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Pathological implication of S-adenosylmethionine increase by MAT2A up-regulation in tamoxifen-resistant breast cancer cells

Chosun University Graduate School

College of Pharmacy

By

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타목시펜 저항성 유방암 세포에서 MAT2A 과다발현 에 의한 S-adenosylmethionine 증가 및 병리학적 의미

Pathological implication of S-adenosylmethionine increase by MAT2A up-regulation in tamoxifen-resistant breast cancer cells

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약학과

응구엔띠뚜이퐁

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ABSTRACT

Pathological implication of S-adenosylmethionine increase by MAT2A up-regulation in tamoxifen-resistant breast cancer cells

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Development of acquired resistance to anti-estrogen tamoxifen (TAM) is a serious clinical problem for the treatment of breast cancer patients. Methionine adenosyltransferase (MAT) encoded by two genes *MAT1A* and *MAT2A* is a key enzyme in cellular metabolism which catalyzes the synthesis of S-adenosylmethionine (SAM). It has been also known that increase in MAT2A expression facilitates cell growth, whereas SAM controls cellular fate to growth or apoptosis. Here, we discovered that the MAT2A expression and SAM level were increased in TAM-resistant breast cancer cells (TAMR-MCF-7) cells compared to control MCF-7 cells. The enhanced expression of MAT2A in TAMR-MCF-7 cells was suppressed by NF-κB inhibition or Nrf2 knockdown. Increase in SAM with the activation of DNA methyltrasferase (DNMT) could cause an aberrant hypermethylation in gene promoter and subsequently silences gene expression. Methylation-specific PCR analyses revealed that two sites of PTEN promoters were methylated in TAMR-MCF-7 cells, which resulted in the down-regulation of PTEN expression and the increase in Akt phosphorylation. Both the loss of PTEN expression and the increased Akt phosphorylation in TAMR-MCF-7 cells were completely reversed by 5-aza-2′-deoxycytidine (5-Aza, a DNMT inhibitor). In addition, 5-Aza treatment inhibited the basal cell proliferation rate of TAMR-MCF-7 cells and intra-peritoneal injection of 5 mg/kg 5-Aza significantly suppressed TAMR-MCF-7 tumor growth in xenograft study. These results suggest that MAT2A-mediated SAM

increase and methylation in PTEN promoter are potential therapeutic targets to reverse TAM resistance in breast cancer.

Key word: Tamoxifen resistance, PTEN, methylation, S-adenosylmethionine, MAT2A.

1. Introduction

Breast cancer is the most common malignancy in Western woman and grows under hormone-dependent control. Estrogen is the main stimulant in the development and growth of breast cancer (1, 2). Over the past three decades, the estrogen receptor antagonist tamoxifen (TAM) has been mainstay of hormonal therapy and has used worldwide for women with estrogen receptor (ER) positive breast cancer (3). Although most patients are initially responsive, acquired TAM resistance is a critical problem for anti-estrogen therapy (4-7) and the mechanism occur remains elusive. To mimic TAM-resistace in ER-positive breast cancer patients, we and others have established an MCF-7 derived TAM-resistant cell line (TAMR-MCF-7 cells) by long-term (> 9 months) culture of MCF-7 cells with 4-hydroxytamoxifen (8, 9).

Methionine adenosyltransferase (MAT) gene is one of genes required for survival of an organism and is the only enzyme responsible for the formation of S-adenosylmethionine (SAM), a principal biological methyl donor, from methionine and ATP (10, 11, 12). MAT is a product of two genes: *MAT1A* and *MAT2A* which encode for two homologous MAT catalytic subunits, α1 and α2 (13). MAT1A is expressed mostly in liver and is a marker for normal differentiated or mature liver phenotype (10), whereas MAT2A is widely distributed and predominate in the fetal liver and is gradually replaced by MAT1A during liver development. In contrast to MAT1A, MAT2A is a marker for rapid growth and dedifferentiation (10). In hepatocytes, increased MAT2A expression was reported to associate with cancer growth and malignant degeneration (10, 14-17). In addition, the role of SAM was known as a key metabolite that regulates hapatocyte growth and differentiation (17). Outside of the liver, MAT2A expression and SAM levels have been also reported to increase in activated T-lymphocyte (18) and in human colon cancer cells where MAT2A expression was shown to be important for cell proliferation and was required for the mitogens to induce cell growth (19). However, studies about MAT2A expression and SAM levels as well as their roles to tumor growth and chemo-resistance in TAM-resistant breast cancer cells have been uncovered.

In the liver, up to half of the daily intake of methionine is converted to SAM and up to 85% of all methylation reactions takes place (20). DNMTs are enzymes that catalyze transfer methyl group from SAM to DNA, including DNMT1, DNMT3a and DNMT3b. Methylation of DNA occurs on cytosine residues of the CpG dinucleotides in DNA. This epigenetic alteration in DNA is heritable but does not alter nucleotide sequence, in contrast to genetic changes (21, 22). It has been reported that gene silencing by methylation may be an important mechanism of carcinogenesis whereby critical genes normally involved in tumor suppression may be switched off. On the other hand, overexpression of DNMT1 and DNMT3b has been also found to associate with DNA hypermethylation in malignant cell and transcriptional silencing of tumor suppressor genes (23). Thereby, DNA methylation plays a crucial role in the development of nearly all types of cancer (21, 22).

PTEN is a product of tumor suppressor gene and functions as a phosphatase negatively regulate intracellular levels of phosphatidylinositol-3,4,5-triphosphate and phosphatidylinositol 3-kinase (PI3-kinase)/Akt signaling pathway (24) which plays a fundamental role in cell proliferation and survival when aberrantly activated in tumorigenesis (25, 26). One of the silencing mechanisms of *PTEN* gene was reported to relate to methylation in this gene promoter in diverse cancer types (27-34). And it was also documented that aberrant methylation and hence silencing of the *PTEN* gene was associated to the activating genetic alterations in the PI3K/AKT pathway and contributed to progression of thyroid tumors (35). Persistent activation of PI3-kinase/Akt was a common phenomenon and was required for anti-estrogen resistance in human breast cancer cells (36, 37). Although role of DNA methylation associated to inactivation of *PTEN* gene has been reported in invasive breast carcinomas (38), influence of methylation on loss of PTEN expression in TAM-resistant breast cancer cells has not been studied.

In this study, we focused on the gene expression of PTEN to further clarify the relationship between the increased SAM levels and the methylation status in TAMR-MCF-7 cells, whether increased SAM could lead to aberrant methylation in this cell type, which could contribute partly in finding the underlying mechanism of PTEN expression loss and TAM resistance in this cell type. Here, we revealed for the first time

that MAT2A gene expression was up-regulated in TAMR-MCF-7 cells and consequently SAM synthesis increased. Moreover, increased SAM synthesis with the enhanced DNA methyltransferase 1 (DNMT1) expression leaded to methylation of *PTEN* gene promoter, and caused to silencing of this gene which coexists with activating alternation of PI3-kinase/Akt pathway and then contributed to cell proliferation and tumor growth of TAMR-MCF-7 cells.

2. Materials and methods

2-1. Materials

5-aza-2'-deoxycytidine was obtained from Sigma (St. Louis, MO). Antibodies against MAT2A, PTEN, NF-E2-related factor2 (Nrf2), P65, c-Jun, DNMT1 and DNMT3b were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies targeting phosphorylated Akt and Akt were obtained from Cell signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated donkey anti-rabbit, anti-goat IgG, and alkaline phophatase-conjugated donkey anti-mouse IgG were supplied from Jackson Immunoresearch Laboratories (West Grove, PA). Anti-actin antibody and most of the reagents used for molecular studies were obtained from Sigma (St. Louis, MO). MAT2A-luc reporter plasmid was kindly donated from Dr. Shelly C. Lu (University of Southern California, CA). Some plasmids were used in this study such as I-κBα overexpression plasmid and pGL-ARE minimal reporter (containing three copies of the ARE region of the quinone oxidoreductase promoter) was donated from Dr. KY Lee (Chonnam National University, Korea) and Dr. MK Kwak (Yeungnam University, Korea), respectively. pNF-κB-luc and pAP-1-luc reporter plasmids were purchased from Stratagene (La Jolla, CA).

2-2. Cell culture and establishment of TAMR-MCF-7 cells

MCF-7 cells were cultured at 37°C in 5% CO_2/95% air in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 units/ml penicillin, and 100μg/ml streptomycin. TAMR-MCF-7 cells were established by using the

methods previously reported (8). Briefly, MCF-7 cells were washed with PBS, and the culture medium was changed to phenol-red-free DMEM containing 10% charcoal-stripped, steroid-depleted FBS (Hyclone, Logan, UT) and 4-hydroxytamoxifen (0.1 μM). The cells were continuously exposed to this treatment regimen for 2 weeks and the concentratin of 4-hydroxytamoxifen was gradually increased to 3 μM over a 9 month period. Initially, the cell growth rates were reduced. However, after exposed to the medium for 9 months, the rate of cell growth gradually increased, showing the establishment of a tamoxifen-resistant cell line (9).

2-3. Immunoblot Analysis

After washing with sterile PBS, the cells were lysed in lysis buffer containing 20 mM Tris-Cl (pH 7.5), 1% Triton X-100, 137 mM sodium chloride, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 25 mM β-glycerolphosphate, 2 mM sodium inorganic pyrophosphate, 1 mM phenylmethylsulfonylfluoride, and 1 μg/ml leupeptin. Total cell lysates were centrifuged at 10,000*g* for 10 min to remove the debris, and the proteins were fractionated using a 10% separating gel. The fractionated proteins were then transferred electrophoretically to nitrocellulose paper, and the proteins were immunoblotted with specific antibodies. For tumor tissues from nude mice, tumor tissues were homogenized in PBS, and then centrifuged at 10,000g for 20 min and supernatants were used for immunoblottings.

2-4. Reported Gene Analysis

A dual-luciferase reporter gene assay system (Promega, Madison, WI) was used to determine promoter activity. Briefly, cells were plated in 12 well plates and transiently transfected with 1 μg/ml reporter plasmids and phRL-SV plasmid (*hRenilla* luciferase expression for normalization) using Hillymax reagent (Dojindo Molecular Technologies, Gaithersburg, MD). The cells were then incubated in culture medium without serum for 18 h. Firefly and hRenilla luciferase activities in the cell lysates were measured using a luminometer (LB941, Berthold Technologies, Bad Wild, Germany). The relative luciferase activities were calculated by normalizing the promoter-driven firefly luciferase activity to the hRenilla luciferase.

2-5. Methylation-specific PCR

Methylation status of PTEN promoter region was determined by methylation-specific PCR after bisulfite-modification. The methylation targets of PTEN gene promoter were three sites (A, B and C region) (39). Genomic DNA was isolated and was modified by bisulfite reaction using an EpiTect Bisulfite kit (Qiagen, US). Methylated and unmethylated genomic regions can be amplified by PCR using each sequence-specific pairs of primers (39).

2-6. Measurement of SAM

MCF-7 and TAMR-MCF-7 cells were harvested by scraping into 5% perchloric acid. Cell lysates were centrifuged at 14,000 g and 4°C for 20 min. The supernatant was then removed, and SAM concentration was determined using the HPLC method of She et al. (1999) (40). HPLC equipment was from Shimadze: pump, model LC-10AT; system controller, model SCL-10A; injector, Rheodyne equipped with a 20-µl loop; detector, model SPD-20A. The supernatant was directly applied to the HPLC system with a TSK-GEL ODS-80TM column (4.6 × 250 mm) (Tosoh). To determine protein concentrations, the pellets were dissolved in 1 ml 0.1 M NaOH. Protein concentrations were determined using a BCA^{TM} Protein Assay Kit.

2-7. Measurement of cell proliferation

After exposure of cells to 10% FBS containing medium for the indicated time, viable adherent cells were stained with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] (2 mg/mL) for 4 h. Media were then removed and the formazan crystal-stained cells were dissolved in 200 µl dimethylsulfoxide. Absorbance was assayed at 540 nm using a microtiter plate reader (Berthold Tech., Bad Wildbad, Germany).

2-8. Flow cytometry

TAMR-MCF-7 cells were incubated in serum-free DMEM with 10 µM 4-Hydroxytamoxifen in the presence or absence of 5-Aza (30µM) for 24hrs. The cells

were harvested with trypsin treatment, stained with both annexin V –FITC and propidium iodize according to the manufacture's protocol (Invitrogen) and analysed by flow cytometry (FACStar, BD Biosciences) set for FL1 (annexin V) and FL2 (propidium iodide). A total of 10^4 cells were counted for each sample.

2-9. Xenograft Study

6 weeks old BALB/c athymic nude mice (Joongang Experimental Animal, Seoul, Korea) were inoculated subcutaneously with 5×10^6 TAMR-MCF-7 cells. When tumors reached ~200 mm^3 (about 14 days), the mice were randomly allocated to control and 5-Aza-treated group. 5 mg/kg 5-Aza was intraperitoneally injected every 6 days for 21 days. Tumor volumes were measured as described previously (41). Animal care was maintained in accordance with Pusan University institutional guidelines.

After sacrificing the mice, excised tumors were fixed in 10% buffered formalin and embedded in paraffin. For the pathology examination, 4 μm thick tissue sections were stained with H&E. Immunohistochemical staining was done with the avidin-biotin complex method using an anti-proliferating cell nuclear antigen (PCNA) antibody. Immune reactions were visualized with 3, 3- diaminobenzidine and counterstained with Mayer's hematoxylin. Tissue TUNEL assays were performed using ApopTag Plus Peroxidase In Situ Apoptosis Detection kits (Intergen, Purchase, NY) according to the manufacturer's instructions. Briefly, slides were deparaffinized and immersed in 3% hydrogen peroxide to block endogenous peroxidase. Then, slides were incubated with reaction buffer containing terminal deoxynucleotidyl transferase at 37°C for 1 h. Slides were then incubated with peroxidase-conjugated anti-digoxigenin antibody for 30 min, and the reaction products were visualized with 0.03% 3,3-diaminobenzidine solution containing 2 mmol hydrogen peroxide. Counterstaining was achieved with 0.5% methyl green. The PCNA and TUNEL-positive cells were counted and represented as the average of the five highest areas within a single x200 field. A portion of tumor tissues was homogenized and subjected to immunoblotting for PTEN and phosphorylated Akt.

2-10. Immunohistochemistry for human cancer tissues

Blocks for all the sample were consecutively cut in 4μm sections and mounted on poly-l-lysine coated glass slides. Xylene was used to remove the paraffin from the sections, and the samples were rehydrated. Antigen retrieval was performed by boiling sections for 5minutes three times in a 10^{-3} M sodium citrate buffer (pH 6.0) in microwave oven. After natural cooling, endogenous peroxidase activity was blocked using 3% hydrogen peroxide in methanol for 10minutes, followed by washing three times with PBS (pH 7.6). Sections were then incubated with anti-PTEN antibody (Santa Cruz, CA) at 4^0C overnight. Following washing three times with PBS, sections were incubated with HRP-Polymer anti mouse IgG, respectively for 30minutes and washed three times with PBS. The color was developed by incubation with DAB solution using DAB detection kit (Golden Bridge International, Inc. Mukilteo, WA). Finally sections were counterstained with hematoxylin, dehydrated, mounted, and observed. PTEN expression level between Tam-resistant and sensitive human breast cancer tissues were compared.

2-11. Statistical Analysis

Scanning densitometry was done using LAS-3000 mini (Fujifilm). Paired Student's t test was used to examine the significant intergroup differences. Statistical significance was accepted at either $P < 0.05$ or $P < 0.01$.

3. Results

3-1. Up-regulation of MAT2A leads to increased synthesis of SAM in TAMR-MCF-7 cells

SAM functions as the principal methyl donor and the precursor for polyamine biosynthesis in body. Moreover, recent studies have revealed that SAM is a critical regulator to determine cellular fate, growth or apoptosis in liver (17). Because increased MAT2A expression facilitates cancer cell growth (17) and relevant to SAM synthesis, we first compared the MAT2A expression levels in control MCF-7 and TAMR-MCF-7 by western blot analyses. MAT2A protein levels in TAM-MCF-7 cells were distinctly higher than those in MCF-7 cells (Fig. 1A). Next, we performed reporter gene analysis using a MAT2A-Luc reporter plasmid containing the luciferase structural gene and -570/+61 bp human MAT2A promoter. MAT2A-luc reporter activity was significantly increased in TAMR-MCF-7 cells compared to MCF-7 cells (Fig. 1B), which suggests that the enhanced MAT2A expression results from the transcriptional activation of *MAT2A* gene in TAMR-MCF-7. Consistent with these, SAM levels in TAMR-MCF-7 is also higher than those in MCF-7 cells (Fig. 1C).

3-2. NF-κB and Nrf2 are required for MAT2A overexpression in TAMR-MCF-7

We were then interested in which transcription factor(s) was responsible for the enhanced *MAT2A* gene transcription in TAMR-MCF-7 cells. The promoter region of MAT2A gene contains several transcription factor binding sites including c-Myb, specific protein1, nuclear factor-κB (NF-κB), activator protein-1 (AP-1) and Nrf2. DNase protection assay showed that the protein binding to specific promoter region of human *MAT2A* gene (-354 to -312 bp) was increased in hepatocarcinoma as compared with normal liver (42) and four consensus elements responsible for NF-κB, AP-1, c-Myb and Nrf2 were located in this region. Among them, NF-κB and AP-1 play pivotal role in tumor necrosis factor-α stimulated transactivation of *MAT2A* gene in HepG2 cells (43). Hence, we assessed activation of each transcription factor in TAMR-MCF-7

cells and tested their possible involvements in MAT2A expression. Reporter gene and western blot analyses confirmed that the basal reporter activities of AP-1, NF-κB and Nrf2/antioxidant response element (ARE) and the nuclear levels of c-Jun, p65 and Nrf2 were all enhanced in TAMR-MCF-7 cells, compared to those in control MCF-7 cells (Fig. 2A). We previous revealed that c-Jun was selectively up-regulated in TAMR-MCF-7 cells and the enhanced AP-1 activity was dependent on c-Jun (44). When TAMR-MCF-7 cells were transfected with c-Jun siRNA, the increased MAT2A expression and MAT2A-Luc reporter activity were not changed in this cell type (Fig. 2B). However, exposure of the cells with tosyl phenylalanyl chloromethyl ketone (TPCK, 5 or 10 μM), a NF-κB inhibitor for 24 h, significantly suppressed the basal expression and reporter activity of MAT2A in TAMR-MCF-7 cells (Fig 2C). To confirm the role of NF-κB in MAT2A up-regulation in TAMR-MCF-7 cells, the cells were co-transfected MAT2A-luc with I-KBα-overexpression plasmid (IKBα-WT). As shown in Fig. 2C, MAT2A reporter activity was significantly diminished by IKBα overexpression plasmid. Noticeably, Nrf2 knock-down by Nrf2 shRNA also reduced the basal expression of MAT2A in TAMR-MCF-7 cells, however MAT2A-Luc reporter activity was not changed by Nrf2 inhibition (Fig. 2D). To clarify the role of Nrf2, MCF-7 cells were treated with *tert*-butylhydroquinone (t-BHQ) (1, 3, 10, 30 μM), a representative pro-oxidant which have been reported to induce diverse phase II detoxifying enzyme through Nrf2 activation (45). T-BHQ also induced MAT2A in a concentration-dependent manner (Fig. 2E). These implicate that persistent activation of both NF-κB and Nrf2 in TAMR-MCF-7 cells is critical for the up-regulation of MAT2A.

3-3. PTEN down-regulation by hypermethylation of PTEN gene promoter causes sustained activation of PI3-kinase in TAMR-MCF-7 cells

Since SAM is a key mediator for biological methylation reactions, increased SAM in TAMR-MCF-7 cells could lead to hypermethylation in promoter region of some genes and consequently results in silencing of these genes. DNMTs mediate transferring methyl group from SAM to DNA. Moreover, DNMT1 and DNMT3b are mainly involved in the DNA methylation reactions of breast cancer cells (46). When we determined the expression levels of DNMT1 and DNMT3b in TAMR-MCF-7 cells, the

basal expression of DNMT1 was enhanced in TAMR-MCF-7 compared to control MCF-7 cells; however DNMT3b was not detected in both MCF-7 and TAMR-MCF-7 cells (Fig. 3A).

The activation of PI3-kinase/Akt pathway is considered as a key event for TAM resistance acquisition in breast cancer cells. PI3-kinase inhibition restores anti-estrogen responsiveness in both long-term estrogen depleted xenograft model and anti-estrogen resistant breast cancer cell lines (37, 47). Given both the importance of PTEN as a potential tumor suppressor gene and the function of PTEN as a specific phosphotase against PI3-kinase, we further studied the PTEN expression and the methylation status of *PTEN* gene promoter in TAMR-MCF-7 cells. Western blot analyses showed that the basal PTEN expression as well as *PTEN* gene reporter activity was diminished in TAMR-MCF-7 cells, reversely Akt phosphorylation were highly enhanced in the cells (Fig. 3B). It has been reported that PTEN inactivation is closely related with epigenetic silencing, which has been reported in diverse malignancies and promoter region of *PTEN* gene contains three methylation sites (28, 39). Methylation-specific PCR analysis revealed that the frequency of methylation of the PTEN gene promoter in sites A and B in TAMR-MCF-7 cells was higher than that in MCF-7 cells. The methylation of site C was at equal frequency in both cell types (Fig. 3C). These could be reason of loss of PTEN expression and increased PI3-kinase activity in TAMR-MCF-7 cells.

To further confirm the role of DNMT-dependent methylation of *PTEN* promoter, we used 5-aza-2'-deoxycytidine (5-Aza), a demethylation agent that inhibits the recruitment of DNMTs to DNA. Treatment of TAMR-MCF-7 cells with 5-Aza for 24 h decreased aberrant hypermethylation of PTEN gene at both site A and site B (Fig. 3C), and reversed both the loss of PTEN expression and Akt phosphorylation in TAMR-MCF-7 cells (Fig. 3B). Moreover, cycloleucine (2 mM), a MAT inhibitor, partially restored PTEN expression in TAMR-MCF-7 cells (Fig. 3D), which suggest that MAT2A-dependent SAM increase with DNMT1 overexpression is crucial for the PTEN promoter methylation and the subsequent decrease in PTEN expression in TAMR-MCF-7 cells.

3-4. 5-Aza treatment inhibits proliferation of TAMR-MCF-7 cells and induces apoptosis in TAMR-MCF-7

We then examined whether 5-Aza suppresses cell proliferation rate of TAMR-MCF-7 cells. Pretreatment of 30 μM 5-Aza for 24 h significantly inhibited cell proliferation rate of TAMR-MCF-7 cells but not of MCF-7 cells (Fig. 4A). In addition, to determine whether growth inhibition was associated with increased apoptosis, we performed flow cytometry analysis after annexin-V staining. TAMR-MCF-7 cells revealed resistance to 10 μM 4-hydroxytamoxifen treatment (Fig. 4B). However, concomitant treatment of 30 μM 5-Aza with 10 μM 4-hydroxytamoxifen in TAMR-MCF-7 cells for 24 h increased the annexin V-positive apoptotic cell population compared with the 4-hydroxytamoxifen alone-treated cells (Fig. 4B). These results indicate that 5-Aza could enhance TAM responsiveness in TAMR-MCF-7 cells.

3-5. 5-Aza treatment inhibits tumor growth in TAMR-MCF-7 cells bearing athymic nude mice

To determine *in vivo* effect of 5-Aza, we used athymic nude mice bearing TAMR-MCF-7 cells. Intraperitoneal injections of 5 mg/kg 5-Aza (every 6 days for 21days) reduced significantly tumor growth about by 42% in comparison to vehicle-treated control mice (Fig. 5A). Proliferating cell nuclear antigen (PCNA) is a representative marker for the cancer cell proliferation. Immunohistochemistry analysis showed that most of the tumor cells in the vehicle-treated control were PCNA-positive, while the number of PCNA-positive cells was significantly decreased in the tumor tissues from 5-Aza-treated mice (Fig. 5B). Moreover, 5-Aza treatments enhanced the amount of TUNEL-positive cells (a representative apoptosis index) (Fig. 5C). We further measured the expression levels of PTEN in the tumor tissue lysates. The PTEN protein expression was obviously enhanced in the 5-Aza treated tissue samples (Fig. 5D). These data confirm that DNMT-mediated methylation plays a pivotal role in the expression regulation of *PTEN* gene, which is related with proliferation of TAM-resistant breast cancer cells.

3-6. Expression changes of PTEN in TAM-resistant and –sensitive human breast cancer tissues

We finally compared the expression levels of PTEN in human breast cancer tissues. Tumor tissues were obtained from two groups of patients, depending on the occurrence of relapse after TAM therapy. The four TAM-resistant cases relapsed within 3 years from surgery, while 8 TAM-sensitive cases were disease free. As shown in Fig. 6 that PTEN immunoreactivity was significantly higher in TAM-sensitive cases than TAM-resistant cases. The result confirms that loss of PTEN expression may be related with TAM resistance acquisition in clinical situation.

4. Discussion

DNA methylation is one of two types of epigenetic mechanisms affecting transcriptional regulation. Alterations in DNA methylation has been common and has been extensively studied in many cancers including breast cancer where it has been suggested as one of causes of genes inactivation such as *p16INK4A*, *ERα*, *progesterone receptor*, *BRCA1*, *GSTP1*, *TIMP-3*, and *E-cadherin* (48, 49). A recent study showed association between DNA methylation and TAM resistance in breast cancer patients that concomitant treatment of procainamide, a DNA methyaltion inhibitor with tamoxifen restored ER-β expression and increased the TAM response in ER-α-positive and ER-β-negative breast cancers cell line (50). Our present study reveals a crucial role of methylation in transcriptional inactivation of *PTEN* gene which causes the sustained activation of PI3-kinase/Akt pathway and finally guarantees cell proliferation and resistance to TAM. Moreover, we also suggest that MAT2A induction and subsequent SAM synthesis are responsible for PTEN down-regulation in TAMR-MCF-7 cells.

MAT2A expression relates to rapid growth and ultimately carcinogenesis (10, 14-17). The knowledge about MAT2A has been mainly reported in liver and liver cancer. MAT2A is progressively replaced by MAT1A during liver development, reversely switch from MAT1A to MAT2A expression could play a pivotal pathogenetic role in liver cancer (10). One of the representative phenotypes of TAMR-MCF-7 cells is higher cell proliferation rate than parental MCF-7 cells. Thus, aberrantly increase in

MAT2A expression was discovered in TAMR-MCF-7 cells could contribute to the ability of rapid growth of this cell type. Little was known about the molecular mechanism in transcriptional regulation of MAT2A, specially there have been no reports on MAT2A regulation in TAMR-MCF-7 cells. Because activation of both NF-κB and AP-1 is essentially required for MAT2A up-regulation in TNF-α stimulated HepG2 cells (43), we first assumed that NF-κB and c-Jun/AP-1 might be potential transcription factor for MAT2A induction in TAMR-MCF-7 cells. However, the results showed that inhibition of c-Jun, a major active form of AP-1 in TAMR-MCF-7 cells (44) did not change MAT2A expression or the gene promoter reporter activity (Fig 2B). As expectation, NF-κB was found to play important role in MAT2A up-regulation in TAMR-MCF-7 (Fig. 2C). Rather, we unexpectedly found that Nrf2 shRNA efficiently reversed the increase of MAT2A protein in TAMR-MCF-7 cells. As suggested in a previous paper, an Nrf2/ARE binding region was located in -354 to -312 bp region of human MAT2A promoter (51), however Nrf2 blocking did not change the activity of reporter plasmid containing -570/+61 bp human MAT2A promoter. This raises a possibility that Nrf2 binding site(s) located in the distal region of MAT2A promoter would be functional for *MAT2A* gene transactivation in TAMR-MCF-7 cells.

Another major role of SAM is the ultimate source in polyamine biosynthesis, is required for cellular growth and repair. Increase in SAM level was believed to be necessary for polyamine biosynthesis during rapid growth period (11). Here, we showed that concomitant with MAT2A up-regulation, SAM levels were enhanced in TAMR-MCF-7 cells. SAM synthetic reaction was undertaken by MAT enzymes. First, methionine receives an adenosine group from ATP, to give SAM, SAM then transfers the methyl group to an acceptor molecule such as DNMTs in the process of DNA methylation reaction or norepinephrine in epinephrine synthesis to produce S-adenosyl homocysteine. This product is hydrolysed to homocysteine and adenosine by S-adenosylhomocystein hydrolase. L-homocysteine has two primary fates: conversion back to L-methionine which can then be converted back to SAM or conversion to L-cysteine (52, 53). In our previous study, we showed that cysteine contents in TAMR-MCF-7 cells were decreased to 21% of control MCF-7 cells (54). This may result from

decrease in biosynthesis of cysteine and reversely increase in SAM synthesis to respond for hypermethylation reactions in TAMR-MCF-7.

PTEN is an upsteam antagonistic phosphatase of PI3K. It removes the 3' phosphate of PIP3 and inhibits downsteam signaling of activated PI3-kinase (24-26, 36, 37). Previous studies well documented that decreased expression of PTEN was associated with increased phosphorylation of AKT and inhibition of apoptosis of cancer cells and loss of PTEN caused aberrant activation of the PI3K/AKT pathway and consequent tumorigenesis (25, 26, 36, 37). The PI3K/Akt pathway activated by growth factor, cytokine and integrin signalings is important for the regulation of growth, survival and inhibition of apoptosis of both normal cells and cancer cells (55, 56). Increased level of PI3K and Akt may lead to anti-estrogen resistance (57) and is required for anti-estrogen-resistant cell growth (36, 37). In this study, we confirmed that loss of PTEN expression leaded to constitutive activation of Akt in TAMR-MCF-7. Moreover, we have found down-regulation of PTEN expression in TAMR-MCF-7 is the occurrence of aberrant hypermethylation in PTEN gene promoter, which subsequently activates AKT and affects cell proliferation as well as resistance to tamoxifen.

Because DNMTs are critical regulators for DNA methylation, we compared the expression change of DNMT1 and DNMT3b in MCF-7 and TAMR-MCF-7 cells. DNMT1 was selectively overexpressed in accordance with hypermethylation occurrence in TAMR-MCF-7. We hypothesized that increased DNMT1 was responsible for DNA methylation in PTEN gene in TAMR-MCF-7. DNMT1 is the best known member of the DNMT family. Increased activity of DNMT1 is related to increase in cell proliferation (58), tumorigenesis (59), and tumor progression (60). On the other hand, inhibition of this enzyme can induce global DNA demethylation and reverse the malignant phenotype (61). It has been also reported that DNMT1 played a key role in maintenance of methylation and DNMT3B might act as an accessory DNA methyltransferase to epigenetically siclence CXCL12 gene expression in MCF-7 cells. And the demethylation agent 5-Aza exhibited the strongest effect on promoter demethylation, sequentially restored expression level of CXCL12 transcript and protein in this cell type (46). In present study, we also used 5-Aza to assess the role of DNMT1

methylation reaction in PTEN expression and subsequent proliferation of TAMR-MCF-7 cells. 5-Aza treatment reversed methylation of PTEN gene and caused PI3K inhibition as well as PTEN restoration in TAMR-MCF-7 cells. As our expectation, 5-Aza not only inhibited cell proliferation in TAMR-MCF-7 but also increased TAM-dependent cytotoxicty when concomitantly treated. Xenograft study further clarified the role of methylation and 5-Aza *in vivo*. 5-Aza efficiently reduced tumor growth in athymic nude mice bearing TAMR-MCF-7. Expression of PTEN and phosphorylation of AKT in the tumor tissue lysates was also reversed by 5-Aza treatments. In addition, 5-Aza increased TUNEL- positive apoptotic tumor cells. The data implicate that methylation plays an important role in down-regulation of PTEN expression and tumor growth of TAMR-MCF-7 cells. In fact, 5-Aza has been evaluated in clinical trials as a cancer therapeutic agent for the treatment of patients with acute myeloid leukemia and myelodysplastic syndrome (62). Besides, 5-Aza could inhibit cell growth, and induce cell cycle arrest and apoptosis in human endometrial cancer cell lines (63). Hence, 5-Aza as demethylated agent would be a potential agent to attenuate the TAM resistance of breast cancer.

In summary, we have revealed that SAM increase via MAT2A overexpression and higher DNMT1 activity in TAMR-MCF-cells contributes to both rapid proliferation and drug resistance presumably through aberrant methylation in PTEN promoter. DNMT1 may serve as a new therapeutic target for the treatment of anti-estrogen resistant breast cancer.

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6. Figure legends

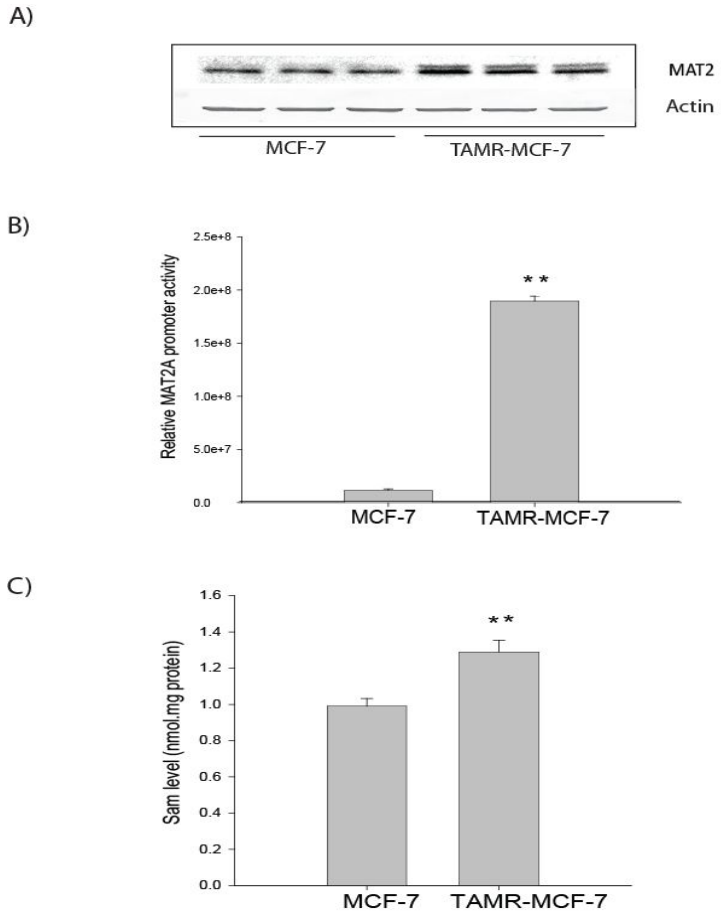
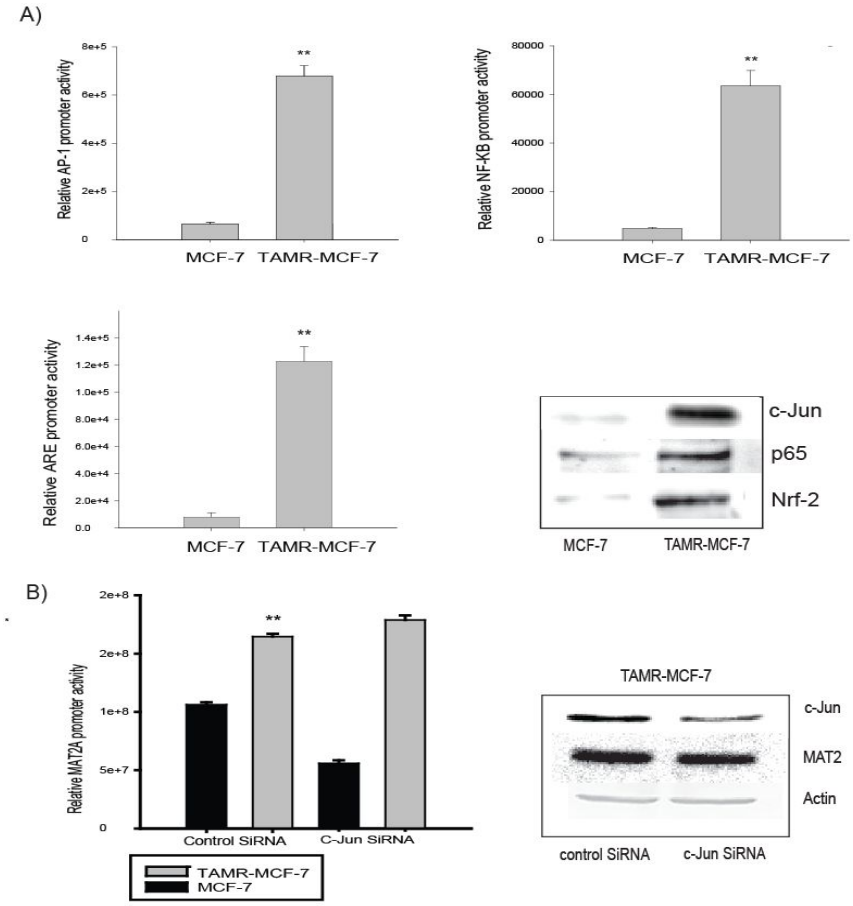


Figure 1. MAT2A expression and SAM level in MCF-7 and TAMR-MCF-7 cell lines. (A) Immunoblot analysis of MAT2A in MCF-7 and TAMR-MCF-7 cell lines. (B) Basal reporter activity of MAT2A promoters (MAT2A-luc) in MCF-7 and TAMR-MCF-7 cell lines. Each cell type was transiently co-transfected with MAT2A-luc reporter plasmid containing -570/+61 bp human MAT2A promoter (1µg/ml) and phRL-SV (hRenilla) (1ng/ml). Dual luciferase reporter assays were performed on the lysed 18h after transfection. Reporter gene activation was calculated as a relative ratio of firefly luciferase to hRenilla luciferase activity. Data represent means +/- SD with 6 different

samples (significance versus MCF-7 cells, **P< 0.01) C). SAM level in MCF-7 and TAMR-MCF-7 cell lines (**P< 0.01).



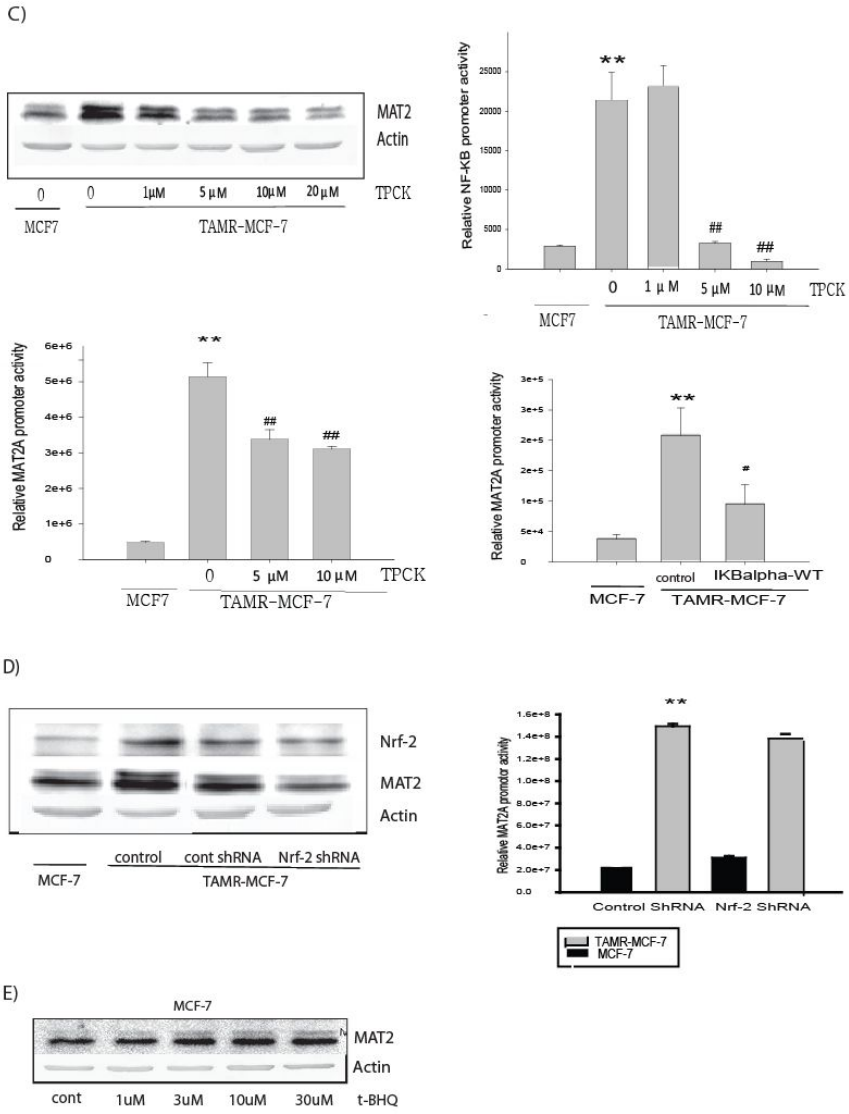


Figure 2. Role of NF-κB and Nrf-2 in up-regulation of MAT2A in TAMR-MCF-7 cells. A) Basal reporter activities of AP-1, NF-kB and Nrf-2/antioxidant response element (ARE). Each cell type was transiently transfected with above each type of plasmid (1μg/ml). Dual luciferase reporter assays were performed on the lysed cells co-

transfected with phRL-SV (*hRenilla*) (1ng/ml) 18h after transfection. Reporter gene activation was calculated as a relative ratio of firefly luciferase to hRenilla luciferase activity. Data represent means +/- SD with 6 different samples (significant versus MCF-7 cells, **P< 0.01). And lower, in the right hand, immunoblot analysis in the nucleus of c-Jun, p65 and Nrf-2 in MCF-7 and TAMR-MCF-7 cell lines. B) Role of AP-1 in MAT2A regulation in TAMR-MCF-7. Each cell type was co-transfected or transfected with c-Jun siRNA 20 *p*mole (for reporter gene assay, left figure) and 60 *p*mole (for immunoblot, right figure). C) Inhibition of MAT2A transactivation by TPCK for 24h. Upper, left: MAT2 expression after treatment with TPCK for 24h. Upper, right: Decrease in relative NF-κB promoter activity by TPCK (5, 10μM). Lower, left: Reduction of *MAT2A* reporter activity in TAMR-MCF-7 by TPCK (5, 10 μM). Lower, right: Inhibition of MAT2A transactivation by IKBα-over-expression plasmid (0.5μg/ml) (IKBα-WT). D) Decrease in MAT2A expression in TAMR-MCF-7 after transfection with Nrf-2 shRNA (5μl/ml). E) Induction of MAT2A expression in MCF-7 by t-BHQ exposition (1, 3, 10, 30μM) for 24h.

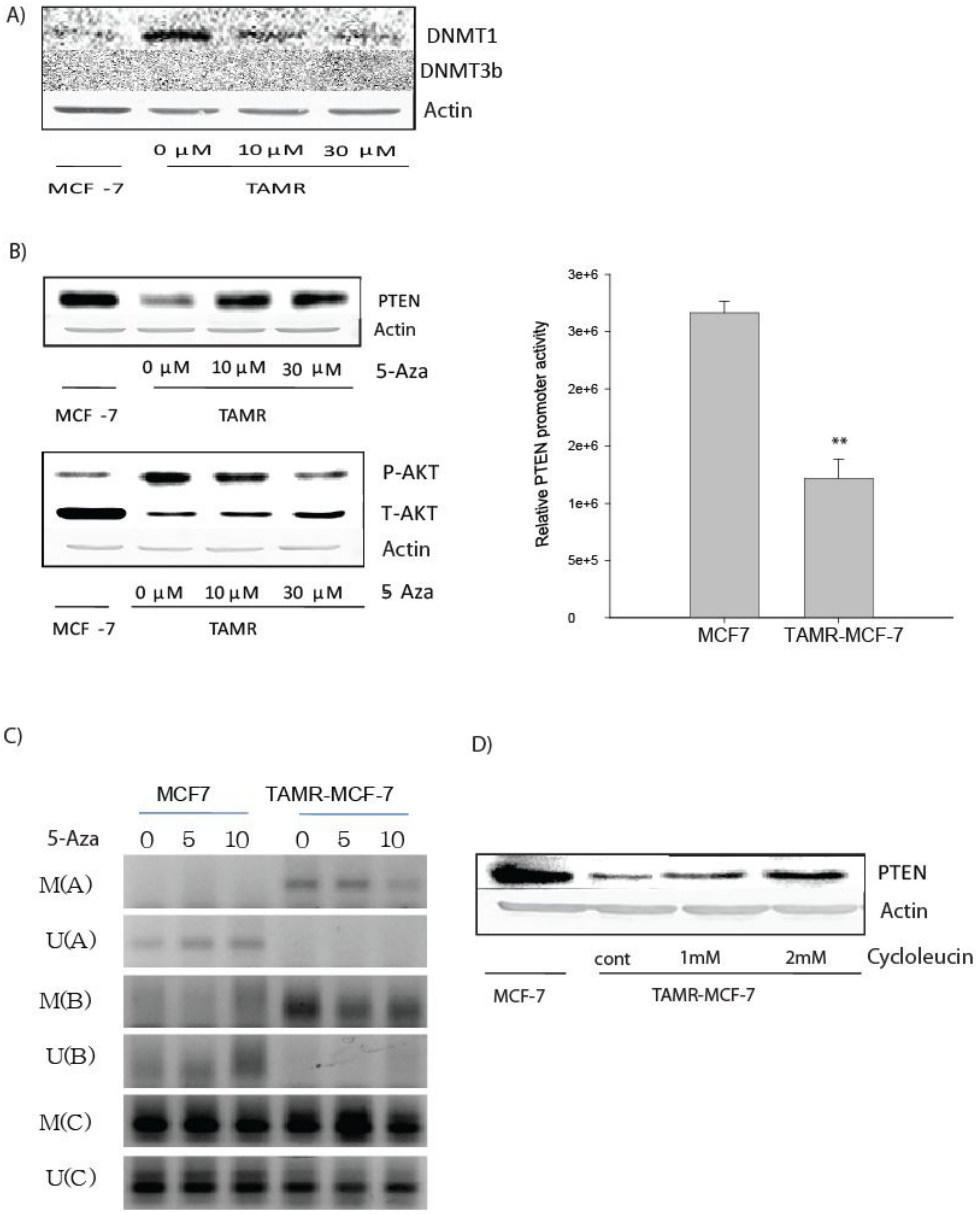
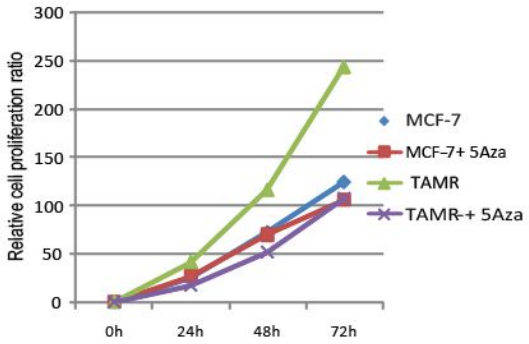


Figure 3. (A) Immunoblot analysis of DNMT1 and DNMT3b in MCF-7 and TAMR-MCF-7 cell lines. (B) Immunoblot analysis for PTEN, p-AKT and AKT in MCF-7 and TAMR-MCF-7 cell lines. Treatment with 5-Aza (10, 30μM) reversed the PTEN, p-AKT, AKT expression in TAMR-MCF-7. (C) MSP analysis of *PTEN* gene in MCF-7

and TAMR-MCF-7 with and without treatment 5-Aza (5, 10 μM). MSP regions studied in the sites: (A), (B) and (C). *M* and *U*, PCR products of methylated and unmethylated allels, respectively. (D) Treatment TAMR-MCF-7 with Cycloleucin (2mM) reversed PTEN expression.

A)



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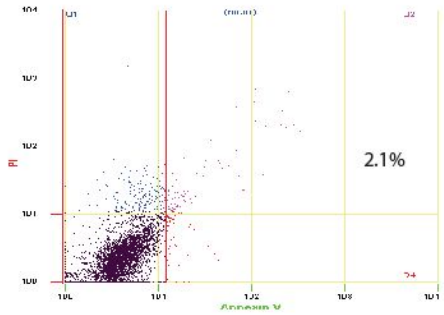
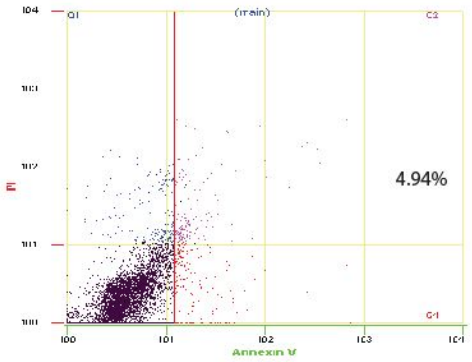
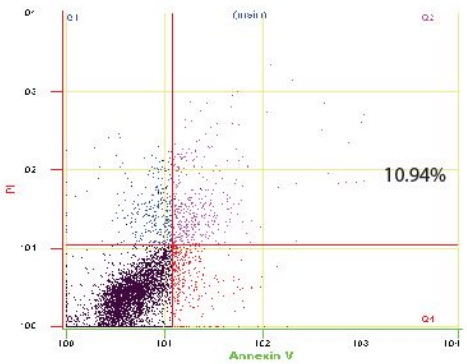


TAMR-MCF-7 cont



4-OH-TAM

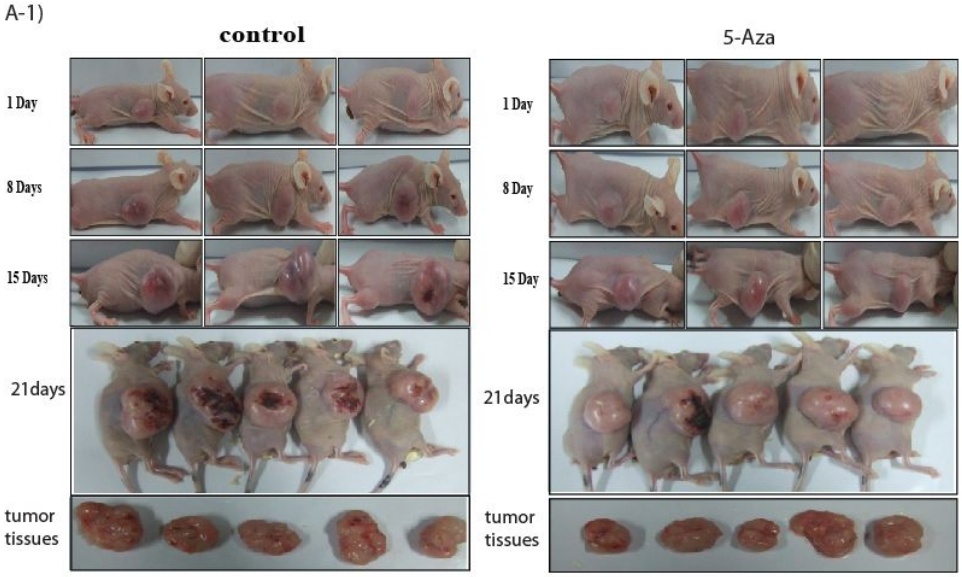


4-OH-TAM + 5-Aza

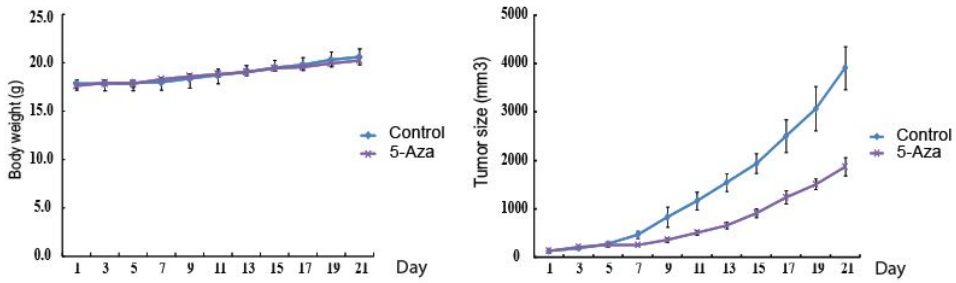
Figure 4. (A) Inhibition cell proliferation rate of TAMR-MCF-7 cells by 5-Aza treatment (30µM) through 24h-72h. (B) Representative dot plots of TAMR-MCF-7 stained with

annexin V-FITC and propidium iodide (PI). The cells were treated with 10µM hydroxy-tamoxifen in the presence or absence of 5-Aza (30µM) for 24h. (Q4) represents early apoptotic cells. (Q1, Q2) represent late necrotic and apoptotic cells, respectively. (Q4) represents survival cells. The percentage counts of cell apoptosis (Q2 + Q4) are indicated.

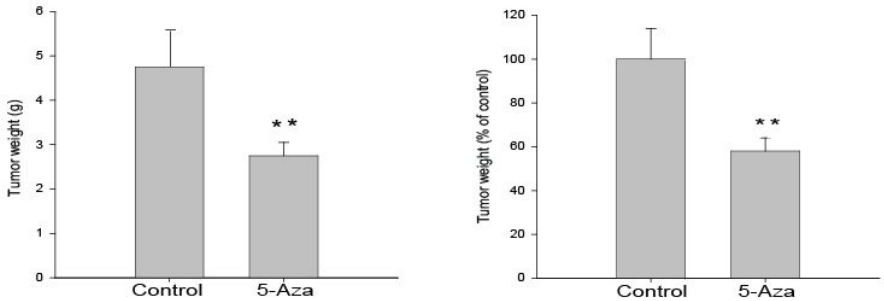
A-1)


control
5-Aza
1 Day
8 Days
15 Days
21days
tumor tissues
1 Day
8 Day
15 Day
21days
tumor tissues

A-2)


Body weight (g)
25.0
20.0
15.0
10.0
5.0
0.0
1 3 5 7 9 11 13 15 17 19 21
Day
Control
5-Aza
Tumor size (mm3)
5000
4000
3000
2000
1000
0
1 3 5 7 9 11 13 15 17 19 21
Day
Control
5-Aza

A-3)


Tumor weight (g)
6
5
4
3
2
1
0
* *
Control
5-Aza
Tumor weight (% of control)
120
100
80
60
40
20
0
* *
Control
5-Aza

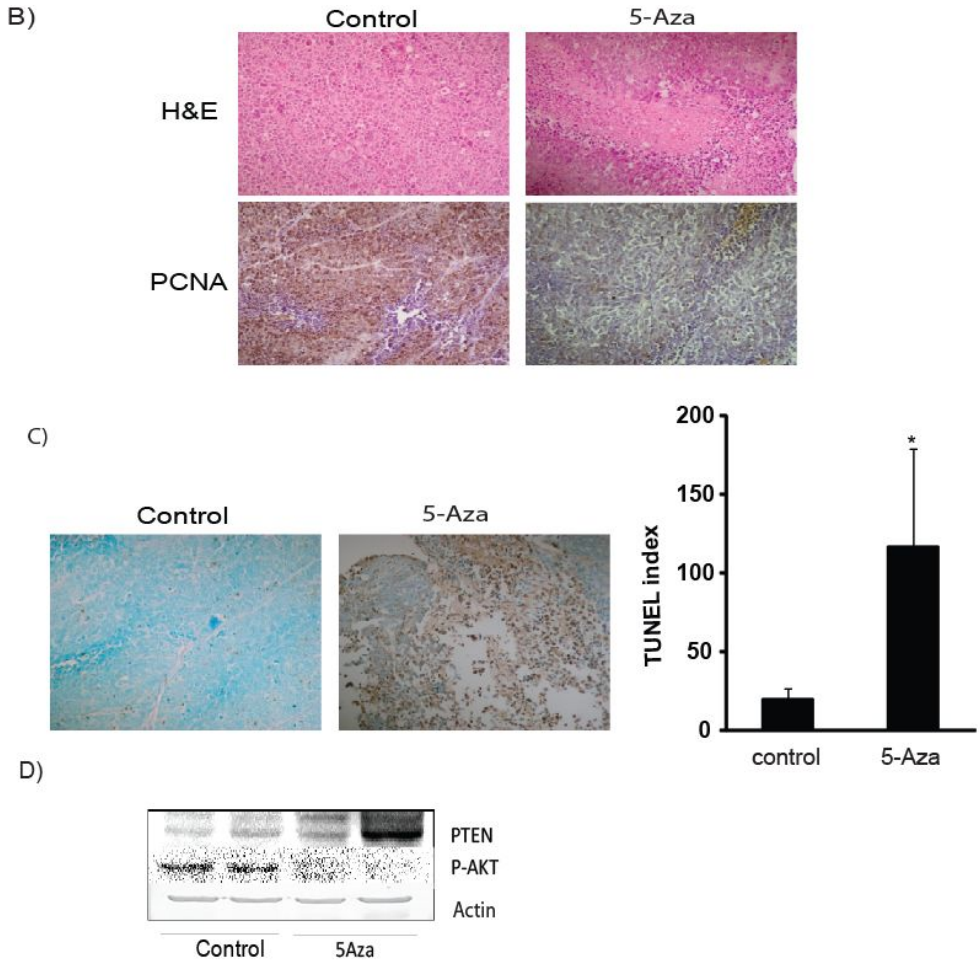
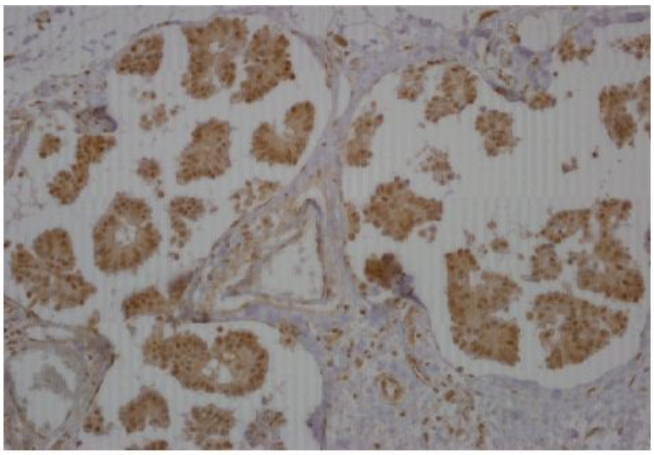


Figure 5. (A) Reduction of tumor growth in athymic nude mice bearing TAMR-MCF-7 cells after intraperitoneal injections of 5 mg/kg 5-Aza (every 6 days for 21days). A-1) Left figures: control group treated with sterile water after 1 day, 8 days and 15 days. After 21days, tumor tissues were cut and were assessed. Right figures: 5-Aza- treated group after 1day, 8 days, 15 days. After 21 days, tumor tissues were cut and were assessed. A-2) Left graph: Body weight of nude mide of each group was observed in 21 days. Right graph: Decrease in tumor size of 5-Aza–treated group compared with sterile water- treated group. A-3) Attenuation of tumor weight of 5-Aza treated group

compared with sterile water- treated group (**P< 0.01). (B) Upper panels, the routine stain for tumor tissues of nude mice bearing TAMR-MCF-7 cells without 5-Aza (control, left panel) or with 5-Aza (right panel) (H: Hematoxylin, E: Eosin). Lower panels, Immunohistochemistry analysis of PCNA protein in vehicle-treated control tissue section and 5-Aza- treated tumor tissue section. C) Increase in apoptotic cells (TUNEL-positive cells) of tumor tissue treated with 5-Aza (*P <0.05). (D) Western blot analysis for PTEN and p-AKT protein in tumor tissue lysates of with or without 5-Aza treated samples.

A)



B)

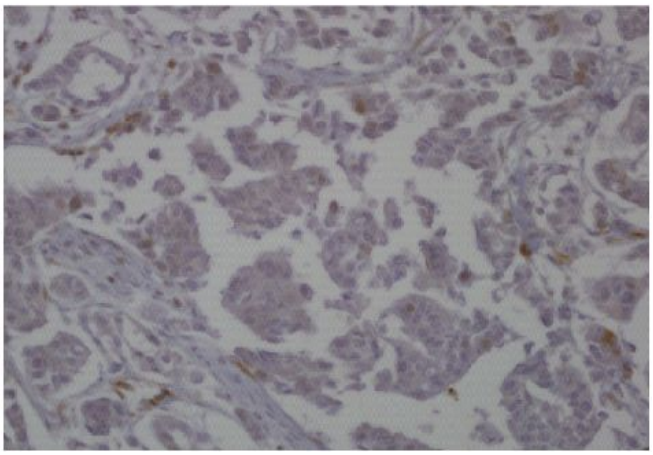


Figure 6. Expression changes of PTEN in human breast cancer tissue. (A), (B) Immunohistochemistry analysis of PTEN in human breast cancer tissues of 2 groups of patients: TAM-sensitive cases and TAM-resistant cases, respectively.

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논문 제목	한글: **타목시펜 저항성 유방암 세포에서 MAT2A 과다발현 에 의한 S-adenosylmethionine 증가 및 병리학적 의미**				
	영문: **Pathological implication of S-adenosylmethionine increase by MAT2A up-regulation in tamoxifen-resistant breast cancer cells**				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건 아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB 구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함.

2. 위의 목적을 위하여 필요한 범위 내에서의 편집과 형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.

3. 배포・전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.

4. 저작물에 대한 이용기간은 5 년으로 하고, 기간종료 3 개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.

5. 해당 저작물의 저작권을 타인에게 양도하거나 출판을 허락을 하였을 경우에는 1 개월 이내에 대학에 이를 통보함.

6. 조선대학교는 저작물 이용의 허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음.

7. 소속 대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송・출력을 허락함.

동의여부 : 동의(○) 반대( )

2010 년 8 월 25 일

저작자: Nguyen Thi Thuy Phuong (인)

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