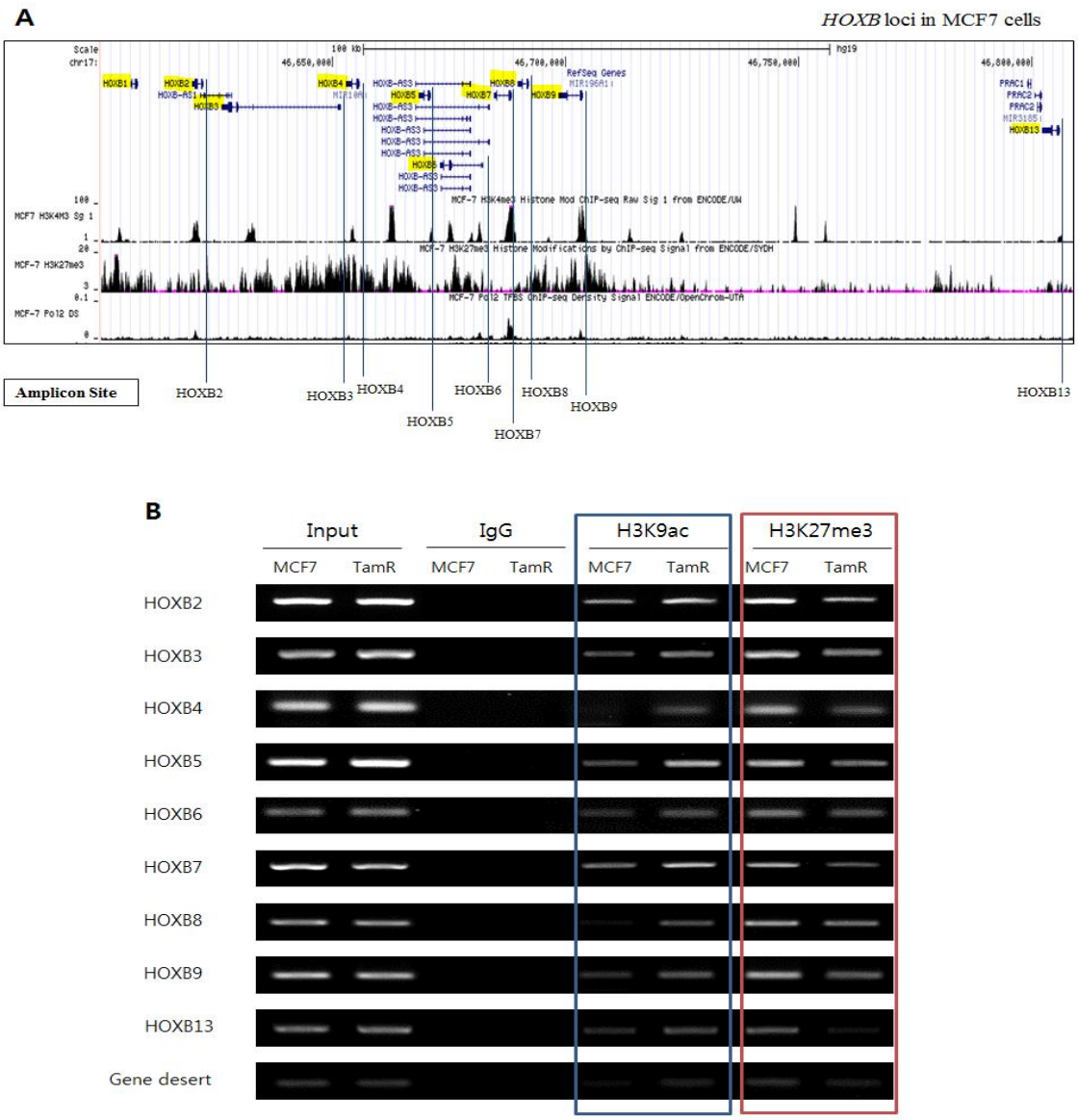

Figure 9. Histone modification in MCF7-TamR cells. (A) A screenshot of HOXB cluster in UCSC genome browser. Amplicon sites of each gene are located at HOXB gene regulatory region. (B) ChIP-PCR analysis for histone modification was performed in MCF7 and MCF7-TamR.

Ⅳ. DISCUSSION

This study provides evidence that HOXB genes are activated in tamoxifen resistant MCF7 breast cancer cells and midcluster HOXB gene expressions have a potential role in the development of tamoxifen resistance. In addition, we demonstrate that an epigenetically controlled modification in chromatin structure is linked to the change in gene expression.

Approximately 70% of breast cancers are classified as ER α positive breast cancers.[2] An ER α positive patient is able to benefit from endocrine therapy, such as tamoxifen and aromatase inhibitor treatments. However, a large number of patients failed to respond to initial therapy or develop resistance after prolonged treatment.[24] Several molecular mechanisms have been associated with endocrine therapy resistance, such as ER gene (ESR1) mutations, epigenetic aberrations, or signaling crosstalk.[25] Recently, convincing evidence has accumulated that several members belonging to the four HOX clusters contribute to endocrine therapy resistant breast cancer and these HOX genes were also associated with several mechanisms. Previous studies showed only single HOX gene effect on endocrine resistance. HOXB13 confers tamoxifen resistance via repression of ER expression, as well as induction of IL6.[17] HOXB7 overexpression renders breast cancer cells resistant to tamoxifen through the activation of receptor tyrosine kinase pathways and regulation of ER target gene expression.[16, 26] Permanent repression of HOXC10 in breast cancer via methylation eventually results in acquisition of aromatase inhibitor resistance.[27] In our results, evidence has been presented to support that the multi HOXB genes are the new regulator of tamoxifen resistance. Not single-HOXB gene expression, but multi-HOXB genes co-expression induced the tamoxifen resistance in MCF7 breast cancer cells (Fig. 7). In contrast, knockdown of multi-HOXB genes re-sensitized MCF7-TamR cells to tamoxifen (Fig. 8).

Recent studies showed that endocrine therapy resistance leads to differential gene methylation in the enhancer regions of estrogen-responsive genes, which are in turn involved in diverse cellular processes, such as apoptosis regulation, endoplasmic reticulum to Golgi trafficking, and DNA damage responses.[28] Furthermore, changes in modified histone protein (e.g., H3K27me3) and ER chromatin-binding events have also been associated with endocrine resistance. Studies investigating breast cancer epigenetic alterations after endocrine therapy have reported dysregulation in the expression of genes involved in key cellular pathways, such as metabolic processes, nucleoside transport, and developmental processes.[29-30] Taken together, these findings showed that epigenetic dysregulation of ER and its responsive genes largely contribute to endocrine resistance, although, other mechanisms do exist. We already observed that the activation of HOXB cluster genes was correlated with histone modification (Fig. 9). Then, we can suggest that the up-regulation of HOXB cluster genes by epigenetic alterations could be related with epigenetic dysregulation of ER. Also there is a possibility that the DNA methylation status was changed at the HOXB cluster locus as the HOXC10 in endocrine resistance breast cancer cell. Then, we could investigate the DNA methylation status at the HOXB locus. Furthermore, we could figure out the ER chromatin-binding events or chromatin conformation structures in MCF-TamR cells, because over-expression of HOXB cluster genes might be affected by these epigenetic events as previously reported in other genes.

Not only HOX genes, but also microRNAs and non-coding RNAs residing in HOX cluster have been identified as important independent predictors of endocrine resistant breast cancer.[15] Changed expression of specific microRNAs has been implicated in tamoxifen resistance development and predicts outcome and therapeutic response in breast cancer.[31-33] The HOX gene clusters harbor five different microRNAs: miR-10a, miR-10b, miR-196a1, miR-196a2 and miR-196b. The miR-10a gene is located between HOXB4 and HOXB5.[34] Using a global

microRNA screen (1,105 human miRNAs) of primary tumors of patients with ER positive breast cancer treated with tamoxifen, who were either recurrence-free or had suffered a recurrence, miR-10a was found as one of top 20 deregulated miRNAs. It is also a major predictor of ER positive tumor relapse in early postmenopausal breast cancer patients who are treated with tamoxifen.[35] As the miR-10a, HoxBlinc (hoxb locus-associated lincRNA) is also located between hoxb4 and hoxb5 and regulates hoxb gene transcription by modulating local chromatin alterations in mouse ES cells. HoxBlinc RNA recruits Setd1a/MLL1 complexes and facilitates the organization of a specific 3D chromatin architecture that activates the anterior hoxb genes, resulting in cardiogenic/hemogenic mesoderm differentiation.[36] Taken together, the up-regulation of HOXB cluster genes in MCF7-TamR cells might be associated with miR-10a or HoxBlinc. We can hypothesize that HoxBlinc RNA recruits the polycomb-Trithorax protein complex and re-organizes 3D chromatin architecture that activates the HOXB genes, or the altered expression of miR-10a could regulate the transcriptional activity at the HOXB locus.

In this study, we have evidence that the up-regulation of HOXB cluster genes has a role in the development of tamoxifen resistance in breast cancer cell. In addition, we demonstrate that the activation of the HOXB cluster was correlated with an epigenetic modification in gene regulatory regions. Furthermore, probably many mechanisms of the activation of HOXB genes in tamoxifen resistant breast cancer might be related with epigenetic aberrations, changes in microRNAs or noncoding RNAs.

Ⅴ. CONCLUSION

- In MCF7-TamR cell line, HOXB genes (HOXB2, 3, 4, 5, 6, 7, 8, 9, 13) were significantly up-regulated.
- Survival analysis of breast cancer patients showed a correlation between high expression of HOXB with a poor response to endocrine therapy.
- Overexpressing midcluster HOXB genes (HOXB5, 6, 7) in the MCF7 cells renders tamoxifen resistance.
- Reducing at least two HOXB levels in the MCF7-TamR cells confers tamoxifen sensitivity.
- The activation of HOXB genes in MCF7-TamR might be related to the gain of H3K9ac and loss of H3K27me3 at gene regulatory regions.

HOXB genes are activated in tamoxifen resistant MCF7 breast cancer cells and mid-cluster HOXB have a potential role in development of tamoxifen resistance. In addition, epigenetically controlled modification in chromatin structure is linked to the change in HOXB gene expression.

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ABSTRACT (IN KOREAN)

타목시펜 저항성이 있는 유방암 세포에서의 혹스 유전자의 역할

<지도교수 김 명 희>

연세대학교 대학원 의과학과

양 서 연

호르몬 수용체 양성의 조기 또는 진행성 유방암 환자는 거의 모두 타목시펜과 아로마타제 억제제와 같은 내분비치료를 받는다. 치료방법이 발전되고 있으나 내분비치료제의 저항성으로 인해 병이 재발하는 경우가 25%에 달하여 문제가 되고 있다. 내분비 저항성이 생기는 기전에 대하여 여러 가지 메커니즘이 알려지고는 있지만 분자적 수준에서 어떻게 약제에 저항성이 생기는지에 대해서는 완벽하게 밝혀지지 않은 상태이다. 그렇기에 내분비 저항성을 이겨내는 방법을 연구하는데 있어 타목시펜에 저항성이 생긴 유방암세포에서 어떤 유전자들의 발현이 변화되지를 살펴보고 이를 토대로 연구를 진행하는 것이 필요하다. HOX유전자는 발생 과정 동안 일어나는 여러 종류의 세포 내 반응을 조절하는 전사조절인자로 특히 동물 배아의 형태 형성 과정에서 매우 중요한 역할을 하며 최근에는 여러 종류의 암의 진행과 전이를 조절하는데 관련 있다고 알려져 있다. 이전 연구를 통해 HOXB5, HOXB7 그리고 HOXB13와 같은 HOX 유전자가 유방암에서 타목시펜 저항성을 유도하는데 있어 중요한 역할을 한다는 많은 증거들이 제시되어 있다. 다른 HOX 유전자 또한 타목시펜 저항성에 관련 있는

지를 알아보기 위하여 본 연구에서는 타목시펜에 저항성이 있는 MCF7 유방암 세포를 만들고 (MCF7-TamR) 이 세포 내에서의 HOX 유전자 발현의 변화를 살펴보고자 했다. 만들어진 MCF7-TamR 세포는 MCF7 세포에 비해 타목시펜에 더 저항성이 있으며 세포 내에 HOXB 유전자가 up-regulation되어 있다. 한편, Kaplan-Meier 분석을 통해 타목시펜 치료를 받은 에스트로겐 수용체 양성 환자의 무원격전이생존율(DMFS) 을 살펴보았을 때, HOXB 유전자의 발현이 높은 환자 군에서 타목시펜 치료의 예후가 좋지 않았다. 이러한 결과를 토대로 하여 본 연구에서 MCF7 세포 내에 여러 HOXB 유전자를 동시에 과 발현 시킨 후 살펴보았을 때, HOXB 유전자의 과 발현이 타목시펜 저항성을 유도된다는 것을 알 수 있었으며 반대로 MCF7-TamR 세포 내에서 여러 HOXB 유전자를 knockdown 하였을 때는 MCF7-TamR 세포가 타목시펜에 좀 더 민감성을 갖게 된다. MCF7-TamR 세포 내에서 HOXB 유전자의 활성은 H3K9ac이 증가되고 H3K27me3이 감소하는 히스톤 변화와 관련되어 있다. 이러한 결과들을 종합하여 봤을 때 유방암 세포 내에서 타목시펜 치료의 저항성을 갖게 되는데 있어서 HOXB 유전자들은 기능적 역할을 하며 이는 후성적인 조절을 받는다.

핵심 되는 말 : 유방암, 타목시펜 저항성, 혹스 유전자

PUBLICATION LIST

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