

Original Article

Inhibition of S-adenosylhomocysteine hydrolase decreases cell mobility and cell proliferation through cell cycle arrest

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Abstract: S-adenosylhomocysteine hydrolase (AHCY) hydrolyzes S-adenosylhomocysteine to adenosine and L-homocysteine, and it is already known that inhibition of AHCY decreased cell proliferation by G2/M arrest in MCF7 cells. However, the previous study has not indicated what mechanism the cell cycle arrest is induced by. In this study, we aimed to investigate the different cell cycle mechanisms in both p53 wild-typed MCF7 and p53 mutant-typed MCF7-ADR by suppressing AHCY. We extensively proved that AHCY knockdown has an anti-proliferative effect by using the WST-1 assay, BrdU assay, and cell cytometry analysis and an anti-invasive, migration effect by wound-healing assay and trans-well analysis. Our study showed that down-regulation of AHCY effectively suppressed cell proliferation by regulating the MEK/ERK signaling pathway and through cell cycle arrests. The cell cycle arrest occurred at the G2/M checkpoint by inhibiting degradation of cyclinB1 and phosphorylation of CDC2 in MCF7 cells and at the G1 phase by inhibiting cyclinD1 and CDK6 in MCF7-ADR cells. Finally, we determined that AHCY regulates the expression of ATM kinase that phosphorylates p53 and affects to arrest of G2/M phase in MCF7 cells. The findings of this study significantly suggest that AHCY is an important regulator of cell proliferation through different mechanism in between MCF7 and MCF7-ADR cells as p53 status.

Keywords: S-adenosylhomocysteine, breast cancer, cell proliferation, cell cycle, p53, ataxia telangiectasia mutated kinase

Introduction

Breast cancer is one of the most frequently diagnosed cancers and a heterogeneous disease that is the second most lethal cancer in women worldwide [1]. It is also the most characterized human cancer using genome-wide technologies [2]. Various human breast cancer cell lines divided four subtypes such as luminal A, luminal B (MCF7 and T47D), HER2+ (MCF7-ADR and SK-BR-3) and Triple negative breast cancer (MDA-MB-231 and MDA-MB-435S) [3]. The adriamycin-resistant cell line, MCF7-ADR, was prepared in 1986 by incubating the MCF7 cells with high concentrations of the anthracycline antibiotic ADR [4-6]. This cell line is widely known as a stable multidrug-resistant (MDR) model because the cells have a high expression of P-glycoprotein and it is reported that the cells have a considerable hormone-independent metastatic potential [7, 8].

S-adenosylhomocysteine hydrolase (AHCY) is a critical enzyme in the S-adenosyl methionine (SAM) cycle that plays a role in making, expending, and regenerating SAM [9]. AHCY strongly inhibits trans-methylation because it converts S-adenosylhomocysteine (SAH)- created from transferring a methyl group from SAM-into adenosine and homocysteine that are required for generating SAM [10, 11]. AHCY knockdown has biochemical effects on trans-methylation reactions that affect alcohol-induced liver disease and exerts an anti-cancer effect in breast cancer cell lines [12-14], AHCY has an important role as it regulates DNA methylation in many physiological states and thus mediates gene expression [15, 16]. AHCY is up-regulated by c-MYC for removing inhibitory product, RNA guanine-7 methyltransferase (RNMT), of cap methylation [17]. We hypothesize that AHCY knockdown inhibits the uncontrolled cell cycle pro-

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gression of breast cancer cells and finally decreases cell proliferation in breast cancer.

Almost all breast tumors have some defect in cell cycle regulatory molecules, including cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors (CDKIs) that regulate each phase of the cell cycle progression [18, 19]. In cancer cells, many CDKs have alternative substrates and functions and causes un-regulated cell proliferation [20, 21]. CDK4 and CDK6 are particularly early G1 phase kinases and they strongly regulate homologous interactions with the D-type cyclins and CDKIs [22]. Once CDK4, CDK6, or their regulatory D-type cyclins are over-expressed in cultured cells, they lead to excessive cell growth and confer cancer characteristics on the cells [23]. On the other hand, CDC2 is phosphorylated on threonine-14 and tyrosine-15, which detaches it from cyclin B1 during G2 phase. The phosphorylation of CDC2 inhibits the activity of the CDC2/cyclin B1 kinase complex restricting entry into mitosis. CDC2 is suppressed by the transcriptional targets of p53, Gadd45, p21, and 14-3-3 δ . The p53 is phosphorylated by ATM kinase upon DNA damage [24].

This study substantiates that knockdown of *AHCY* decreases cell proliferation through both MEK/ERK pathway down-regulation and p53-induced cell cycle arrest by activated-ATM kinase. These data propose that *AHCY* has the potential to be a regulator related to cell proliferation in breast cancer.

Materials and methods

Cell culture and transfection

MCF7, MCF7-ADR, MDA-MB-231, MDA-MB-435s and SK-BR-3 cells were cultured in high glucose Dulbecco's modified eagle's medium (DMEM) (Welgene, Republic of Korea) supplemented with 10% FBS (Fetal bovine serum, qualified, Canada origin, Gibco); MCF10A cells were cultured in DMEM/F12 medium added with 5% horse serum, 20 ng/ml EGF, 0.5 mg/ml hydrocortisone, 100 ng/ml cholera toxin and 10 μ g/ml insulin; T47D cells were cultured in RPMI-1640 medium including 10% FBS and 10 μ g/ml bovine insulin in a humidified chamber with 5% CO₂ at 37°C. Approximately 2 \times 10⁵ MCF7-ADR cells were seeded per well in 6-well culture plates with 10% FBS-DMEM for 1 day. When the cells were approximately 50% conflu-

ent, control [multiplicity of infection (MOI) 1:5, Santa Cruz, sc-108080] and *AHCY* shRNA lentiviral particles (MOI 1:5, Santa Cruz, sc-62972-V) were transduced into the cells with 10% FBS-DMEM containing 10 μ g/ml polybrene (Santa Cruz, sc-134220). After 12 h, the cells were incubated with medium without polybrene for 24 h and then the clones stably expressing the shRNA were selected using 10 μ g/ml puromycin (Santa Cruz, sc-108071) and maintained in culture in 10% FBS-DMEM including 2 μ g/ml puromycin. MCF7 cells were seeded at a density of 5 \times 10⁵ cells in a 60-mm culture dish and they were transfected with 25 nM *AHCY* siRNA using Lipofectamine RNAi MAX (Invitrogen, 13778-150) with 0.5% FBS-DMEM.

Real-time quantitative PCR

RNA was isolated from breast cancer cell lines using NucleoSpin® RNA/Protein kit (Macherey-Nagel) using the manufacturer's instruction. Complementary DNA (5 μ g) was made by reverse-transcribing RNA using M-MLV reverse transcriptase (Promega, M170A), RNasin® ribonuclease inhibitor (Promega, N211A), 100 nM oligo-dT, and 2.5 mM dNTP mixture. Approximately 0.1 μ g cDNA was used for real-time qPCR that was conducted using HiFast SYBR Lo-Rox (Genepole, Q100240) and primers in LightCycler® thermocycler (Roche). The primers were as follows: human *AHCY* (forward: 5'-ATC CTT GGC CGG CAC TTT GAG-3', reverse: 5'-TCC ACC TGC GGC TTG ATG TTC-3') and human 18 s rRNA (forward: 5'-GTC GGC GTC CCC CAA CTT CTT-3', reverse: 5'-CGT GCA GCC CCG GAC ATC TA-3'). Primers were produced by Bioneer (Daejeon, South Korea), and human 18 s rRNA was used for normalization.

Western blotting

Proteins were isolated from breast cancer cell lines using NucleoSpin® RNA/Protein kit (Macherey-Nagel) following the manufacturer's instructions. The protein concentrations were determined using the bicinchoninic acid solution (Sigma, B9643) and copper (II) sulfate solution (Sigma, C2284). Proteins were separated on sodium dodecyl sulfate-polyacrylamide gels [10% resolving gel, 5% stacking gel; H₂O, 30% acrylamide (Bio-Rad, #161-0156)], 1.5 M pH 8.8 Tris, 10% SDS, 10% ammonium persulfate, TEMED (Sigma, T9281) and transferred to a Clear Blot membrane (Atto, AE-6667-P). Membranes with the transferred pro-

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Table 1. Information about antibodies used to western blotting analysis

Name	Cat No.	Company
AHCY	ab134966	Abcam
Ras (27H5)	#3339	Cell signaling
Phospho-B-Raf (Ser445)	#2696	Cell signaling
B-Raf (55C6)	#9433	Cell signaling
Phospho-C-Raf (Ser259)	#9421	Cell signaling
C-Raf	#9422	Cell signaling
Phospho-MEK1/2 (Ser221) (166F8)	#2338	Cell signaling
MEK1/2 (L38C12)	#4694	Cell signaling
phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (197G2)	#4377	Cell signaling
p44/42 MAPK (Erk1/2)	#9102	Cell signaling
cyclinD1 (92G2)	#2978	Cell signaling
CDK6 (DCS83)	#3136	Cell signaling
cyclinE (HE12)	#4129	Cell signaling
CDK2	sc-163	Santa cruz
cyclinB1 (D5C10) XP®	#12231	Cell signaling
phospho-cdc2 (Tyr15) (10A11)	#4539	Cell signaling
cdc2 (POH1)	#9116	Cell signaling
p21 waf1/cip1 (DCS60)	#2946	Cell signaling
chk2	NB100-500	Novus Bio
ATM	NB100-309	Novus Bio
phospho-MDM2 (Ser166)	sc-293105	Santa cruz
MDM2 (SMP14)	sc-965	Santa cruz
phospho-p53 (Ser15)	sc-101762	Santa cruz
p53 (DO-1)	sc-126	Santa cruz
Gadd45α (D17E8)	#4632	Cell signaling
14-3-3ζ/δ (D7H5)	#7413	Cell signaling
cytoskeletal actin	A300-291A	Bethyl
goat anti-rabbit IgG, pAb	ADI-SAB-300-J	Enzo
goat anti-mouse IgG, pAb	ADI-SAB-100	Enzo
anti-biotin HRP-linked Ab	#7075	Cell signaling

teins were blocked with a 5% solution of skim milk (BD, 232100) and washed with $1 \times$ PBS (Welgene, LB204-01) with 0.1% Tween-20 (Sigma Aldrich, P9416), and the bands were visualized using chemiluminescence on an X-ray film (Fujifilm). Used-antibodies were organized in **Table 1**.

Cell proliferation assay

Approximately 1×10^4 cells were seeded per well in 12-well cell culture plates in 10% FBS-DMEM. Five hours after seeding, 100 μ l/well of the cell proliferation reagent WST-1 (Roche, 644807001) was added to the zero-hour wells and incubated in the dark for 1 h. Approximately 100 μ l of the supernatant from each well was transferred to a 96-well culture plate and the absorption was measured at 450 nm using an

ELISA plate reader (Bio-tek instruments, Power-WaveX340). The medium was changed to 1% FBS-DMEM and 100 μ l/well WST-1 was added for 1 h and the supernatant was moved to a 96-well plate and the absorbance measured from 12 h post-incubation up to 48 h post-incubation in 12-h intervals in MCF7 cells and from 24 h post-incubation up to 96 h post-incubation in 24-h intervals in MCF7-ADR cells. Three independent experiments were carried out in triplicates.

BrdU assay

Approximately 1×10^4 cells were seeded per well in 96-well plates and incubated in a humidified incubator at 37°C for 24 h. To analyze the cells' proliferation capacity, 10 μ M BrdU (Roche, 11647229001) was added to each well. After 2

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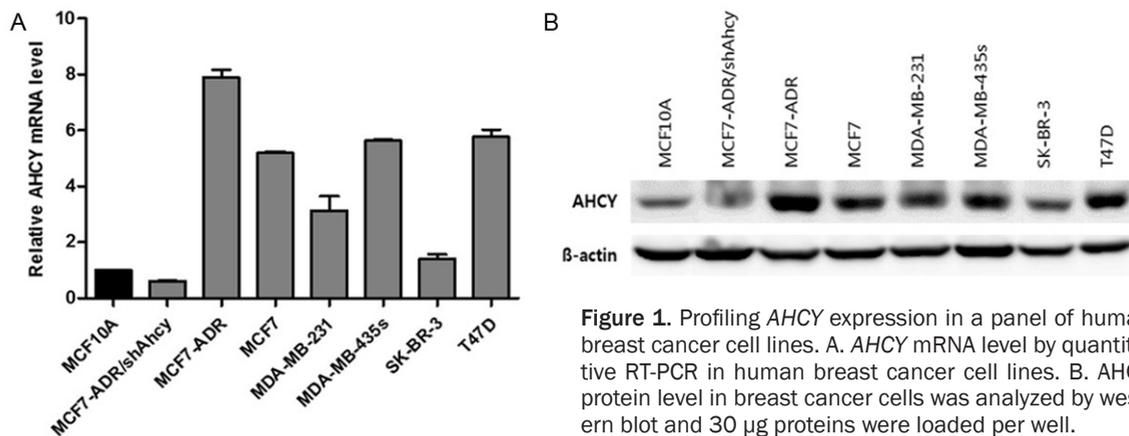


Figure 1. Profiling *AHCY* expression in a panel of human breast cancer cell lines. **A.** *AHCY* mRNA level by quantitative RT-PCR in human breast cancer cell lines. **B.** *AHCY* protein level in breast cancer cells was analyzed by western blot and 30 μ g proteins were loaded per well.

h, the cells were fixed and stained according to the manufacturer's instruction. Colorimetric analysis was carried out using an ELISA plate reader (Bio-tek instruments, PowerwaveX340).

Wound healing analysis

Approximately 1×10^5 cells per milliliter were seeded in 10% FBS-DMEM in 60-mm dishes and kept in a humidified incubator at 37°C for 24 h. The medium was changed to 1% FBS-DMEM for an additional 24 h. When the cells were confluent, the cells on the dish's midline were removed using a 10- μ l pipette tip. After 0, 24, and 48 h of culture, the plate was photographed under a microscope at 40 \times magnification.

Trans-well invasive analysis

Cell invasion was assessed using the Biocoat Matrigel Invasion Chamber (Becton Dickinson, 354480) according to the manufacturer's instructions. Invasion chambers (24 wells) were rehydrated by adding 500 μ l of warmed serum-free DMEM on each upper and lower chamber for 2 h. DMEM in the upper chamber was replaced with 500 μ l of 3×10^4 cells/ml in 1% FBS-DMEM, and in the lower chamber, DMEM was replaced with 750 μ l of 10% FBS-DMEM with 5 μ g/ml fibronectin (Sigma, F2006) as a chemoattractant. The plates were incubated for 24 h at 37°C with 5% CO₂. Non-invasive cells were blotted dry from the upper chamber using cotton swabs. The cells that had invaded through the matrigel membrane were fixed with 100% methanol and stained with crystal violet (Sigma, C0775). The upper chamber was washed with H₂O and the non-invading cells that had remained in the staining solution were

removed by using cotton swabs. Invaded cells were visualized at 100 \times magnification using a light compound microscope. Subsequently, 10% acetic acid was added to the upper chamber for quantifying the invading cells by using an ELISA plate reader (Bio-tek instruments, PowerwaveX340).

Cell cycle analysis

Approximately 1×10^5 cells/ml were seeded in 10% FBS-DMEM in 100-mm dishes and incubated overnight. The medium was changed to FBS-free DMEM for 24 h and then changed to 1% FBS-DMEM for an additional 24 h. The cells were trypsinized and washed twice with phosphate-buffered saline (1 \times PBS), autoclaved, and then fixed in 70% cold ethanol overnight at -20°C. Immediately before the assay, the cells were stained using PI/RNase staining buffer (Becton Dickinson, 550825) in the dark on ice until cell cycle analysis was carried out using a flow cytometer (Becton Dickinson, BD FACS Canto II). The flow cytometer equipped with a 488-nm laser was used for the analysis and 10,000 cells were analyzed in each measurement. Data were acquired and analyzed using the BD FACS Diva software (Becton Dickinson). The relative change in the mean fluorescence intensity was calculated as the ratio between mean fluorescence intensity in the channel of the treated cells and that of the control cells.

Statistical analysis

All *in vitro* experiments were repeated at least three times and presented as the mean \pm standard deviation (SD). Statistical significance was analyzed using Student's *t* tests at a *P* < 0.05.

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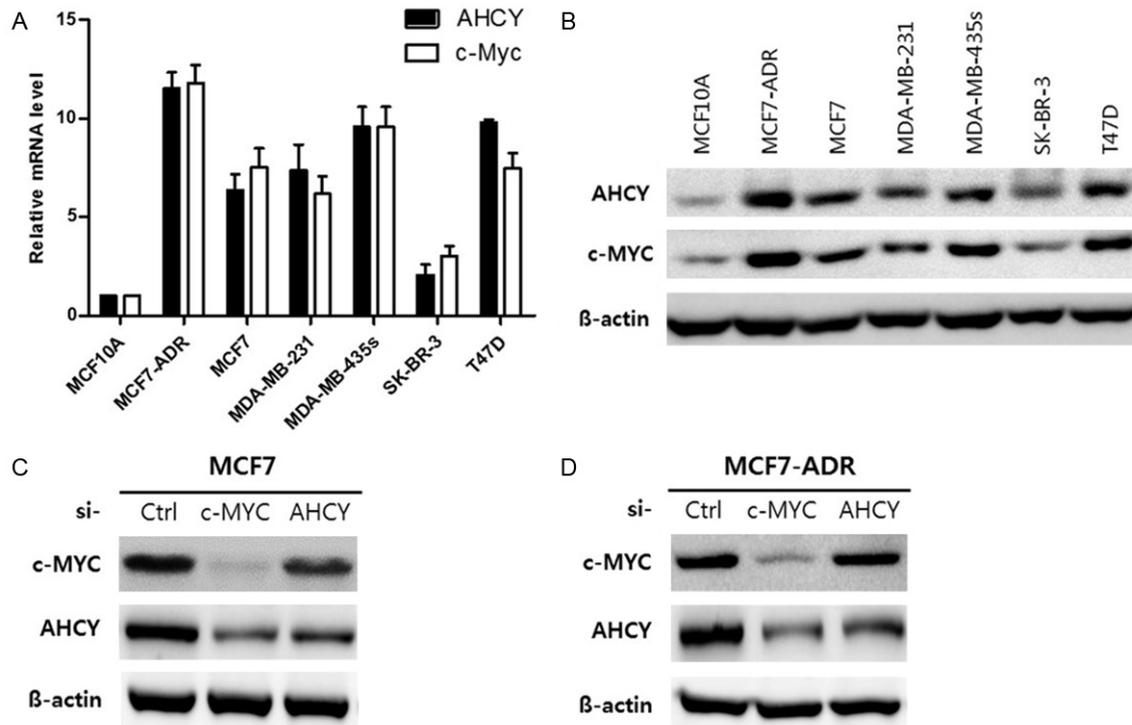


Figure 2. The expression of *AHCY* is determined by *c-MYC* activation in human breast cancer cell lines. **A.** *c-MYC* mRNA level by quantitative RT-PCR in human breast cancer cell lines. **B.** *AHCY* and *c-MYC* protein levels in breast cancer cells were analyzed by western blot and 30 μ g proteins were loaded per well. **C, D.** 25 nM control, *c-MYC* and *AHCY* siRNA were treated in both MCF7 and MCF7-ADR cells for 48 h and the 30 μ g protein levels were loaded per well and analyzed by western blot.

Ethics statement

This study was officiated by the Biomedical Ethics Committee of the Sookmyung women's university. This study was carried out according to contents of Institutional Review Board (IRB) seminar. The certification of the IRB seminar was assigned by Korea Human Resource Development Institute for Health & Welfare.

Results

AHCY is differentially expressed in human breast cancer cell lines

To screen the expression of *AHCY* in human breast cancer cell lines, we investigated the expression of *AHCY* by quantitative RT-PCR and western blot analysis. The mRNA and protein forms of *AHCY* were highly expressed in most of breast cancer cells including MCF7, MCF7-ADR, MDA-MB-231, MDA-MB-435S and T47D cells compared with human breast MCF10A cells, while they were less expressed in SK-BR-3 cells (**Figure 1**). To confirm the function of *AHCY* in

breast cancer cells, we established stable *AHCY*-knockdown MCF7-ADR cells by using shRNA lentiviral particles (MCF7-ADR/shCon, MCF7-ADR/shAHCY). The mRNA and protein expression levels of *AHCY* in these cells are shown in **Figure 1**.

The expression of AHCY is regulated by c-MYC activation in human breast cancer cell lines regardless of subtypes

Although MCF7-ADR cells and SK-BR-3 cells belong to same subtype of human breast cancer cell lines, they have opposite expression of *AHCY* each other. *AHCY* was over-expressed in MCF7-ADR cells, while less-expressed in SK-BR-3 cells than normal cells. In addition to, MCF7-ADR and MCF7 cells are different subtypes but they had similar expression pattern of *AHCY* (**Figure 1**). To investigate mechanism regulating expression of *AHCY*, we studied expression of *c-MYC* that is known as upstream molecule for *AHCY* expression. The results indicated that *AHCY* expression is dependent to *c-MYC* expression unrelated to subtype of

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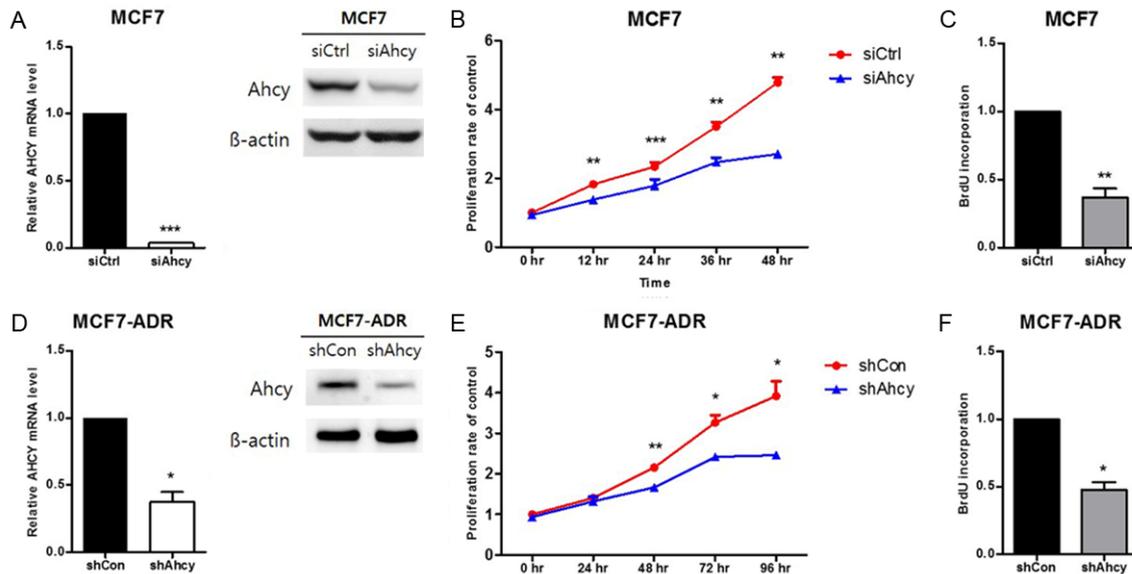


Figure 3. Effect of AHCY-knockdown in breast cancer cells. A, D. The AHCY expression levels in the cells used in the proliferation assay were confirmed by qRT-PCR and western blot and 30 μ g proteins are loaded per well. The graphs show mean \pm SD of three independent experiments. *means $P < 0.05$ and ***means $P < 0.005$. B, E. Cell proliferation rate of MCF7 and MCF7-ADR cells was detected by staining with the 10% solution of WST-1 for 48 and 96 h. The graphs show mean \pm SD of three independent experiments. *means $P < 0.05$, **means $P < 0.01$ and ***means $P < 0.005$. C, F. BrdU incorporation was measured after 2 h of staining by thymidine. The graphs show mean \pm SD of three independent experiments. *means $P < 0.05$ and **means $P < 0.01$.

breast cancer cell lines (Figure 2A and 2B). To study how different cell cycle mechanisms regulated by AHCY in both p53 wild-type and mutant-type breast cancer cell lines, we selected MCF7 as p53 wild-type cells and MCF7-ADR as p53 mutant-type cells. Before using MCF7 and MCF7-ADR cell lines for study, we confirmed that AHCY expression is decided by c-MYC using c-MYC siRNA. In the results, we can know that expression of AHCY is decreased by inhibiting c-MYC and c-MYC expression is little changed by AHCY inhibition in both MCF7 and MCF7-ADR cell lines (Figure 2C and 2D). It suggests that c-MYC effects on expression of AHCY in human breast cancer cells independent of subtypes.

Knockdown of AHCY induces a decrease in cell proliferation in MCF7 and MCF7-ADR cells

To confirm whether the two cell lines we used for the functional studies had a change in cell viability and proliferation upon knock-down of AHCY, we first examined the expression of AHCY by quantitative RT-PCR and western blotting. The mRNA expression of AHCY was inhibited by about 90% in MCF7 cells and 60% in MCF7-ADR cells compared to the cells of origin (Figure 3A and 3D). To demonstrate the effects of

AHCY on cell viability, we examined the cell proliferation rate using the cell proliferation reagent WST-1 in both AHCY-silenced breast cancer cell lines. The results showed that cell viability was decreased in MCF7 cells that were treated with AHCY siRNA starting at 12 h and MCF7-ADR/shAHCY cells starting at 48 h compared to each control groups (Figure 3B and 3E). To accurately assess the cell proliferation inhibitory ability of AHCY knockdown, we used BrdU analysis in both cell lines. The results indicated that the cell proliferation was reduced by about 70% in MCF7/siAHCY cells and by 50% in MCF7-ADR/shAHCY cells cultured in 5% FBS-DMEM (Figure 3C and 3F). According to these results, AHCY silencing causes a decrease in cell viability and the proliferation rate in both MCF7 and MCF7-ADR cells.

AHCY inhibition suppresses cell invasion and migration in MCF7 and MCF7-ADR cells

To confirm whether AHCY inhibition has the ability to regulate invasion and migration in breast cancer cells, we examined the invasive and metastatic activities of both MCF7 and MCF7-ADR cell lines using the wound-healing and trans-well assays. As shown in Figure 4A, the migration of AHCY siRNA-transfected MCF7

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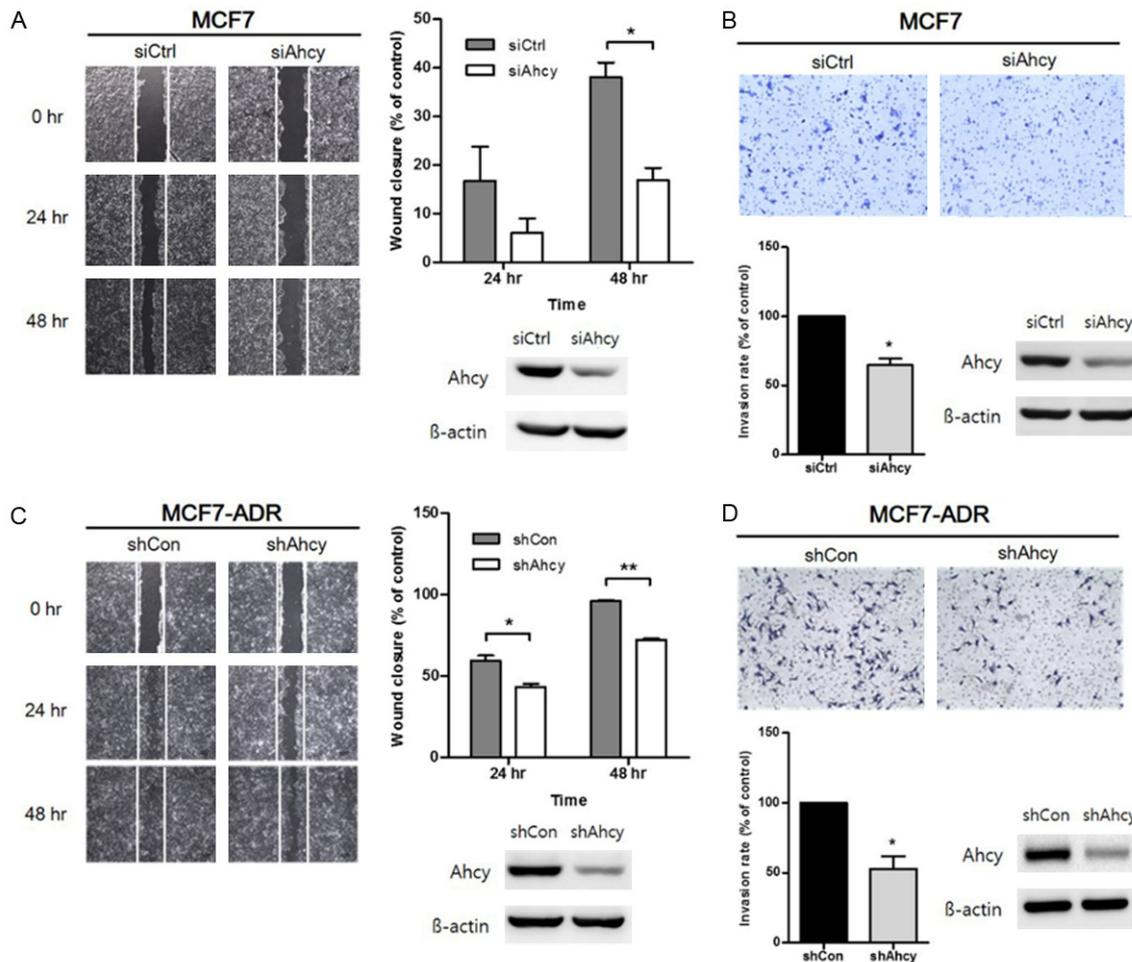


Figure 4. Effect of inhibiting *AHCY* in breast cancer cells on cell invasion and migration. A, C. Effect of *AHCY* on cell migration was studied by wound-healing assay with both MCF7 and MCF7-ADR cell lines. A wound was made by scraping with a pipet tip and the plates were observed after 24 h and 48 h using microscope with 40 × magnification. The empty area was measured for quantification of wound closure rate using the Image J program. The expression of *AHCY* in these cells was confirmed by western blotting analysis. The graphs show mean ± SD of three independent experiments. *means $P < 0.05$ and **means $P < 0.01$. B, D. Invasive cells were observed after 24 h of seeding under a microscope with 100 × magnification. Quantification of the relative cell invasion was detected by 10% acetic acid solution. The expression of *AHCY* of cells used in each experiment was confirmed by western blotting analysis. The graphs show mean ± SD of three independent experiments. *means $P < 0.05$.

cells was reduced compared with control cells. Similarly, when MCF7 cells had low expression of *AHCY*, the invasive ability of the cells was also decreased (Figure 4B). In MCF7-ADR cells, the wound closure rate was lower in MCF7-ADR/shAHCY cells at 24 h post-wound induction and beyond (Figure 4C). The difference in the wound size between control groups and experimental groups was bigger in MCF7 cells than in MCF7-ADR cells (Figure 4A and 4C). The movement capability of cells was suppressed by about 50% in MCF7-ADR/shAHCY cells compared to the non-transduced cells (Figure 4D). The western blotting data in Figure 4 show that the expression of *AHCY* in both breast cancer

cells used to each experiment. Therefore, we conclude that down-regulation of *AHCY* plays a role in interrupting the invasion and migration potential of breast cancer cells.

Silencing of AHCY regulates cell proliferation through the MEK/ERK signaling pathway in MCF7 and MCF7-ADR cells

The effect of *AHCY* on Ras, phospho-B-Raf (P-B-Raf), phospho-C-Raf (P-C-Raf), MEK, and ERK protein expression in breast cancer cells was studied by western blot analysis because these proteins are important for cell proliferation of breast cancer cells. A decrease in the

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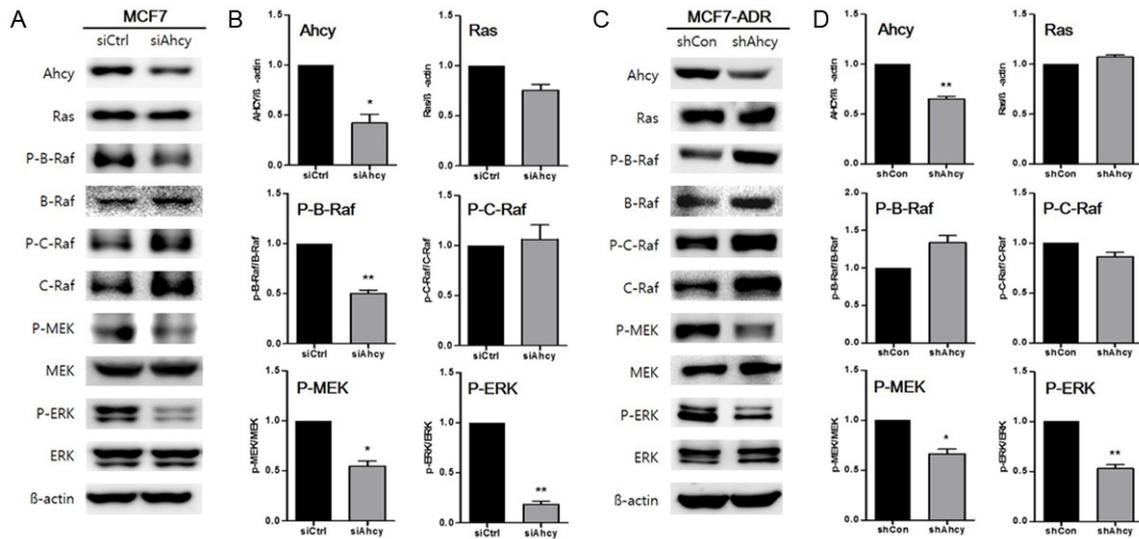


Figure 5. Regulation of MEK/ERK signaling in *AHcy*-knockdown breast cancer cells. A, C. Expression levels of Ras, C-/B-Raf, MEK, and ERK pathway molecules were detected by western blotting analysis in both MCF7 and MCF7-ADR cells and 30 μ g of the proteins were loaded per well. B, D. Protein levels were quantified by densitometry using Multi gauge. The graphs show mean \pm SD of three independent experiments. *means $P<0.05$ and **means $P<0.01$.

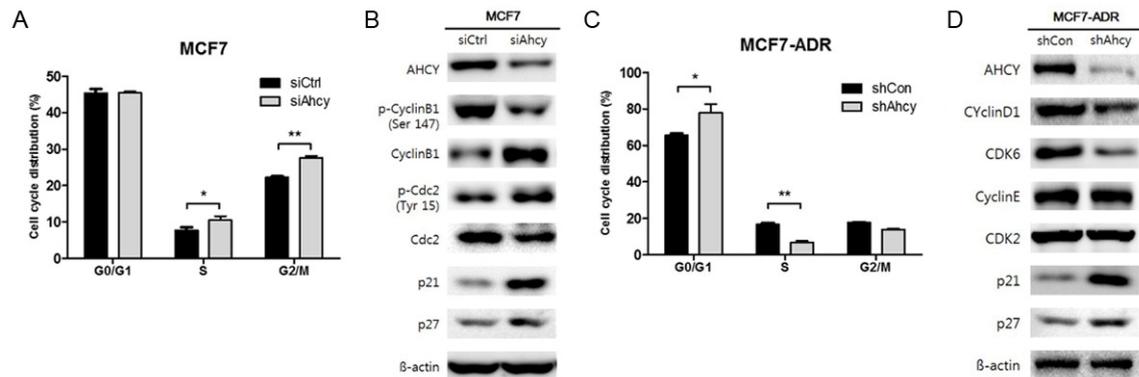


Figure 6. Cell cycle arrest by *AHcy* inhibition in breast cancer cells. A, C. The number of cells treated with propidium iodide (PI) for DNA staining in various stages of the cell cycle were quantified by measuring the area under the peaks. The cells in each phase were normalized to the total cells stained with PI. The graphs show mean \pm SD of three independent experiments. *means $P<0.05$ and **means $P<0.01$. B, D. The expression of cell cycle proteins was detected by western blotting analysis in both MCF7 and MCF7-ADR cells and 30 μ g proteins were loaded per well.

protein levels of P-B-Raf, P-MEK, and P-ERK was observed in *AHcy*-knockdown MCF7 cells but the protein levels of Ras and P-C-Raf were not changed by *AHcy* down-regulation (Figure 5A and 5B). Similar to the results in MCF7 cells, phosphorylation of MEK and ERK was also inhibited in MCF7-ADR/shAhcy cells but the phosphorylation of B-Raf was slightly elevated as opposed to the MCF7 cells (Figure 5C and 5D). These results suggest that the proliferation of breast cancer cell lines is regulated by

the expression of *AHcy* through the MEK/ERK signaling independent to Ras and Raf.

Reduction of AHcy causes G2/M phase arrest in MCF7 cells and G0/G1 phase arrest in MCF7-ADR cells

To study the mechanism behind *AHcy*'s cell proliferation-inhibitory activity, we carried out cell cycle analysis using flow cytometry. The results show that the cell cycle was arrested at

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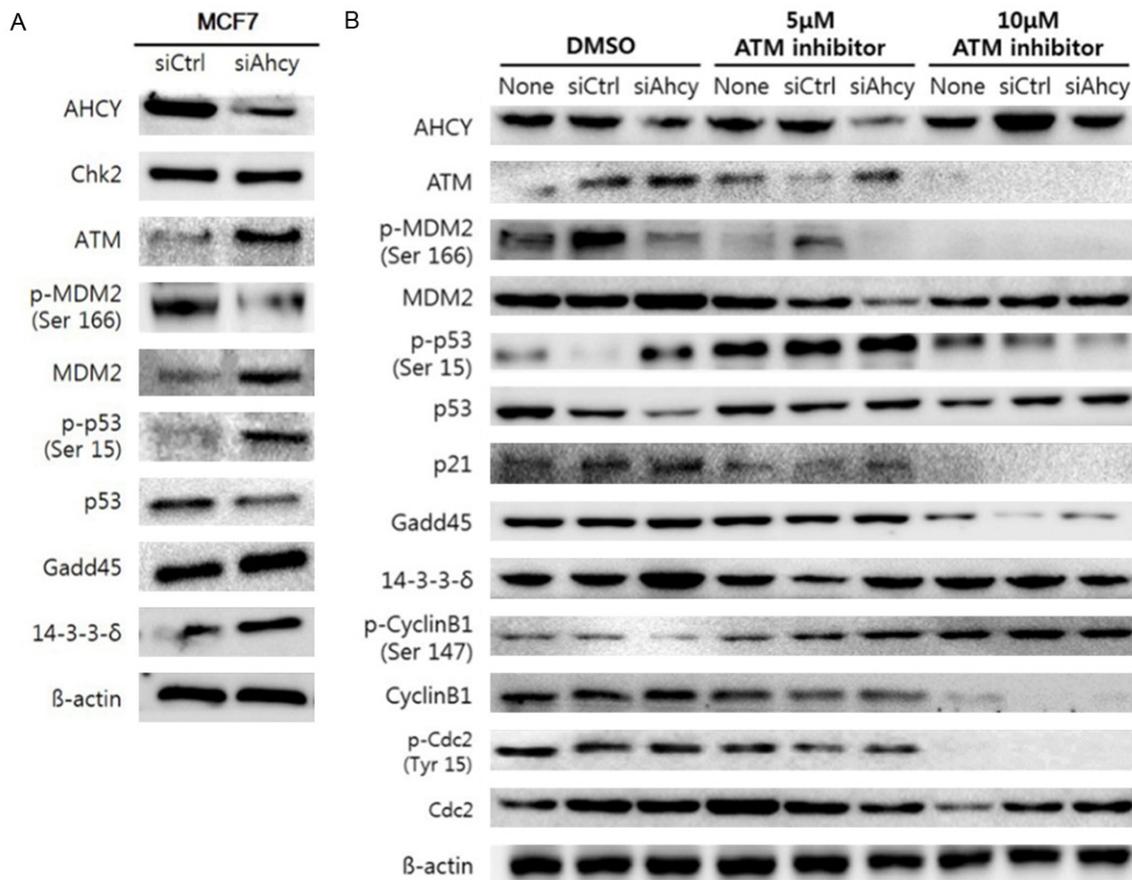


Figure 7. Phosphorylation of p53 through ATM kinase is controlled by AHCY in breast cancer cells. **A.** The expression of proteins related to the p53/MDM2 pathway were detected by western blotting analysis in MCF7 cells and 30 µg proteins were loaded per well. **B.** The expression of proteins related to both the p53/MDM2 pathway and G2/M checkpoint in the cell cycle were detected by western blotting analysis in MCF7 cells and 30 µg of the proteins were loaded per well. The ATM inhibitor was dissolved in DMSO and 10 µl of each solution was added to 2 ml DMEM. The ATM inhibitor was added after 1 h of AHCY siRNA transfection and the cells were harvested after 24 h of adding the ATM inhibitor.

the G2/M checkpoint in AHCY-knocked down MCF7 cells (**Figure 6A**). To elucidate the molecular mechanism behind the cell cycle arrest, we examined the expression of regulatory proteins of G2/M phase including cyclinB1 and phospho-CDC2 in AHCY-suppressed MCF7 cells. We determined that cyclinB1 was not degraded but was upregulated because CDC2 could not be de-phosphorylated and thus was unable to phosphorylate cyclinB1, which would cause the G2/M arrest of the cell cycle (**Figure 6B**). When AHCY was knocked down in MCF7-ADR cells, the cell cycle was arrested at the G0/G1 checkpoint (**Figure 6C**). The expression of cyclinD1 and CDK6-modulators of the G0/G1 cell cycle checkpoint-was reduced in MCF7-ADR/shAHCY cells as opposed to the levels of cyclinE and CDK2, which were not changed (**Figure 6D**). We

also confirmed that the expression of p21 and p27, two CDK inhibitors, was increased in both MCF7 and MCF7-ADR cells treated with AHCY siRNA and shRNA (**Figure 6B** and **6D**). The results demonstrated that AHCY knockdown induced cell cycle arrest at the G2/M checkpoint in MCF7 cells and at the G0/G1 checkpoint in MCF7-ADR cells.

AHCY inhibition indirectly affects phosphorylation of p53 by regulating ATM kinase in MCF7 cells

We confirmed that the expression of p53, an inducer of p21, increased in MCF7 cells treated with AHCY siRNA. To confirm whether phosphorylation of p53 is indirectly induced by regulation of AHCY in MCF7 cells, we studied the

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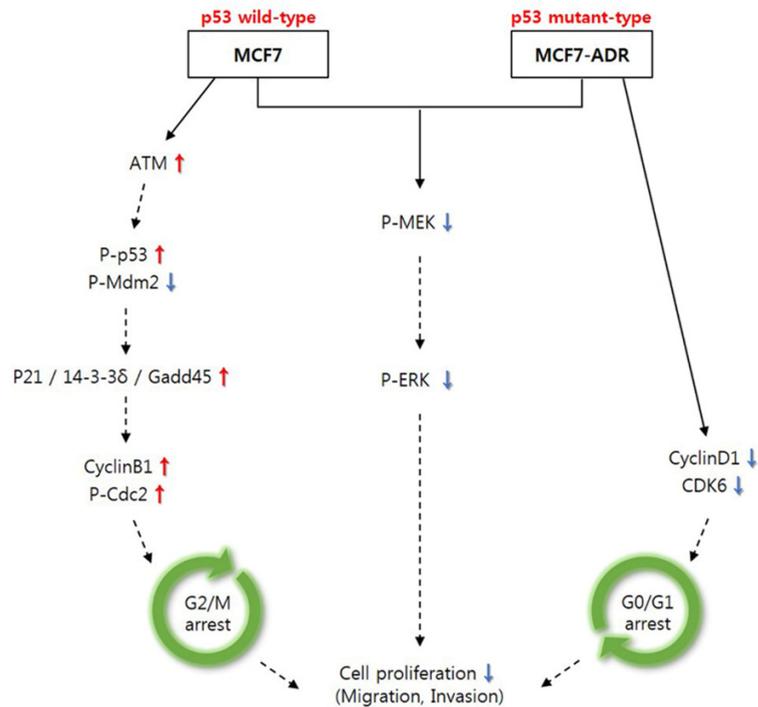


Figure 8. Summary of function of silenced-*AHCY* in MCF7 and MCF7-ADR cells. Brief sketch about function of *AHCY*-silencing on cell proliferation in both MCF7 and MCF7-ADR cells *in vitro*. Solid line means novelty processes and dotted line means to be already known.

expression of phospho-p53 and phospho-MDM2, an inhibitor of p53. Additionally, we also confirmed the expression of ATM—a kinase that directly phosphorylates p53- and CHEK2—an up-regulator of ATM- and molecules downstream of p53 such as Gadd45 and 14-3-3 δ . When *AHCY* was suppressed in MCF7 cells, ATM was hyper-expressed independent of CHEK2 and p53 was actively phosphorylated by the de-phosphorylation of MDM2. Furthermore, the expression of Gadd45 and 14-3-3 δ , two modulators of G2/M cell cycle arrest, were upregulated (**Figure 7A**). These results support the data of flow cytometry shown in **Figure 6**. To verify that ATM is directly regulated by *AHCY*, we used an ATM inhibitor. Although MDM2 was slightly phosphorylated by the ATM inhibitor, other molecules such as phospho-p53, p21, Gadd45, 14-3-3 δ , cyclinB1 and phospho-CDC2 that were upregulated by *AHCY* siRNA, were down-regulated by the ATM inhibitor (**Figure 7B**). We thus conclude that *AHCY* regulates the cell cycle and proliferation in an ATM-dependent manner.

Discussion

In this study, inhibition of *AHCY* in both MCF7 and MCF7-ADR cells suppressed cell invasion and migration as well as proliferation via the inhibition of the phosphorylation of MEK/ERK and cell cycle arrest. The cell proliferation was regulated by arrested at the G2/M checkpoint through the over-expression of ATM in *AHCY*-deficient MCF7 cells and at the G0/G1 checkpoint through the down-regulation of cyclinD1 and CDK6 in *AHCY*-silenced MCF7-ADR cells (**Figure 8**). Especially, our study demonstrated that the arrest of G2/M phase resulted from ATM kinases which was regulated by *AHCY* in MCF7 and it can be novel fact.

Despite MCF7 and MCF7-ADR cell lines have common origin, the two cell lines are very different.

The MCF7-ADR cells were more resistant to doxorubicin than MCF7 cell line and expression of genes that are important in drug resistance significantly change about 49% [25]. However, the expression pattern of *AHCY* was same in both MCF7 and MCF7-ADR cells (**Figure 1**). These expression patterns may result from c-MYC-overexpressed in two cell lines because it is known that c-MYC induces up-regulation of *AHCY* for mRNA cap methylation in cells [26]. We determined that the *AHCY* is expressed dependent of c-MYC in various subtypes of breast cancer cell lines including luminal (MCF7 and T47D), HER2 positive (MCF7-ADR and SK-BR-3) and triple-negative (MDA-MB-231 and MDA-MB-435s). This finding suggests that breast cancer cells negatively expressing *AHCY* such as SK-BR-3 may have c-MYC mutation, but it remains unclear whether c-MYC directly regulates expression of *AHCY*.

The Ras-Raf-MEK-ERK is responsible for many phenotypes in breast cancer cells, including cell proliferation, cell cycle progression, apoptosis, and senescence [27]. Consequently, our

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study confirms that breast cancer cell proliferation is inhibited by a decrease in the phosphorylation of MEK and ERK through the silencing of *AHCY* and independent of Ras and B-/C-Raf. This suggests that the *AHCY* knockdown may induce activation of some molecules that suppress the phosphorylation of MEK independently of the levels of Ras and Raf. PP2A regulates MEK activation Raf-independent in response to ERK silencing [28], we supposed that *AHCY*-defected induces activation of PP2A for inhibiting MEK phosphorylation but we confirmed that PP2A was not a target of *AHCY* (not shown data). More research is needed on the molecules that are regulated by *AHCY* and suppress MEK independently of Ras and Raf.

While MCF-7 human breast carcinoma cells have wild-type p53, MCF7-ADR subline cells have mutant p53 [29]. We found that cell cycle arrest occurred at different phases of the cell cycle in the breast cancer cells, MCF7 and MCF7-ADR (**Figure 6**). These studies suggest that *AHCY* may influence on cell cycle in other p53-wild typed breast cancer cells through same mechanism as that in MCF7 cells. Furthermore, silenced-*AHCY* may induce cell cycle arrest in breast cancer cells regardless of p53 status although we could not find accurate mechanisms yet. Therefore, we expect that unnatural cell proliferation could be inhibited by suppressing *AHCY* in various breast cancer cell lines.

ATM is a kinase that activates p53 by de-phosphorylating MDM2 [30], and a crucial substrate of ATM is CHEK2 that results in the activation and, in turn, the phosphorylation of a number of substrates such as p53, breast cancer 1 (BRCA1), and others [31]. However, we found that ATM levels were increased in *AHCY*-deficient breast cancer cells while CHEK2 levels were not changed (**Figure 7**). Therefore, we conclude that the effects of *AHCY* are independent of CHEK2 activation or *AHCY* may have other substrates for ATM activation in breast cancer cells. *AHCY*-dependent substrates about ATM should be more researched in the future.

In conclusion, our study extensively evaluated the anti-proliferative effects of *AHCY*-knockdown breast cancer cells, which was mediated by G2/M arrest via the ATM-kinase activated p53/MDM2 pathway in p53 wild-type MCF7

cells, and G1/S arrest in p53-mutated MCF7-ADR cells. Finally, our findings significantly contribute to the understanding of the anti-cancer properties of *AHCY* as well as to the future development of therapeutic agents against breast cancer.

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Disclosure of conflict of interest

I have disclosed those interests fully to Sookmyung women's university, and have in place an approved plan for managing any potential conflicts arising from this arrangement.

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