약학석사학위논문

유방암 세포주에서 MTA1에 의한 자가포식작용과 타목시펜 내성 유발

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이 논문을 약학석사 학위논문으로 제출함

2015년 7월

서울대학교 대학원

약학과 병태생리학전공

고 다 혜

고다혜의 석사 학위논문을 인준함

2015년 7월

위 원 장 오 우 택 (인)

부위원장 이 호 영 (인)

위 원 이 미 옥 (인)

ABSTRACT

MTA1 Promotes Autophagy and Resistance to Tamoxifen in ERα-Positive Breast Cancer Cell Line

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College of Pharmacy
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Seoul National University

Autophagy, a cellular recycling process in which long-lived proteins and organelles are degraded, has recently been recognized to be associated with drug resistance in various cancers. Several studies have reported an increase in autophagy in tamoxifen-resistant breast cancer. Interestingly, we found metastasis-associated protein 1 (MTA1) to be overexpressed in tamoxifen-resistant MCF7 breast cancer cell line, raising a potential link between MTA1 overexpression and the autophagy-induced tamoxifen resistance. To investigate the involvement of MTA1 in tamoxifen resistance, we used RNA interference against MTA1 followed by 4-hydroxytamoxifen (4OHT) treatment in MCF7 cells. Our data

showed that MTA1 knockdown sensitized cells to 4OHT. Moreover, we observed that the depletion of MTA1 resulted in the decrease in autophagic flux. The reduction was accompanied with the downregulation of LC3-II level, a well-known marker for autophagy. 4OHT is known to induce autophagy in cells, which is a stress response in order to maintain cellular homeostasis. However, 4OHT-induced autophagy is known to ironically lead to the resistance to the drug. Our data showed that MTA1 knockdown led to a decrease in 4OHT-induced autophagy. Through qRT-PCR experiment, we observed that MTA1 depletion correlated with the decrease in the upregulation of ATG9B, an autophagy-related gene involved in the recycling of LC3 protein, in the presence of 4OHT. Our observations may help to understand the role of MTA1 in the development of resistance to endocrine therapy for patients with ERα-positive breast cancer.

keywords : MTA1, Autophagy, Tamoxifen resistance, Breast cancer
Student Number : 2013-23452

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Ⅰ. INTRODUCTION

As the most commonly diagnosed cancer in women in 2013, the incidence of breast cancer continues to incline (Yeo *et al.*, 2014). Due to the struggle for improvements in the treatment of the cancer, the mortality rate is falling. Nonetheless, breast cancer remains the second most common cause of death from cancer in women (Matsen *et al.*, 2013). Efforts for better prognosis put a focus on targeted therapies used to individualize anticancer therapy according to tumor biology and molecular subtypes.

Invasive breast cancer is divided by its molecular status; luminal A, luminal B, HER2, and basal-like types (Schnitt, 2010). These molecular subtypes are classified by the expression of estrogen receptor alpha (ERα), progesterone receptor, and HER2. Almost 80% of invasive breast cancer has been diagnosed to be estrogen receptor-positive (ER+) (Yeo *et al.*, 2014). Tamoxifen is a selective estrogen receptor modulator (SERM) that remains as the standard adjuvant therapy for ER+ breast cancer. It competitively blocks the binding of estradiol to estrogen receptor, hence inhibiting cancer cell proliferation through ER-mediated transcription by estrogen response elements of various genes (Figure 1) (Huber-Keener *et al.*, 2012).

However, the efficacy of tamoxifen treatment in ERα-positive breast cancer patients has been limited due to innate or acquisition or

resistance to the drug (Dixon, 2014). There have been a number of studies on mechanisms to tamoxifen-resistance mechanisms. A few of them include hypersensitivity of ER to circulating estrogens (Gururaj *et al.*, 2005), abolishment of apoptotic cell death through STAT3-RANTES autocrine signaling (Yi *et al.*, 2012), and attenuation of reactive oxygen species by Sirtuin 3 upregulation (Zhang *et al.*, 2013). Autophagy has been investigated lately as a strong potential target for drug resistance in cancer.

Autophagy is a cellular recycling system that degrades long-lived proteins or organelles when the cell is under metabolic or cytotoxic stress (Figure 2; Klionsky *et al.*, 2012). Autophagic process is performed in three stages; initiation stage, elongation stage, and late stage. ULK1 (Atg1) complex is activated in response to stress such as nutrient deprivation, leading to the initiation of autophagy. Membrane nucleation involves Beclin-1 and autophagy-related proteins including Atg5, Atg12, etc. The phagophore encircles intracellular compartments and closes up to become autophagosome. LC3-II protein, a homoloque of yeast Atg8, is a representative marker for autophagy. LC3 is processed into LC3-I form by Atg4. LC3-I is conjugated with phosphatidyl- ethanolamine (PE) to yield LC3-II, a lipidated form of LC3-I. The process involves Atg3 and Atg7. Atg9B is known to participate in the elongation process, while LC3-II is sequestered onto the membrane of autophagic vesicle. In the late stage of autophagy, autophagosome is fused with lysosome to form autolysosome. As lysosome consists of hydrolases and proteases,

intracellular compartments are degraded, and LC3-II is recycled back to the cytosol. Accordingly, autophagy maintains cellular homeostasis and promotes cell survival in the context of stressful conditions.

However, autophagy has ironically been reported as one of the main contribution to drug resistance in many types of cancer (Chen *et al.*, 2010). Anticancer therapy induces autophagy, which subsequently protects a cell by maintaining its energy, leading to a reduced sensitivity against the drug (Figure 3). Hydroxychloroquine (HCQ), an autophagy inhibitor, was found to restore sensitivity to tamoxifen treatment in tamoxifen-resistant ER+ breast cancer (Cook *et al.*, 2014). Another autophagy inhibitor, clomipramine, was also reported to increase sensitivity to enzalutamide, an inhibitor to androgen receptor frequently used in the treatment of prostate cancer, in castration-resistant prostate cancer cells (Nguyen *et al.*, 2014). In line with in vitro studies, autophagy inhibitors have also been used in clinical studies. The study of the adjuvant therapy of HCQ with paclitaxel in malignant lung cancer was in phase II, and HCQ with ixabepilone in metastatic breast cancer was in phase I/II (Jiang *et al.*, 2014).

Metastasis-associated protein 1 (MTA1) is a transcription factor widely known for its repressive function as a part of the nucleosome remodeling and deacetylation (NuRD) complex (Figure 4; Toh *et al.*, 2009). MTA1 was first found to be associated with metastasis in highly metastatic cancer *in vivo* (Toh *et al.*, 1994). Later studies

reported MTA1 to be overexpressed in hepatocellular carcinoma (Moon *et al.*, 2004), prostate cancer (Fan *et al.*, 2012), and breast cancer tissue (Jang *et al.*, 2006). In line with these studies, increase in MTA1 expression level in invasive breast cancer tissue compared to normal cancer was analyzed from The Cancer Genome Atlas database (Figure 5A; TCGA). It was also shown that the overexpression of the protein had increased metastatic function in *in vitro* studies (Mazumdar *et al.*, 2001). Moreover, MTA1 was found to be a downstream target of heregulin-β1 (HRG) and repress ER-mediated transcription by recruiting HDACs in breast cancer. The phenomenon renders ERα-positive breast cancers to obtain more invasive and aggressive phenotypes as in ER-negative breast cancers.

At the same time, MTA1 upregulation was reported to induce resistance to cisplatin in nasopharyngeal carcinoma, suggesting the role of MTA1 in anticancer drug resistance (Feng *et al.*, 2014). According to the database in which relapsed breast tumor to tamoxifen treatment is considered as tamoxifen-resistant (GSE9893; Chanrion *et al.*, 2008), the expression level of MTA1 was significantly upregulated compared to that of relapse-free tumor (Figure 5B). This raised a potential link between MTA1 and tamoxifen resistance.

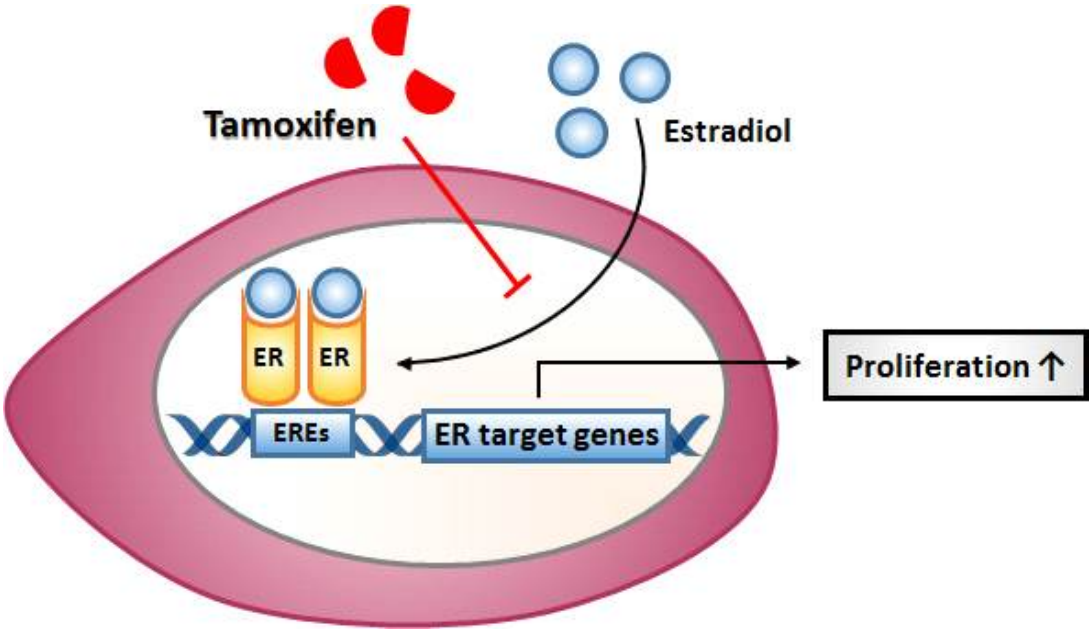


Figure 1. Effect of tamoxifen on estrogen receptor signaling in ERα-positive breast cancer cell

In ERα-positive breast cancer cells, estradiol binds to and activates estrogen receptor (ER). Estradiol-bound ER forms dimers that translocate into the nucleus. They bind on estrogen response elements (EREs) in DNA and promote transcription of ER target genes, leading to cancer cell proliferation. Tamoxifen is a selective estrogen receptor modulator (SERM) that competitively binds to ER, blocking the binding of estradiol on ER. Consequently, estrogen-mediated transcription of ER target genes is abolished, thus inhibiting cancer cell proliferation.

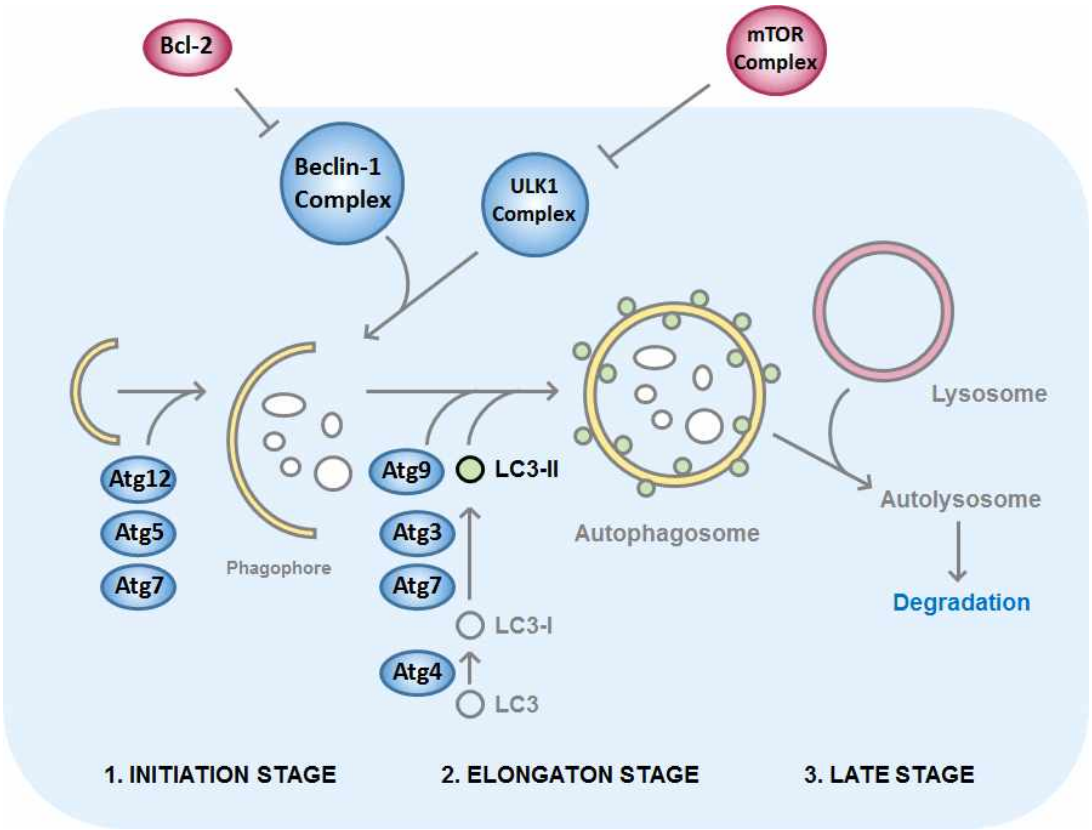

Bcl-2
mTOR Complex
Beclin-1 Complex
ULK1 Complex
Atg12
Atg5
Atg7
Phagophore
Atg9
LC3-II
Atg3
Atg7
LC3-I
Atg4
LC3
Autophagosome
Lysosome
Autolysosome
Degradation
1. INITIATION STAGE
2. ELONGATON STAGE
3. LATE STAGE

Figure 2. Schematic diagram of autophagic process

Autophagy maintains cellular homeostasis through recycling proteins and organelles. Under cytotoxic or metabolic stress, mTOR complex is inactivated, leading to the activation of ULK1 complex. In the initiation stage, beclin-1 (Atg6) complex induces membrane nucleation along with Atg12, Atg5, and Atg7. In the elongation stage, LC3 is processed by Atg4 to LC3-I immediately after synthesis. LC3-I is conjugated with phosphatidylethanolamine to yield LC3-II form, which is sequestered on isolation membranes and autophagosomes. Atg9 also participates in the elongation stage to make autophagosomes. In the late stage of autophagy, autophagosomes fuses with lysosomes to become autolysosomes. Intracellular compartments of autolysosomes are then degraded by proteases and hydrolases in lysosomes. LC3-II proteins are recycled from the autophagosome membrane back to the cytosol.

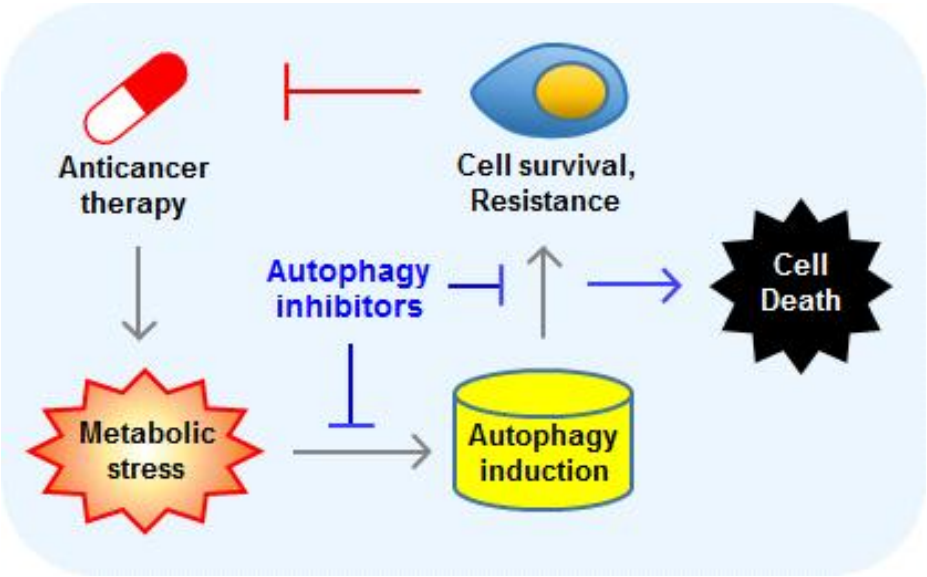


Figure 3. Autophagy promotes the resistance to anticancer therapy

Anticancer therapy stimulates metabolic stress in ERα-positive breast cancer cell. In response to the stress, autophagy is induced to maintain cellular homeostasis. Cell survival promoted by autophagy confers resistance to the anticancer therapy. Several studies have reported the treatment of autophagy inhibitors leads to cancer cell death.

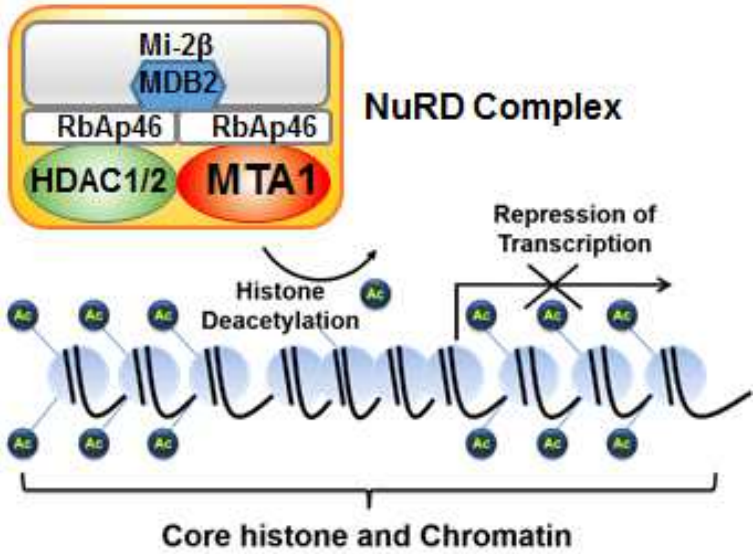


Figure 4. Transcription repressive function of MTA1 in nucleosome remodeling and deacetylation (NuRD) complex

MTA1 protein exerts its strong transcription repressing activity through NuRD complex. Along with MTA1, NuRD complex contains histone deacetylase (HDAC) 1, HDAC2, the histone binding proteins RbAp46/48 and the dermatomyositis-specific autoantigen Mi-2.

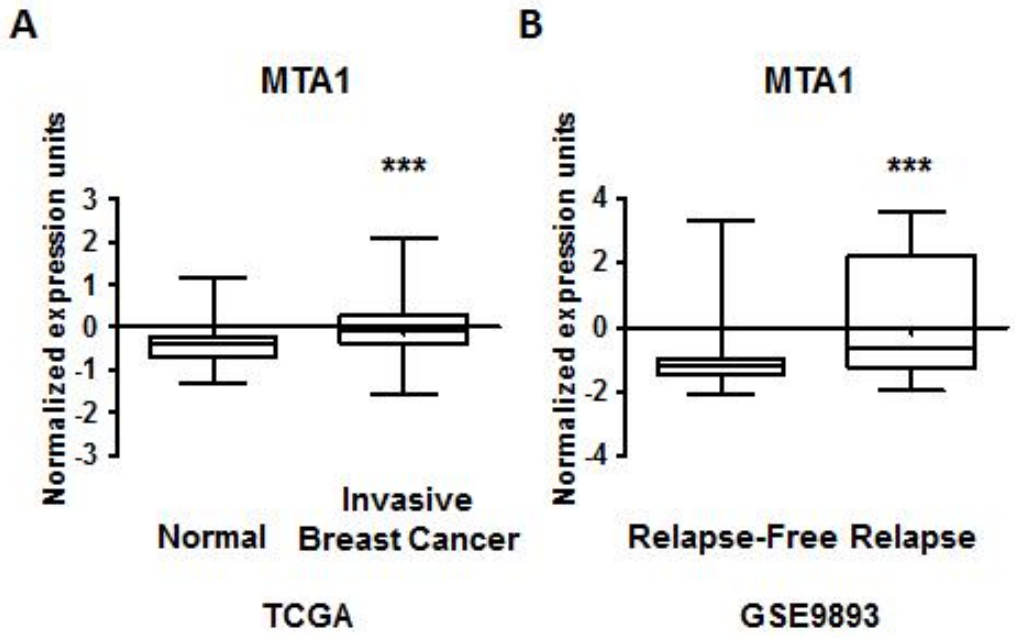


Figure 5. Elevated MTA1 level in invasive breast cancer and tamoxifen-resistant breast cancer

The public microarray datasets were analyzed for MTA1 expression level in human breast tissue. The y-axis shows normalized expression units. Data are median centered and the 25th-75th percentiles are indicated by the closed box. (A) MTA1 expression level is upregulated in invasive breast cancer tissue. The microarray dataset was obtained from The Cancer Genome Atlas (TCGA). (B) Correlation between MTA1 expression and recurrence in tamoxifen treated ER-positive breast cancer patients showed elevation of MTA1 expression level in relapsed breast cancer. Breast cancer that have not relapsed to tamoxifen treatment was classified as 'relapse-free', while relapsed breast cancer was classified as 'relapse'.

Ⅱ. PURPOSE of the STUDY

Resistance to endocrine therapy is a major obstacle in the treatment of ER+ breast cancer. As tamoxifen resistance had largely contributed to poor prognosis, researchers have struggled to investigate the mechanism of the resistance acquisition. A number of studies have recently highlighted autophagy as one of the major contributors to drug resistance. As the keeper of cellular homeostasis, autophagy was found to counteract the effects of anticancer therapy and promote cell survival. As shown in the database (Figure 5B), MTA1 was overexpressed in tamoxifen-relapsed breast tumor. Although well-known for its metastatic characteristics, few studies have reported the association of MTA1 and autophagy. In this study, we aimed to find the role of MTA1 in the autophagy-induced tamoxifen resistance in ER+ breast cancer. To investigate the relevance between MTA1 and tamoxifen resistance, we used siRNA approach for MTA1 knockdown in the ERα-positive breast cancer cell lines, MCF7 and T47D. Moreover, we examined whether MTA1 induced autophagy and how the process was regulated. The present study aims to elucidate the role of MTA1 in the acquisition of resistance to tamoxifen in ERα-positive breast cancer.

Ⅲ. MATERIALS and METHODS

1. Cell lines and materials

MCF7 cells were maintained under 5% CO2 at 37℃ in Dulbecco's modified Eagle's medium (DMEM; SH30243.01; Hyclone; Pennsylvania, USA) supplemented with 10% fetal bovine serum (FBS; SH30919.03; Hyclone) and 1% penicillin and streptomycin (P/S; 15140; Gibco; New Hampshire, USA), and T47D cells were cultured in Roswell Park Memorial Institute (RPMI; SH30027.01; Hyclone) -1640 medium supplemented with 10% FBS and 1% P/S. Tamoxifen-resistant MCF7 (TAMR) cells were generously provided by Dr. Keon-wook Kang (Seoul National University). TAMR cells were maintained in 3 uM 4-hydroxytamoxifen added to the same medium as the parental MCF7 cell line. Bafilomycin A1 (11038) was purchased from Cayman Chemical (Michigan, USA), rapamycin (sc-3504) was from Santa Cruz (Texas, USA), and 4-hydroxy-tamoxifen (3412) was obtained from Tocris Bioscience (Minnesota, USA). Antibodies to MTA1 (sc-10813) and GAPDH (sc-20357) were purchased from Santa Cruz, antibody to α-tubulin (CP06) was from Calbiochem (Darmstadt, Germany), and antibodies to LC3 were from Cell Signaling (2775; Massachusetts, USA) and Sigma-Aldrich (L8919; Missouri, USA).

2. Acridine orange staining

Cells were grown in 6-well plate. After wash with phosphate buffered saline (PBS), cells were incubated in 0.1 ug/ml acridine orange (318337; Sigma-Aldrich) in culture media for 10 minutes. Cells were replaced with a fresh culture medium and observed under a blue light. Images were obtained through Olympus fluorescence microscope (Tokyo, Japan).

3. Cell counting assay

Cells were transfected with siRNA and seeded in 6-well plate. After 48 hours of transfection, they were treated with 1 uM 4OHT upto four days. Cells were trypsinized using trypsin-EDTA (15400; Gibco) and counted everyday with a hemocytometer using trypan blue exclusion assay.

4. Crystal violet staining

Cells were seeded in 24-well plate and transfected with siRNA the next day. After 24 hours of transfection, cells were treated with 1 uM 4OHT and incubated for three days. Cells were then incubated for three hours in thiazolyl blue tetrazolium bromide (Sigma M2128) dissolved in PBS at a concentration of 2 mg/ml. After PBS wash, the stain was dissolved in DMSO for the measurement of optical density.

5. Immunofluorescence assay

Cells grown on cover slips were washed in PBS and fixed with

acetone. After washing off acetone with PBS, cells were incubated with primary antibody for LC3 (2775) in 1% BSA overnight in 4℃. After incubating in secondary antibody for 1 hr in room temperature, DAPI staining was conducted for 30 seconds. The coverslips were mounted on a slide glass for observation. Images were obtained through Zeiss confocal microscope (Oberkochen, Germany).

6. Transfection

For siRNA experiment, MCF7 cells were transfected with siRNA targeting MTA1 or a control siRNA for 6 days using Lipofectamine 2000 (11668-027; Invitrogen; New Hampshire, USA) according to the manufacturer's protocol. Cells were incubated for 24 hours with a transfection mixture and then replaced with fresh medium. The siRNA sequence used are in the table below (Table 1).

7. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated using EASY-BLUE™ Total RNA Extraction Kit (iNtRON Biotechnology; Gyeonggi-do, Korea) according to the manufacturer's instructions. cDNA was synthesized from total RNA in a reaction mixture containing random hexamer (48190-011; Invitrogen) and M-MLV reverse transcriptase (28025-013; Invitrogen). qRT-PCR was performed using SYBR Green PCR mix (4367659; Applied Biosystems; New Hampshire, USA). The resulting ΔCt values were normalized with β-actin.

8. Western blot assay

Cells were washed twice with cold PBS and then harvested with a lysis buffer (10 mM Tris Cl, 100 mM NaCl, 1 mM EGTA, 10% glycerol, 0.5% Triton X-100, 30 mM sodium pyrophosphate, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 5 mM glycerol 2-phosphate) supplemented with a protease inhibitor cocktail (11.836.153.001; Roche Diagnostics; Basel, Switzerland), using a cell scraper. After 30 minutes of incubation on ice, cell lysates were centrifuged at 14,000 rpm for 10 minutes. Supernatant was collected for the protein quantification through BCA Protein Assay Kit (23225; Pierce; New Hampshire, USA). Protein samples were made and loaded onto a 9% or a 15% gel. After SDS-polyacrylamide gel electrophoresis, proteins were transferred onto a 0.45 um (IPVH00010; Millipore; Darmstadt, Germany) or a 0.2 um (ISEQ00010; Millipore) polyvinylidene difluoride (PVDF) membrane via wet-transfer or dry-transfer method. Blocking was done in 5% non-fat dry milk in PBS with 0.1% Tween-20 (PBST) for 30 minutes under room temperature. Then membranes were incubated in primary antibodies in 5% BSA overnight in 4℃. Membranes were washed with PBST and incubated in secondary antibodies for 40 minutes under room temperature. Amersham ECL solution (RPN2106; GE Healthcare; Pennsylvania, USA) was used for detection after washing off residual antibodies with PBST.

9. Statistical analyses

Statistical analyses were calculated using GraphPad Prism (GraphPad Software; California, USA). Significance of the data from cell counting assay was determined by two-way ANOVA test. All other comparisons were made using Mann-Whitney U test. $P < 0.05$ denotes statistical significance.

Table 1. Oligomer sequences of siRNA

Gene		siRNA sequences
GL3	Sense	5′-CUU ACG CUG AGU ACU UCG ATT-3′
	Antisense	5′-TTG AAU GCG ACU CAU GAA GCU-3′
GFP	Sense	5′-GUU CAG CGU GUC CGG CGA GTT-3′
	Antisense	5′-CUC GCC GGA CAC GCU GAA CTT-3′
MTA1	Sense	5′-AAG ACC CUG CUG GCA GAU AAA-3′
	Antisense	5′-UUU AUC UGC CAG CAG GGU CUU-3′

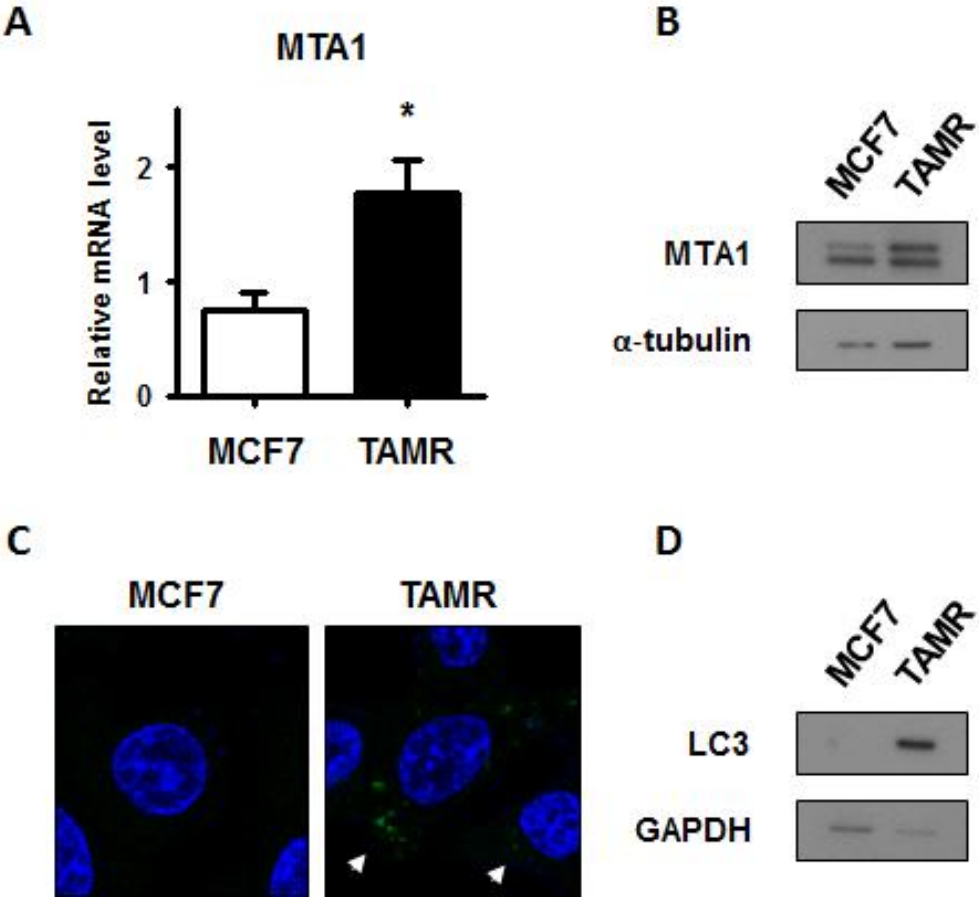
Table 2. Primer sequences used for quantitative RT-PCR analysis

Gene		Primer sequences	Product length (bp)
hMTA1	Forward	5′-CAC GCA CAT CAG GGG CAA-3′	317
	Reverse	5′-GTG CGA AGG TGC CCA CA-3′	
hATG3	Forward	5′-CTG GCG GTG AAG ATG CTA TT-3′	201
	Reverse	5′-GTG GCA GAT GAG GGT GAT TT-3′	
hATG4B	Forward	5′-AGG TGC TAA CAC GGG GCT-3′	134
	Reverse	5′-CGG GAG GCC TTA CTG CTT-3′	
hATG5	Forward	5′-TGG ATT TCG TTA TAT CCC CTT TAG-3′	108
	Reverse	5′-CCT AGT GTG TGC AAC TGT CCA-3′	
hBeclin-1 (ATG6)	Forward	5′-GGA TGG ATG TGG AGA AAG GCA AG -3′	152
	Reverse	5′-TGA GGA CAC CCA AGC AAG ACC -3′	
hATG7	Forward	5′-CAG TTT GCC CCT TTT AGT AGT GC -3′	105
	Reverse	5′-CTT AAT GTC CTT GGG AGC TTC A -3′	
hLC3B (ATG8)	Forward	5′-GCC TTC TTC CTG CTG GTG AAC-3′	91
	Reverse	5′-AGC CGT CCT CGT CTT TCT CC-3′	
hATG9B	Forward	5′-TTG CCA ACC AAC CAA GTA ACC -3′	105
	Reverse	5′-AGC GGA TCC TCT CAG CAC ACT -3′	
hATG12	Forward	5′-TCTATGAGTGTTTTGGCAGT-3′	171
	Reverse	5′-ATCACATCTGTTAAGTCTCT-3′	
β-actin	Forward	5′-CGT GGG CCG CCC TAG GCA CCA-3′	242
	Reverse	5′-TTG GCT TAG GGT TCA GGG GGG-3′	

Ⅳ. RESULTS

1. MTA1 expression and basal autophagy are increased in tamoxifen-resistant MCF7 cell line

In order to investigate whether MTA1 expression level is elevated *in vitro*, we conducted qRT-PCR and western blot assay in tamoxifen-resistant MCF7 (TAMR) cells. MTA1 expression was upregulated in both mRNA and protein level, consistent with the analysis from the database (Figure 6A and 6B). To examine the level of basal autophagy in TAMR cells, we monitored LC3 punctate pattern and the total protein level of LC3-II. Elevated pattern of LC3 punctae in TAMR cells was observed through immunofluorescence assay, indicating increased autophagy flux compared to that of the parental MCF7 cells (Figure 6C). Moreover, LC3-II protein level was significantly upregulated in TAMR cells compared to its control cells (Figure 6D). Taken together, these data indicate that MTA1 expression level and basal autophagy are increased in TAMR cells.


A
MTA1
Relative mRNA level
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*
MCF7
TAMR
B
MCF7
TAMR
MTA1
α-tubulin
C
MCF7
TAMR
D
MCF7
TAMR
LC3
GAPDH

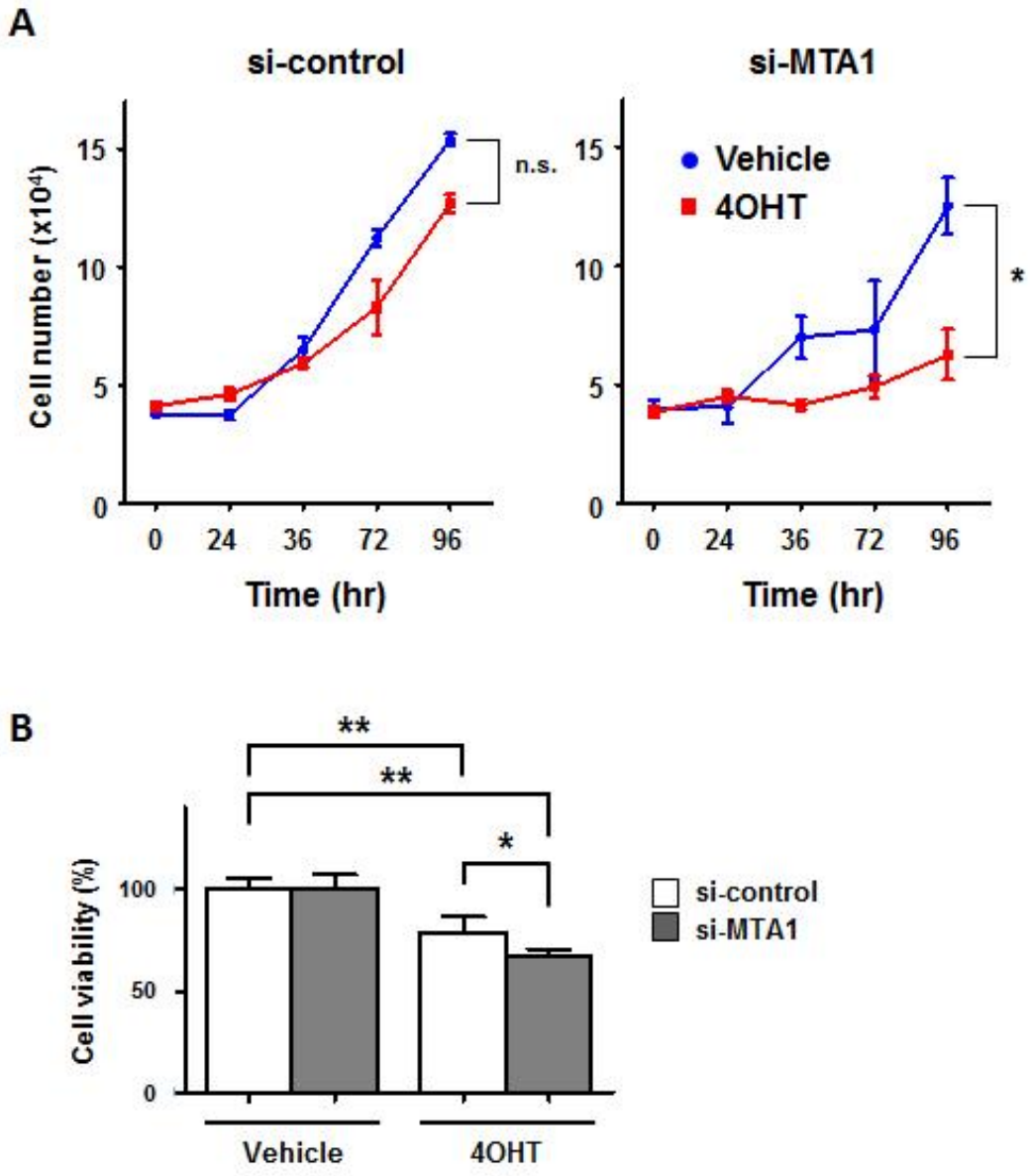
Figure 6. MTA1 expression level and basal autophagy in tamoxifen-resistant MCF7 cells

(A) Total RNA isolated from TAMR and parental MCF7 cells was subjected to quantitative RT-PCR analysis of mRNA for MTA1. *$p < 0.05$.

(B) TAMR and parental MCF7 cells were lysed and subjected to immunoblot analysis with antibodies to MTA1 or the loading control α-tubulin.

(C) TAMR and parental MCF7 cells were immunostained with LC3 antibody. Endogenous LC3 punctate dots were observed by confocal microscopy.

(D) MCF7 and TAMR cells were lysed and subjected to immunoblot analysis with antibodies to LC3 or the loading control GAPDH.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Cell number (x10⁴)
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72
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Time (hr)
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Cell viability (%)
100
50
0
si-control
si-MTA1
Vehicle
4OHT

Figure 7. Knockdown of MTA1 sensitizes ERα-positive breast cancer cells to 4OHT treatment

(A) MCF7 cells were transfected with siRNAs for a control (si-GL3) or MTA1, and then treated with either a vehicle or 1 uM 4OHT for four days. Cells were counted using trypan blue exclusion assay. *$p < 0.05$.

(B) T47D cells were tranfected with siRNAs for a control (si-GL3) or MTA1, and then treated with either a vehicle or 1 uM 4OHT for three days. The cell viability was analyzed by crystal violet staining. Data are representative of three independent experiments. *$p < 0.05$. **$p < 0.01$.

2. MTA1 is involved in the resistance to 4-hydroxytamoxifen

To examine whether MTA1 contributes to tamoxifen resistance, we used RNA interference against MTA1 in MCF7 cells. Depletion of MTA1 sensitized MCF7 cells to 4-hydroxytamoxifen (4OHT) treatment (Figure 7A). Knockdown of MTA1 also sensitized T47D ERα-positive breast cancer cells to 4OHT treatment (Figure 7B). These results suggest a potent involvement of MTA1 in the mechanism of tamoxifen resistance in ERα-positive breast cancer cells.

3. MTA1 induces autophagic flux

To explore the involvement of MTA1 in autophagy, we used siRNA approach for MTA1 knockdown in MCF7 cells. Bafilomycin A1 (Baf. A1) is a chemical that blocks the fusion of autophagosome and lysosome in the late stage of autophagy. It is frequently used to monitor the total flux of autophagy in cells. Depletion of MTA1 significantly decreased autophagy in MCF7 cells, as indicated by LC3 punctate pattern (Figure 8A). However, monitoring punctate dots of endogenous LC3 through immunofluorescence assay has its own limits as the dots also include LC3-I form, which is not actively involved in the process of autophagy. LC3 consists of two forms, LC3-I and LC3-II. LC3-I form is known to be very stable, but the sensitivity of detection through immunoblotting has been shown to be much lower than that of LC3-II (Mizushima *et al.*, 2007). Therefore

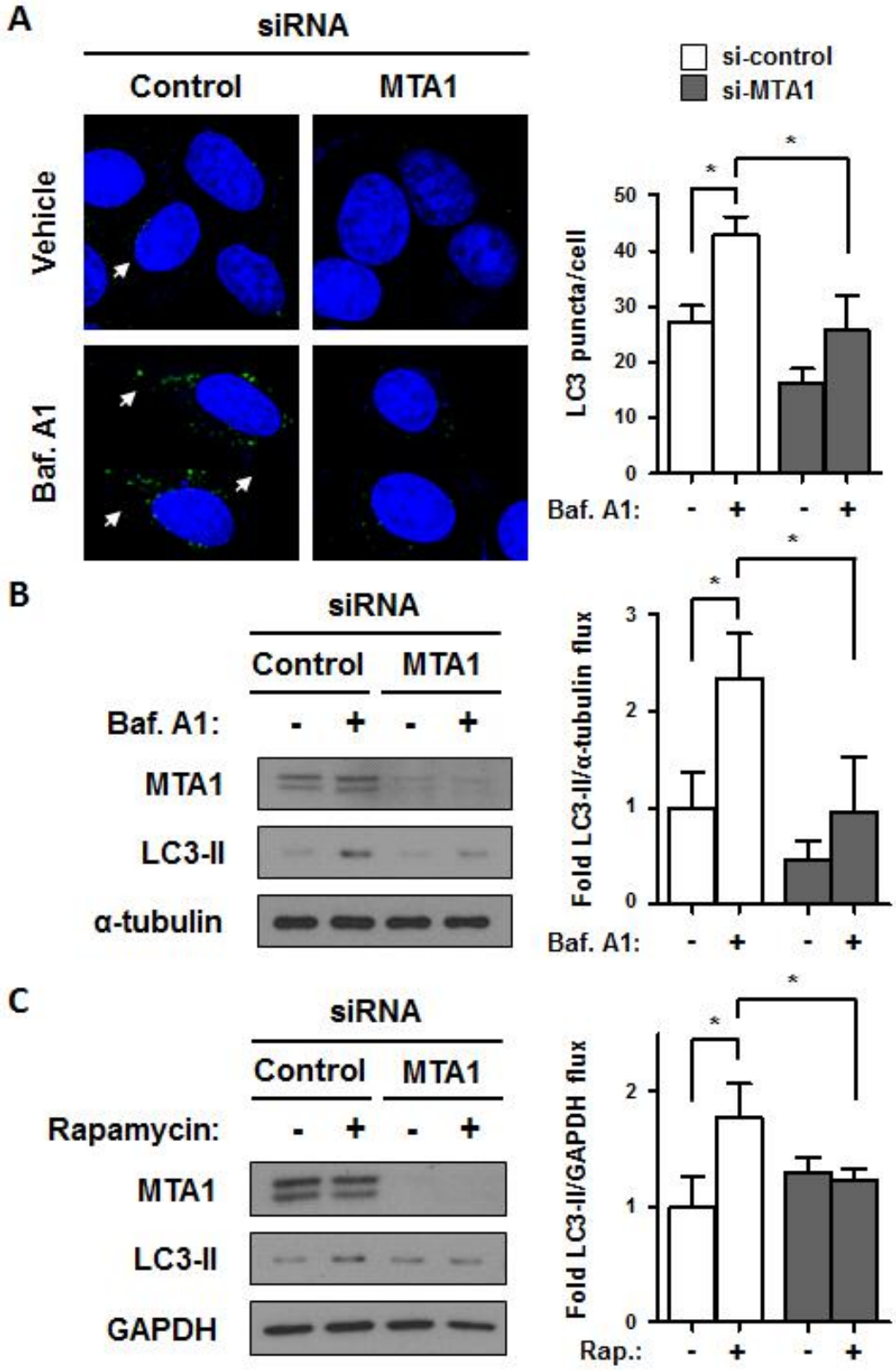
comparing the amount of LC3-II among samples is considered and has been chosen to be a standard method. To further confirm the involvement of MTA1 in autophagy, the autophagic flux by bafilomycin A1 treatment in MCF7 was observed via western blot assay. The protein level of LC3-II accumulated in control cells was greater than that in MTA1 transient knockdown cells, which is in line with the result shown by immunofluorescence assay (Figure 8B). In addition, an autophagy initiation inducer, rapamycin, was used to examine whether MTA1 affects autophagy initiation. The induction of autophagy by rapamycin was observed in the control cells through the increase in LC3-II, whereas the phenomenon was not visible in MTA1 depleted cells (Figure 8C). These data suggest that MTA1 positively regulates autophagy.

4. MTA1 promotes 4OHT-induced autophagy

Tamoxifen is known to induce autophagy, which is a cellular stress response against the anticancer drug. Nonetheless, autophagy acts as a survival process which can eventually lead to the resistance to anticancer therapy. To investigate the role of MTA1 under tamoxifen treatment, the induction of autophagy by 4OHT treatment was first observed as a positive control experiment. Acridine orange staining method visualizes acidic vesicles, including autolysosomes, in bright orange. 4OHT increased the amount of autolysosomes in both MCF7 and T47D cells in a dose-dependent manner (Figure 9A). Since acridine orange also stains lysosomes that are not a part of the

autophagy process, the result from the staining experiment can be misleading. To exclude the possibility of false interpretation, LC3 punctate formation was monitored through immunofluorescence assay. Punctate dots of endogenous LC3 was increased by 4OHT in a dose-dependent manner, confirming the result from the acridine orange staining (Figure 9B).

To examine the involvement of MTA1 in 4OHT-induced autophagy, we used RNA interference against MTA1 in MCF7 cells. The increase in LC3 punctate formation by 4OHT was visible in both control and siMTA1 cells, but the amount of increase was significantly lower in MTA1 knockdown cells (Figure 9C). The induction of LC3-II by 4OHT in control MCF7 cells was observed via western blot assay, but the induction was not significant in MTA1-depleted cells (Figure 9D). Likewise, similar results were obtained from T47D cells (Figure 9E). These results indicate that MTA1 enhances autophagy under 4OHT treatment.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Control
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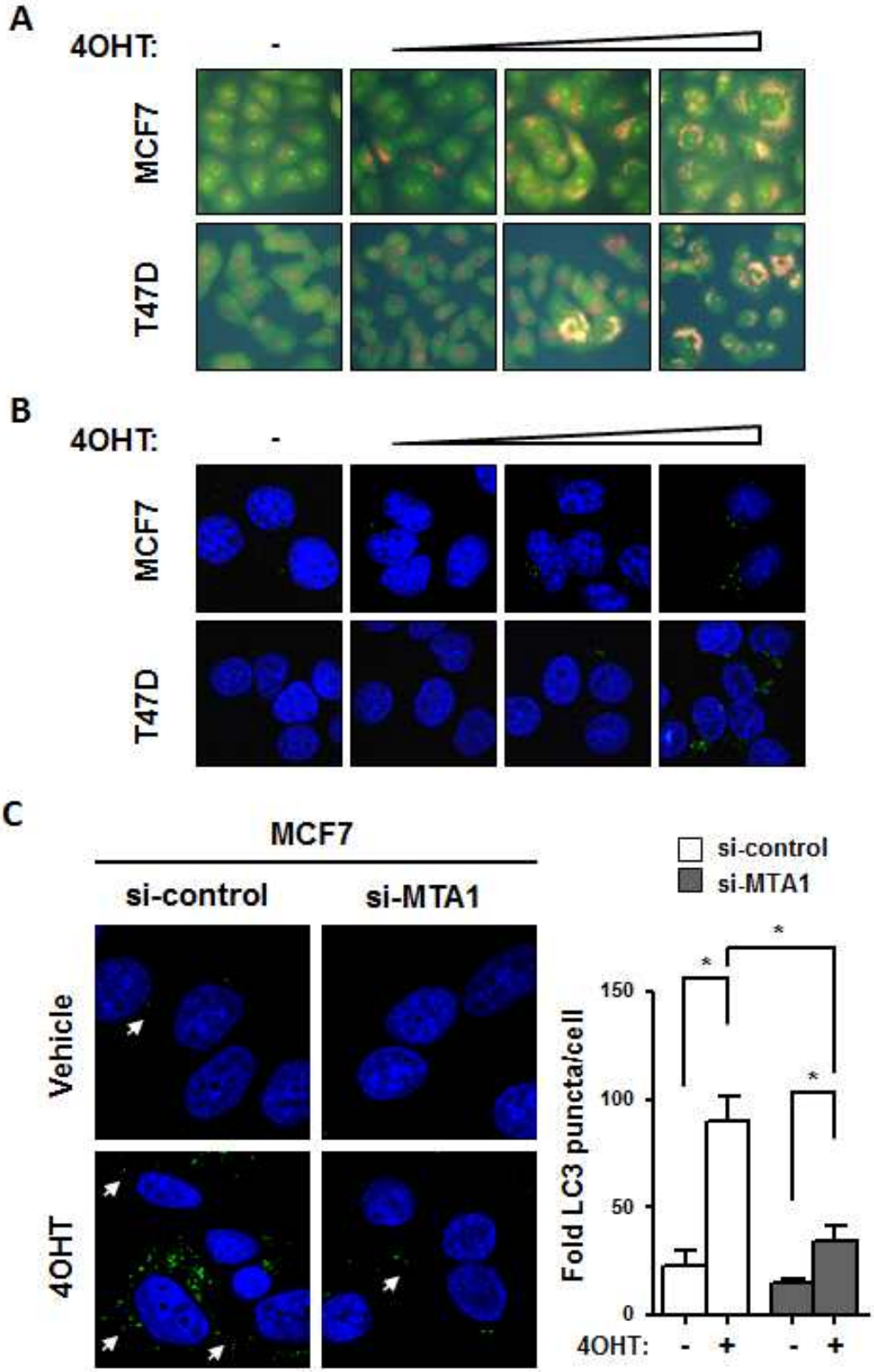
Baf. A1: - + - +
MTA1
LC3-II
α-tubulin
Fold LC3-II/α-tubulin flux
3
2
1
0
Baf. A1: - + - +
C
siRNA
Control
MTA1
Rapamycin: - + - +
MTA1
LC3-II
GAPDH
Fold LC3-II/GAPDH flux
2
1
0
Rap.: - + - +

Figure 8. MTA1 promotes basal autophagy

(A) MCF7 cells were transfected with siRNAs for a control (si-GL3) or MTA1, and then treated with either a vehicle or 20 nM bafilomycin A1 for 1 hour. Then cells were immunostained with LC3 antibody, and the endogenous LC3 punctate dots were observed by confocal microscopy. The quantification of the number of LC3 puncta per cell was obtained by the ImageJ software. Data are means ± SD from four independent experiments. *$p < 0.05$.

(B) Immunoblot analysis of MTA1 and LC3 in MCF7 cells transfected and treated as in (A). The level of LC3-II to α-tubulin was quantified by the ImageJ software. Data are means ± SD from four independent experiments. *$p < 0.05$.

(C) Immunoblot analysis of MTA1 and LC3 in MCF7 cells transfected as in (A) and treated with 100 nM rapamycin for 24 hours. The level of LC3-II to GAPDH was quantified by the ImageJ software. Data are means ± SD from four independent experiments. *$p < 0.05$.


A
4OHT:
-
MCF7
T47D
B
4OHT:
-
MCF7
T47D
C
MCF7
si-control
si-MTA1
Vehicle
4OHT
si-control
si-MTA1
Fold LC3 puncta/cell
150
100
50
0
*
*
*
4OHT:
-
+
-
+

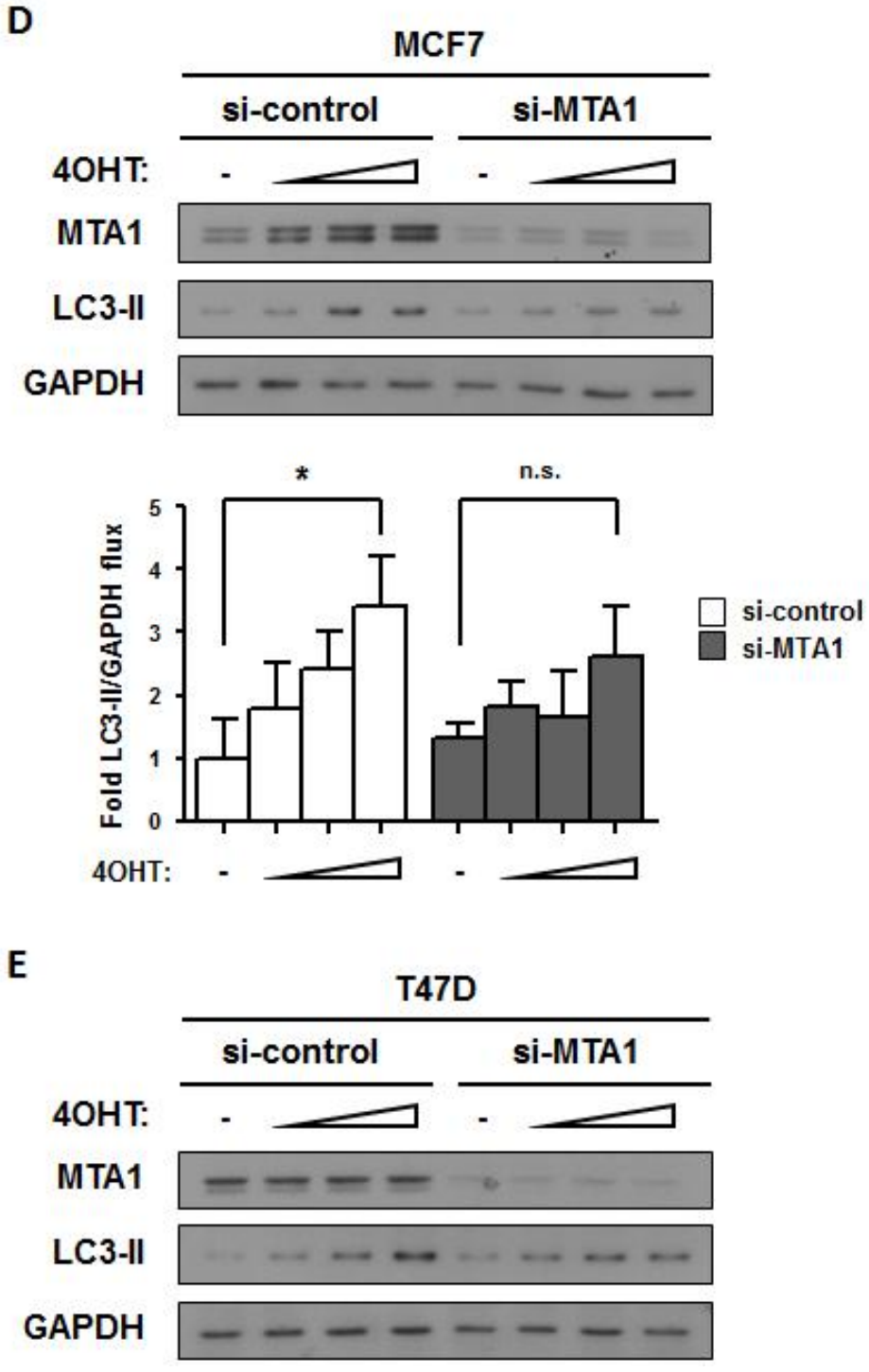

D
MCF7
si-control
si-MTA1
4OHT:
-
-
MTA1
LC3-II
GAPDH
*
n.s.
Fold LC3-II/GAPDH flux
0
1
2
3
4
5
si-control
si-MTA1
4OHT:
-
-
E
T47D
si-control
si-MTA1
4OHT:
-
-
MTA1
LC3-II
GAPDH

Figure 9. MTA1 promotes 4OHT-induced autophagy

(A) MCF7 and T47D cells treated with 4OHT in a dose-dependent manner (1, 2, 5uM) for 24 hours and were subjected to acridine orange staining.

(B) Immunofluorescence analysis of LC3 puncta in MCF7 and T47D cells treated as in (A).

(C) MCF7 cells were transfected with siRNAs for a control (si-GFP) or MTA1, and then treated with 5 uM 4OHT for 24 hours. Cells were immunostained with LC3 antibody, and the endogenous LC3 punctate dots were observed by confocal microscopy. The quantification of the number of LC3 puncta per cell was obtained by the ImageJ software, counted from a minimum of 100 cells per sample. $*p < 0.05$.

(D) Immunoblot analysis of MTA1 and LC3 in MCF7 cells transfected as in (C). 4OHT was treated at a concentration of 1, 2, 5 uM. The level of LC3-II to GAPDH was quantified by the ImageJ software. Data are means ± SD from three independent experiments. $*p < 0.05$.

(E) Immunoblot analysis of MTA1 and LC3 in T47D cells transfected and treated as in (C).

5. MTA1 enhances ATG9B upregulation in the presence of 4OHT

MTA1 is a transcription factor that has both a repressive and inductive function (Wang, 2014). To understand the regulation of the autophagic pathway by MTA1, we examined whether transcription of *Atg* genes is affected by MTA1. Depletion of MTA1 reduced 4OHT-induced ATG9B mRNA expression level (Figure 10). Neither 4OHT nor MTA1 affected the mRNA expression level of other *Atg* genes, such as ATG3, ATG4B, ATG5, ATG6 (Beclin1), ATG7, ATG8 (LC3B), and ATG12. These findings demonstrate that MTA1 enhances the transcription of ATG9B in the presence of 4OHT to promote autophagy.

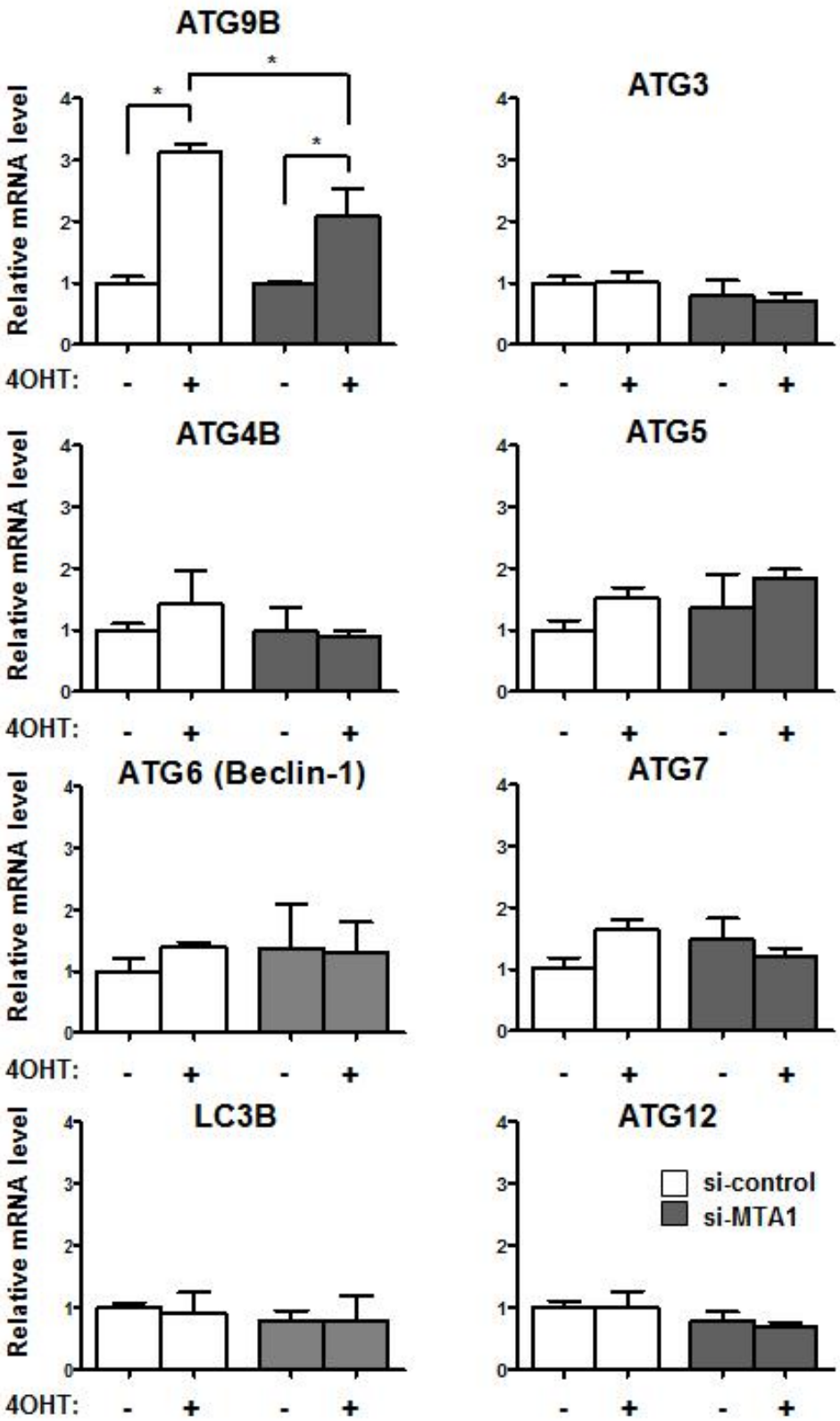

ATG9B
ATG3
ATG4B
ATG5
ATG6 (Beclin-1)
ATG7
LC3B
ATG12
Relative mRNA level
4OHT: - + - +
si-control
si-MTA1
*
*
*

Figure 10. MTA1 promotes 4OHT-induced ATG9B upregulation

MCF7 cells were transfected with siRNAs for a control (si-GL3) or MTA1, and then treated with 2uM 4OHT for three days. Total RNA isolated from cells was subjected to quantitative RT-PCR analysis of mRNA for ATG9B, ATG3, ATG4B, ATG5, ATG6 (Beclin-1), ATG7, LC3B, and ATG12. $*p < 0.05$.

V. DISCUSSION

Treatment of ERα-positive breast cancer has primarily relied on tamoxifen, an anticancer drug used for endocrine therapy (Early Breast Cancer Trialists' Collaborative Group, 2005). However, resistance to tamoxifen treatment has been a major obstacle as the patients have innate or acquired resistance to the therapy (Mandlekar *et al.*, 2001). In an effort to elucidate the mechanism behind the resistance, several studies have identified proteins and pathways that may be involved in endocrine resistance, but thus far, the involvement of MTA1 in tamoxifen resistance has not been investigated.

In the present study, we showed that MTA1 and autophagy was upregulated in tamoxifen-resistant MCF7 cell line (Figure 6). The role of autophagy in cancer is still debatable, but more studies recently proposed that autophagy plays a vital role in the acquisition of resistance to anticancer drugs (Carew *et al.*, 2012). This led us to investigate whether MTA1 contributes to tamoxifen resistance and if autophagy links the acquisition process. Our data revealed that MTA1 depletion increased tamoxifen sensitivity in both MCF7 and T47D ER α-positive breast cancer cell lines (Figure 7), suggesting that MTA1 is involved in the resistance mechanism. Furthermore, we have found

that MTA1 promotes basal autophagy (Figure 8), thereby supporting the hypothesis that MTA1 confers tamoxifen resistance by regulating autophagy.

Cellular survival promoted by autophagy induction under tamoxifen treatment in ERα-positive breast cancer could ironically lead to the resistance to the endocrine therapy (Samaddar *et al.*, 2008). Our data presented that knockdown of MTA1 attenuated 4OHT-induced autophagy through regulating ATG9B transcription (Figure 9 and 10). ATG9B is known to participate in vesicle formation and elongation of autophagosomes in the elongation stage of autophagic process (Settembre *et al.*, 2011). Transcription factor EB (TFEB), a master regulator for lysosomal biogenesis, directly targets ATG9B in the lysosomal-autophagic pathway under starvation (Settembre *et al.*, 2013). Along with other pathways concerning tamoxifen-induced autophagy, we propose that ATG9B may play a vital role in the autophagic pathway under tamoxifen treatment. Whether tamoxifen modulates TFEB to promote ATG9B upregulation needs further investigation.

We also examined whether other signaling pathways towards autophagy, such as AKT, AMPK, Bcl-2, ERK, JNK, and Stat3, were affected by MTA1, but difference in either activation or levels of proteins could not be found (data not shown). Moreover, our data are yet to elucidate the mechanism behind the stimulation of basal autophagy by MTA1. Further experiments using

MTA1-overexpressing ERα-positive breast cancer cell line and tamoxifen-resistant cell line are necessary. Our findings help to understand the potential contribution of MTA1 to the acquisition of resistance via the induction of autophagy (Figure 11).

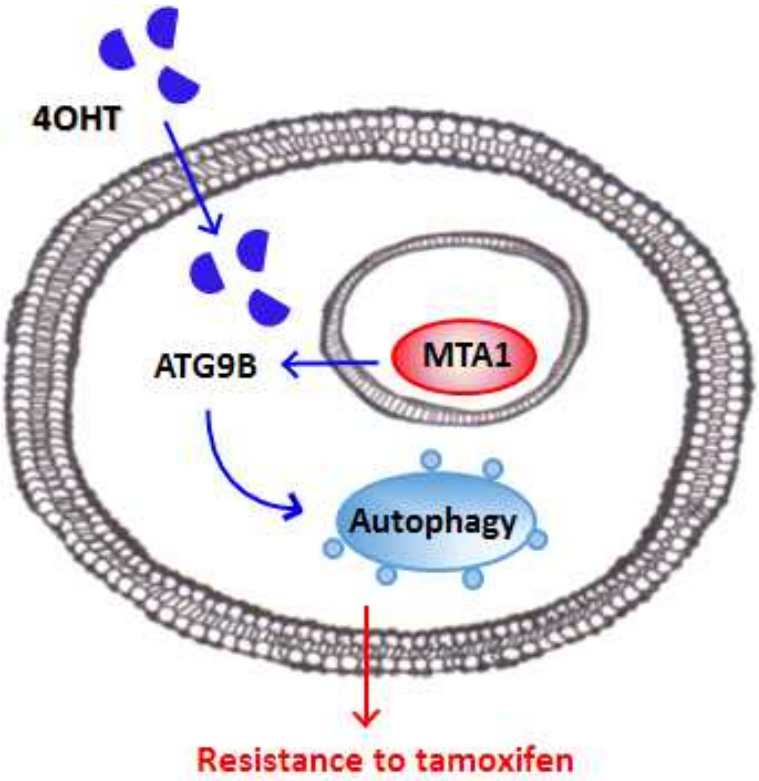


Figure 11. Schematic illustration of MTA1-autophagy pathway under tamoxifen treatment in ERα-positive breast cancer

MTA1 induces ATG9B transcription to promote autophagy under 4OHT treatment, which may lead to tamoxifen resistance.

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국 문 초 록

자가포식작용(Autophagy)은 새포 내의 오래된 단백질이나 세포 내 소기관을 분해하는 세포 재활용 작용으로서 최근 다양한 암에서 약제 내성과 연관되어있다고 알려지고 있다. 다수의 연구에서 타목시펜(tamoxifen) 내성 유방암에서 자가포식작용의 증가가 보고된 바 있다. 흥미롭게도, 본 연구진은 타목시펜 내성 MCF7 유방암 세포주에서 metastasis-associated protein 1 (MTA1)이 과발현되어 있는 것을 확인하였으며, 이로부터 MTA1과 자가포식작용으로 유도된 타목시펜 내성과의 잠재적 연관성을 확인할 수 있었다. 우선, MTA1 타목시펜 저항성에 기여하는지 알아보기 위해서 MCF7 세포주에 RNA interference를 통하여 MTA1의 발현을 감소시킨 후 타목시펜의 활성체(4OHT)를 처리하였다. 그 결과, MTA1 발현 억제가 4OHT에 대한 감수성을 증대시켰고, MTA1의 저발현이 자가포식작용을 감소시킨 것을 확인하였다. 동시에 자가포식작용의 대표적인 지표로 알려진 LC3-II의 단백질량이 감소됨을 확인하였다. 타목시펜은 세포내의 자가포식작용을 유도한다고 알려져 있는데, 이는 세포내 항상성을 유지하기 위한 외부 자극에 대한 반응이다. 하지만, 타목시펜에 의해 유도된 자가포식작용은 역설적이게도 그 약에 대한 내성을 유발한다고 알려져있다. 본 연구 결과 MTA1의 저발현이 4OHT에 유도된 자가포식작용을 감소시키는 것으로 나타났다. 또한, 정량적 RT-PCR 실험을 통해 MTA1 감소가 4OHT에 유도된 ATG9B (자기탐식세포(autophagosome)의 연장 과정에 관여하는 autophagy-related gene (ATG))의 증가를 억제하는 것을 확인하였다.

이상의 결과는 에스트로젠 수용체 (ER) α 양성 유방암 환자의 호르몬 치료시 약제 내성 유발에서 MTA1의 역할에 대한 이해도를 높여준다.

주요어: MTA1, 자가포식작용, 타목시펜 내성, 유방암