의학박사 학위논문

Influence of ATM on DNA methyltransferaseinhibitor-induced radiosensitization

ATM 단백질이 DNA 메틸전이효소 억제제의 방사선감수성 증강에 미치는 영향에 관한 연구

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ABSTRACT

Background: Preclinical studies have demonstrated that inhibition of DNA methyltrasferase (DMNT) induces radiosensitization in human cancer cells. The current study is to investigate the role of ATM in DNMT inhibitor-mediated radiosensitization.

Methods: A pair of isogenic cell lines was constructed from AT fibroblasts: one without ATM and the other expressing functional ATM. The effects of DNMT inhibitor treatment on radiosensitivity were evaluated in these cell lines using two DNMT inhibitors: psammaplin A (PSA) and 5-aza-2'-deoxycytidine (DAC). DNA damage repair was analyzed using γH2AX foci. Western blot of DNA damage foci proteins was done.

Results: DNMT inhibition induced radiosensitization of ATM-expressing cells, but had no effect in ATM-null cells. DNMT inhibitor treatment prolonged expression of both γH2AX foci and phospho-BRCA1 after irradiation in cells with ATM, whereas neither was observed in ATM-null cells treated with DNMT inhibitors. Expression of MRN complex subunits (MRE11, Rad50, and NBS1) was not altered by either of DNMT inhibition or irradiation. DNMT inhibitor treatment or irradiation induced MRE11 phosphorylation in both ATM-null and ATM-expressing cell lines.

Conclusion: DNMT inhibitors enhance radiosensitivity by delaying

DNA damage repairs. Radiosensitization by DNMT inhibition requires functional ATM.

Keywords: DNMT inhibitor, radiosensitization, ATM, BRCA1, MRN complex

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INTRODUCTION

Epigenetic modifications involve heritable information of gene expression regulation, which is not encoded in DNA sequences (1). Epigenetic mechanisms are implicated in human health and disease conditions, such as cancer (1,2). DNA methylation is one of the most important epigenetic mechanisms. Chromatin structure and DNA methylation is closely related and aberrant DNA methylation plays a causal role in tumorigenesis (2). DNA methylation is mediated by DNA methyltransferase (DNMT), which catalyzes the transfer of a methyl moiety from S-adenosyl-L-methionine to cytosines in CpG dinucleotides. It has been suggested that DNMT overexpression and CpG island hypermethylation is associated with tumorigenesis and/or poor prognosis in various cancers (3–6). As DNA methylation is a reversible process, DNMT presents as potential target for cancer treatment. Several DNMT inhibitors are currently under investigation for cancer treatment (7). Recently, DNMT inhibitors have been shown to enhance cell killing effect of ionizing radiation (IR) (8–11). Based on these reports, it has been speculated that DNMT inhibitors potentially enhance the efficacy of radiotherapy.

So far, mechanisms underlying radiosensitization by DNMT inhibitors are not fully elucidated. It has been demonstrated that DNA damage repair is suppressed by DNMT inhibition (8,9). Recent findings

have demonstrated that DNMT1 colocalizes at DNA double-strand break (DSB) sites following genomic insults (12,13), which suggests DNMT1 interacts with proteins recruited at DNA damage foci. Cellular response to DSB involves recruiting of a range of proteins surrounding the lesions. The foci comprise key proteins including ATM, BRCA1, and MRN complex (MRE11, RAD50, and NBS1) (14). ATM, a serine/threonine protein kinase, is one of the proteins recruited in the foci around DNA DSB and plays a key role in sensing and signaling DNA damage (14,15). As ATM status is closely related to cellular sensitivity to ionizing radiation (IR), DNMT inhibitors might enhance cellular radiosensitivity by modulating ATM-dependent pathways. However, the relationship of ATM with DNMT inhibition-induced radiosensitization has not been addressed.

The current study was to investigate the role of ATM in in vitro radiosensitization by DNMT inhibitors. We demonstrated that DNMT inhibition enhanced radiosensitivity by interfering DNA DSB repair only in cells expressing functional ATM. Our findings suggest that aberrant phosphorylation of MRE11 might underlie radiosensitization by DNMT inhibitors.

MATERIALS AND METHODS

Reagents

Two DNMT inhibitors, psammaplin A (PSA) and 5-aza-2'-deoxycytidine (DAC), were obtained from Sigma (St. Louis, MO, USA). Both inhibitors have been demonstrated to effectively radiosensitize human cancer cells (8). DNMT inhibitors were dissolved in DMSO as concentrated stock and diluted in media at the time of experiments. Lipofectamine 2000 reagent and G418 from Invitrogen (Carlsbad, CA, USA). Dulbeco's Modified Eagle Media from Gibco (Carlsbad, CA, USA). RPMI media from Welgene (Daegu, Korea). Fetal bovine serum and gentamicin from Gibco. Anti-ATM antibody from Abcam (Cambridge, MA, USA). Anti-histone γH2AX (Ser139), anti-BRCA1, anti-phospho-BRCA1 (Ser1524), anti-DNA-PK, anti-MRE11, anti-phospho-MRE11 (Ser676), anti-Rad50, anti-NBS1, anti-phospho-NBS1 (Ser343), HRP-linked anti-rabbit IgG antibodies from Cell Signaling Technology (Camarillo, CA, USA). FITC-labeled secondary antibody from Invitrogen.

Cell lines

AT fibroblasts, AT5BIVA, were purchased from Coriell Cell Repositories (GM05849, Camden, NJ, USA). A clone of AT5BIVA stably expressing ATM (AT5BIVA/ATM) was generated by transfecting AT5BIVA with pcDNA3 flag-ATM, which contains wild type ATM

cDNA.AT5BIVA cells were transfected by Lipofectamine and selected with 600 ul/ml G418. A549 and U373MG cells were purchased from the Korean Cell Line Bank (Seoul, Korea). Cell lines were maintained in Dulbeco's Modified Eagle Media (AT5BIVA, AT5BIVA/ATM) and RPMI media (A549, U373MG), supplemented with 10% fetal bovine serum and 12.5 μg/ml of gentamicin. Cultures were maintained saturated with 5% CO_2 at 37 degrees Celsius.

Clonogenic assay

Cells were trypsinized from exponentially growing monolayer cultures and appropriate numbers were seeded in 6-well flasks. After 24-48hrs of incubation, cells were treated with DNMT inhibitors and/or IR. Irradiation was done using 6 MV X-ray from a linear accelerator (Clinac 21EX, Varian Medical Systems, Palo Alto, CA, USA). Cells were treated with DNMT inhibitors for 18 hr prior to irradiation. Following irradiation, cells were incubated in fresh media free from DNMT inhibitors for 14-21 days to form colonies. Colonies were fixed with methanol and stained with 0.5% crystal violet. Colonies containing at least 50 cells were counted to calculate surviving fractions. Each surviving fraction is the mean value from at least triplicate experiments.

Immunocytochemistry

Cells were grown and treated in tissue culture chamber slides (Nalge Nunc International, Naperville, IL, USA). At the specified times, medium was aspirated and cells were fixed in 4% paraformaldehyde

for 10 minutes at room temperature. The paraformaldehyde was aspirated, and the cells were treated for 15 min with a 0.2% NP40/PBS solution. The cells were then washed twice in PBS, and anti-phospho-γH2AX antibody was added at a dilution of 1:200 in 1% bovine serum albumin and incubated overnight at 4 degrees Celsius. Cells were again washed twice in PBS prior to 1 hour of incubation in dark with an FITC-labeled secondary antibody at a dilution of 1:50 in 1% bovine serum albumin. The secondary antibody solution was then aspirated and the cells were washed twice in PBS, followed by 30 minutes of incubation in dark with 4',6-diamindino-2-phenylindole (1 μg/mL) in PBS and two subsequent washings. The coverslips were then mounted with antifade solution (Vector Laboratories, Burlingame, CA, USA). Slides were examined with a Leica DMRXA fluorescent microscope (Leica, Wetzlar, Germany). The images were captured using a PhotometricsSensys CCD camera (Leica) and imported into the IP Labs image analysis software package (Leica). To determine γH2AX-positive cells, we used a method previously reported (16). For each treatment, only foci in nuclei were counted manually in at least 30 cells. Cells were counted as positive for γH2AX when more than 10 foci were detected.

Western blot

Cell lysates were prepared in cell lysis buffer (iNtRON Biotechnology, Seoul, Korea). The total cellular proteins (50 ug) were separated on

SDS-PAGE and transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA). The membranes were blocked with blocking solution in 5% nonfat dry milk (25 mM Tris, pH 7.5; 0.15 M NaCl; 0.05% Tween) for 1 hour and probed with primary antibody at a dilution of 1:1,000 overnight. The membranes were incubated with blocking solution containing appropriate secondary antibody at 1:2,000 for 2 hours. Western blot protein detection was conducted using the ECL kit (Intron Biotechnology, Seongnam, Korea) according to the manufacturers' recommendations. Monoclonal antibody against actin was used as control. The photographs of bands were taken and analyzed using ImageJ version 1.45s (National Institutes of Health, Bethesda, ML, USA).

Statistical analysis

Mean values were calculated at least three independent observations. Error bars represent standard deviations.

RESULTS

Construction of AT5BIVA cell clones expressing ATM

A series of AT5BIVA cells were transfected with a vector containing wild type ATM (Figure 1). The constructed clones varied in ATM expression level. We chose and used the clone with the highest ATM expression in subsequent experiments (AT5BIVA/ATM clone #6 in Figure 1).

Enhancement of cellular radiosensitivity by DNMT inhibitors

To estimate cytotoxicity of DNMT inhibitors, AT5BIVA and AT5BIVA/ATM cells were exposed to varying concentrations of PSA and DAC for 18 hr, and surviving fractions were obtained using clonogenic assay (data not shown). The concentrations killing 50% of cells are defined inhibitory concentration 50% (IC50). Estimated IC50s were 0.5 ug/ml (PSA) and 200 nM (DAC). IC50s were approximately identical between both cell lines. Cells were treated with DNMT inhibitors of respective IC50s and irradiated with graded doses of X-ray. DNMT inhibitors had no effect on radiosensitivity of AT5BIVA cells (Figure 2), while both inhibitors enhanced radiation cell killing in AT5BIVA/ATM cells (Figure 3). These findings demonstrated that DNMT inhibitors induced radiosensitization only in cells expressing functional ATM.

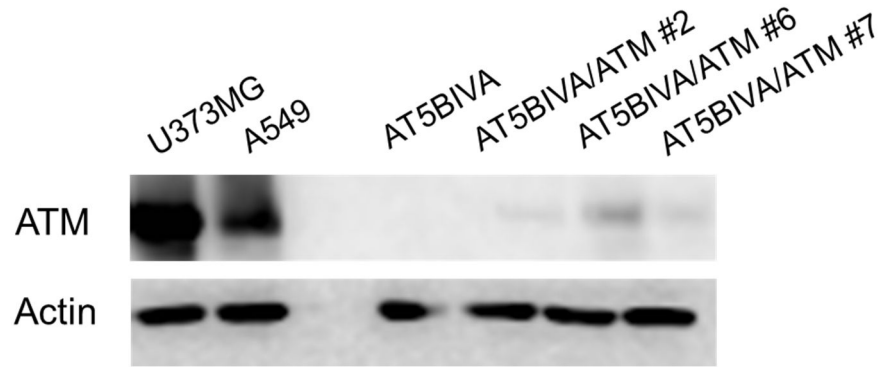


Figure 1. Construction of ATM-expressing clones from AT5BIVA.

Both U373MG and A549 cell lines strongly express ATM, while AT5BIVA cells do not. AT5BIVA/ATM cell clones were constructed by transfecting AT5BIVA cells with an ATM-expression vector. AT5BIVA/ATM clone with the highest ATM expression (AT5BIVA/ATM#6) was used in all subsequent expressions.

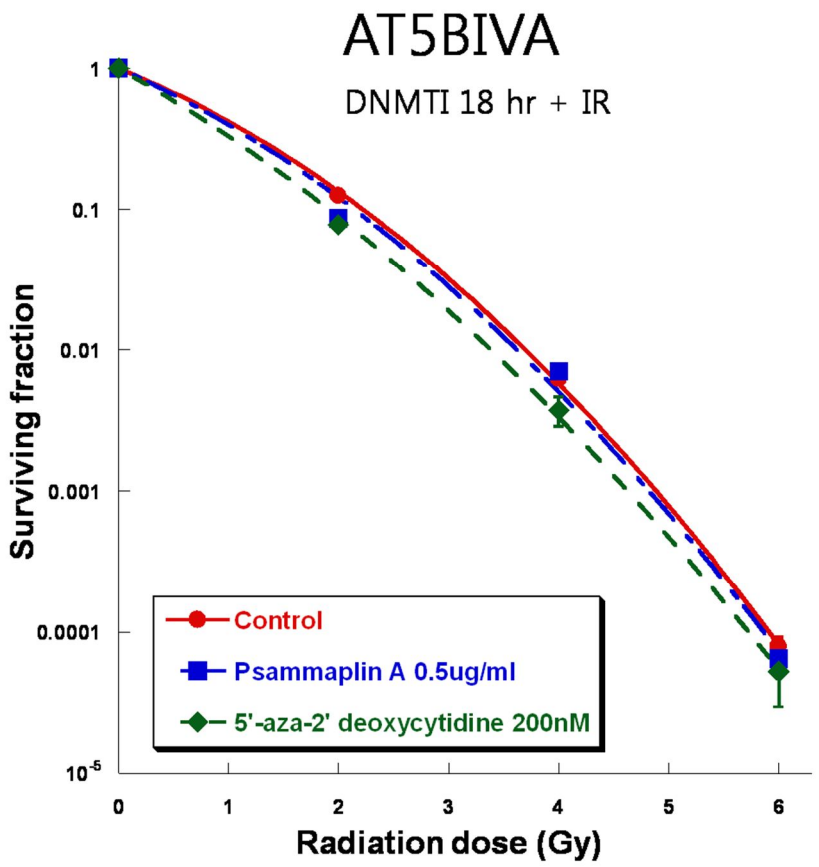


Figure 2. DNMT inhibitors had no effect on radiosensitivity in ATM-null cells.

Cells were treated with DNMT inhibitors for 18 hr prior to IR. Then, drug-containing media were replaced with fresh media, and cells were irradiated.

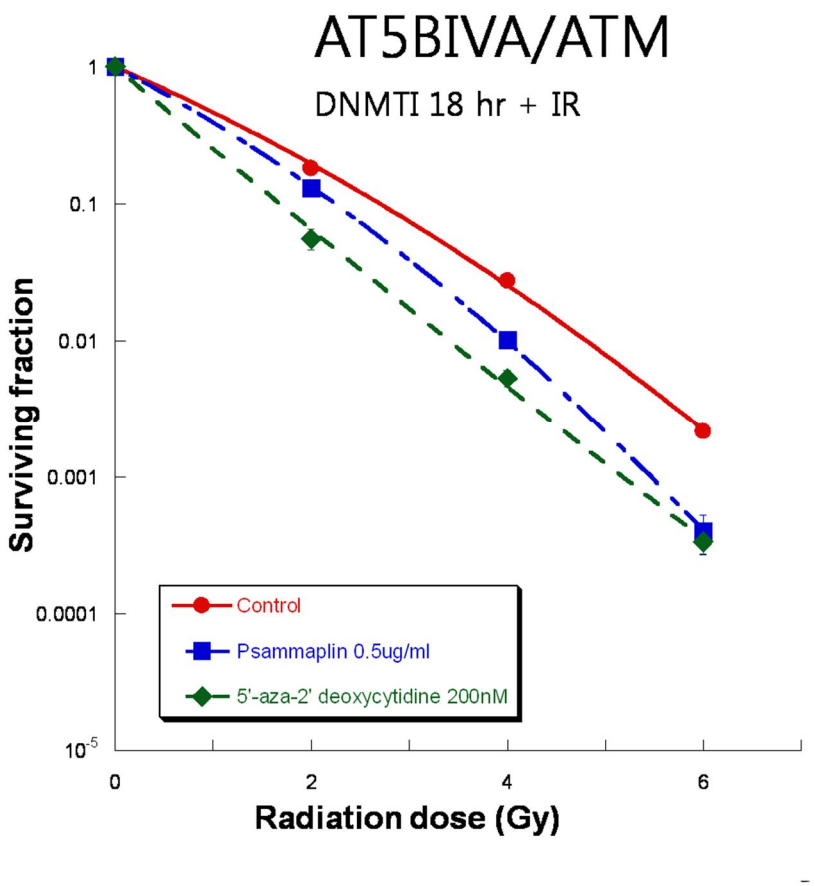


Figure 3. DNMT inhibition enhanced radiosensitivity in ATM-expressing cells.

Cells were treated with DNMT inhibitors for 18 hr prior to IR. Then, drug-containing media were replaced with fresh media, and cells were irradiated.

DNA DSBs after IR

Since ATM is a key regulator of signaling cascades of DNA damage, we chose to investigate DNA damage repair after the combination of DNMT inhibitors and IR. Histone γH2AX has been identified as a marker of DNA DSBs (17). It has been demonstrated that DNMT inhibition delays clearance of IR-induced γH2AX (8). AT5BIVA and AT5BIVA/ATM cells underwent DNMT inhibitor treatment followed by irradiation. Then, immunocytochemistry for γH2AX was performed (Fig 3). In both cell lines, 4-Gy X-ray produced multiple γH2AX foci in the nuclei. While DAC had little effect on the formation of γH2AX foci, PSA treatment induced foci in both cell lines in the absence of IR (Figures 4 and 5). In AT5BIVA cells, γH2AX foci persisted at 24 hr of IR, and DNMT inhibition had little effect on post-IR γH2AX foci formation (Figure 6). In AT5BIVA/ATM cells, γH2AX foci peaked 2 hr following IR and rapidly dropped at 6 hr, and DNMT inhibition delayed disappearance of foci (Figure 7). These findings suggest that DNMT inhibitors delayed IR-induced DNA DSB repair.

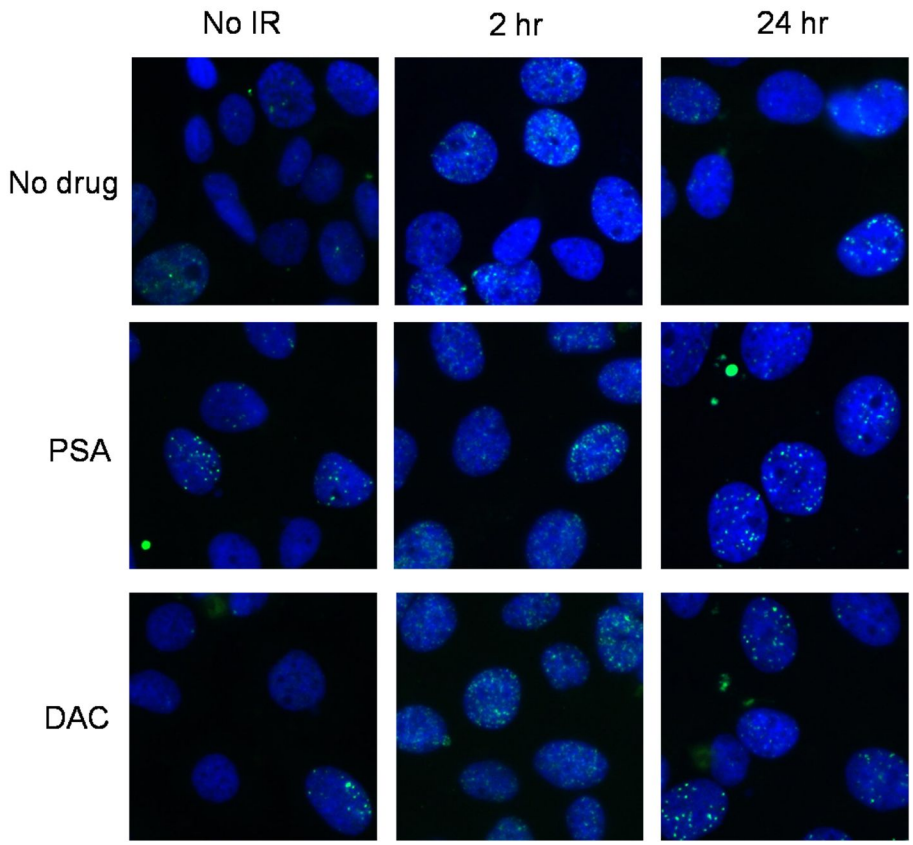


Figure 4. γH2AX following DNMT inhibition and IR in ATM-null AT5BIVA cells.

Cells were treated with DNMT inhibitors for 18 hr, irradiated with 4-Gy X-ray. At specified times after IR, cells were fixed and stained for γH2AX.

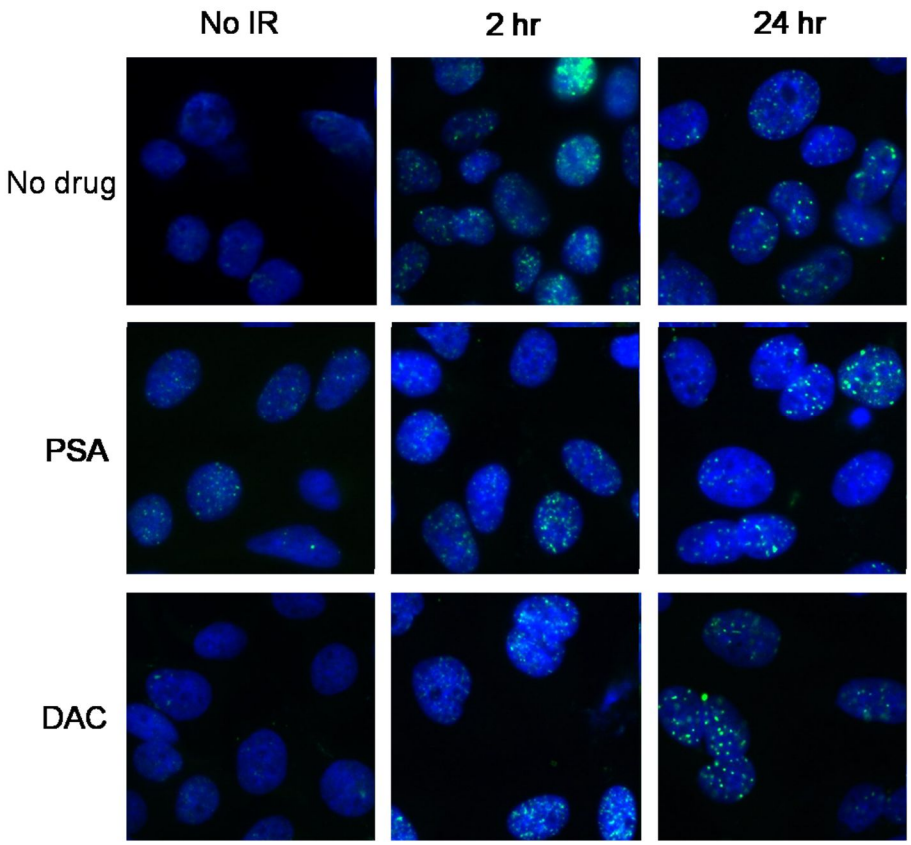


Figure 5. γH2AX following DNMT inhibition and IR in AT5BIVA/ATM cells expressing functional ATM.

Cells were treated with DNMT inhibitors for 18 hr, irradiated with 4-Gy X-ray. At specified times after IR, cells were fixed and stained for γH2AX.

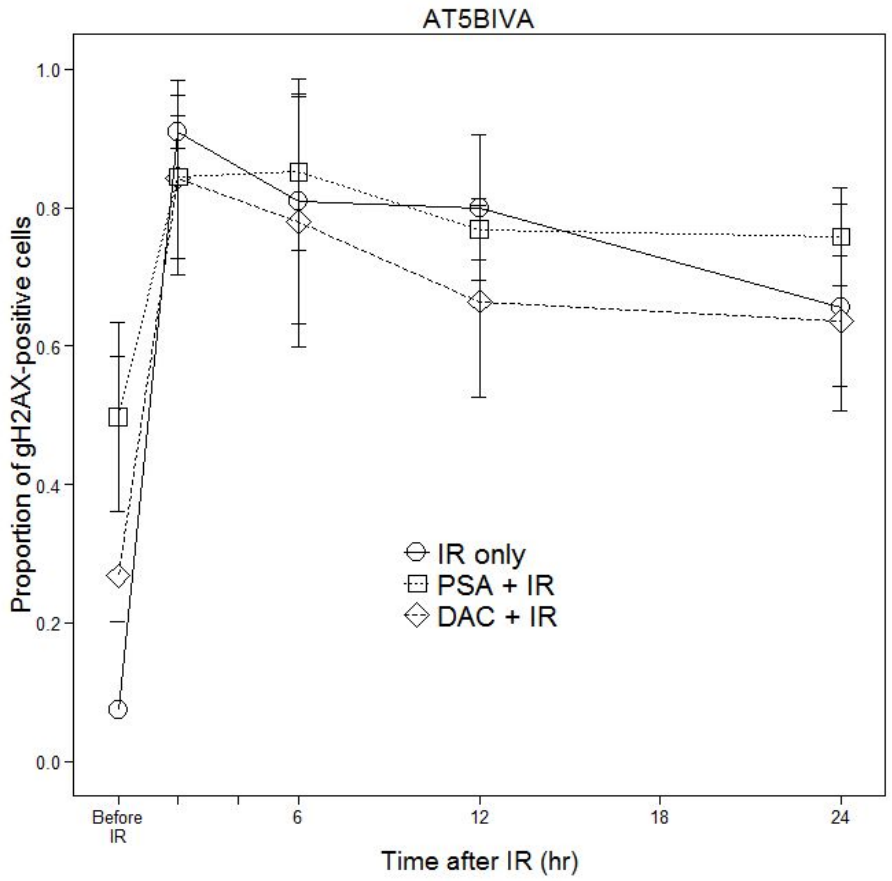


Figure 6. γH2AX change following DNMT inhibition and IR in ATM-null AT5BIVA cells.

Cells were treated with DNMT inhibitors for 18 hr, irradiated with 4-Gy X-ray. At specified times after IR, cells positive for γH2AX foci were counted.

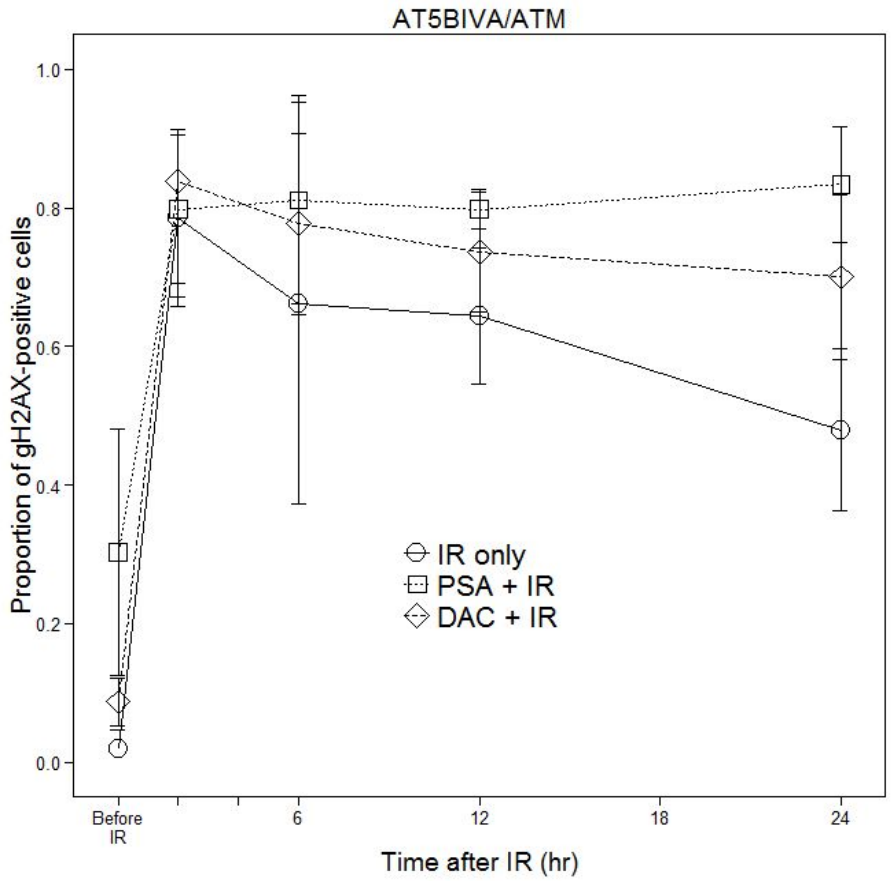


Figure 7. γH2AX change following DNMT inhibition and IR in ATM-expressing AT5BIVA/ATM cells.

Cells were treated with DNMT inhibitors for 18 hr, irradiated with 4-Gy X-ray. At specified times after IR, cells positive for γH2AX foci were counted.

Western blot analyses

Cells respond to DNA DSB by localizing a complex array of proteins to the region surrounding the DNA lesion in a dynamically organized and timely manner (14). As these proteins form a large, multi-component focus around DNA DSB and γH2AX (14), we investigated known components of the foci using Western blot.

Firstly, BRCA1, Rad51, and DNA-PK were investigated. AT5BIVA and AT5BIVA/ATM cells were treated with DNMT inhibitors, irradiated, and analyzed at specified times after IR. Neither DNMT inhibition nor IR had any influence on the expression of BRCA1 and Rad51 in both cell lines (Figure 8A and 8B). Phospho-BRCA1 showed no increase immediately following IR, and its amount decreased at 24 of IR in untreated cells regardless of their ATM expression status. DNMT inhibition had no effect on post-IR phospho-BRCA1 expression in ATM-null cells (Figure 8A). In AT5BIVA/ATM, DNMT inhibitor treatment increased phospho-BRCA1 at 24 hr after IR compared to that of untreated cells (Figure 8B). DNA-PK was not detected in both cell lines in repeated experiments (data not shown).

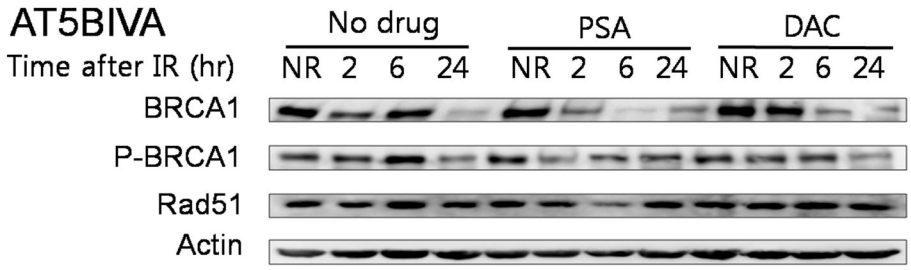
Next, components of MRN complex were analyzed. MRN complex consists of MRE11, Rad50, and NBS1, and plays a key role in sensing, processing, and repairing DNA DSB (14). AT5BIVA/ATM cells were blotted for MRE11, Rad50, and NBS1 after DNMT inhibitor treatment and IR. The expression of MRN subunits was not influenced

by IR, DNMT inhibition, and both (Figure 9). AsMRE11 and NBS1 are known to be phosphorylated following DNA damage, their phosphorylated forms were also investigated. In both AT5BIVA and AT5BIVA/ATM cells, MRE11 phosphorylation was induced by either IR or DNMT inhibitor treatment (Figure 10A and 10B). Combination of DNMT inhibition and IR produced more prominent and prolonged expression of phosphor-MRE11 following IR in both cell lines. Neither IR nor DNMT inhibition induced apparent changes in phospho-NBS1 expression (Figure 10A and 10B). Next, phospho-MRE11 was analyzed in A549 cells, which express wild type ATM. A549 cells showed increased phospho-MRE11 after DNMT inhibitor treatment or IR (Figure 10C). Combination of DNMT inhibition with IR further augmented phospho-MRE11 compared to either of both treatments did. Phospho-NBS1 expression was not affected by DNMT inhibition in A549 cells.

The relative amount of phospho-MRE11 was quantitatively analyzed. Optical density was measured for each band of phopho-MRE11 from AT5BIVA and AT5BIVA/ATM cells. To cancel out the effect of DNMT inhibition on MRE11 phosphorylation, each optical density was divided by that of corresponding unirradiated cells to produce normalized values. MRE11 phosphorylation was observed following IR and PSA treatment in AT5BIVA cells (Figure 11). In AT5BIVA/ATM, IR-induced MRE11 phosphorylation was much less

compared to that in AT5BIVA (Figure 11).

(A)



(B)

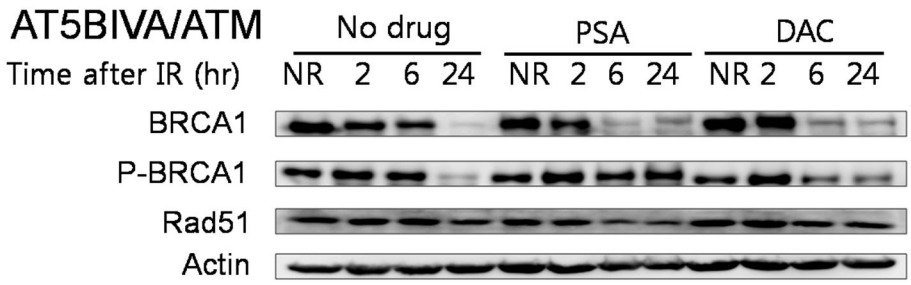


Figure 8. Expression of BRCA1, phospho-BRCA1, and Rad51 following DNMT inhibition and IR.

Cells were treated with DNMT inhibitors for 18 hr, and then irradiated in the absence of DNMT inhibition. At specified times, cells were fixed and analyzed with Western blot. (A) AT5BIVA. (B) AT5BIVA/ATM.

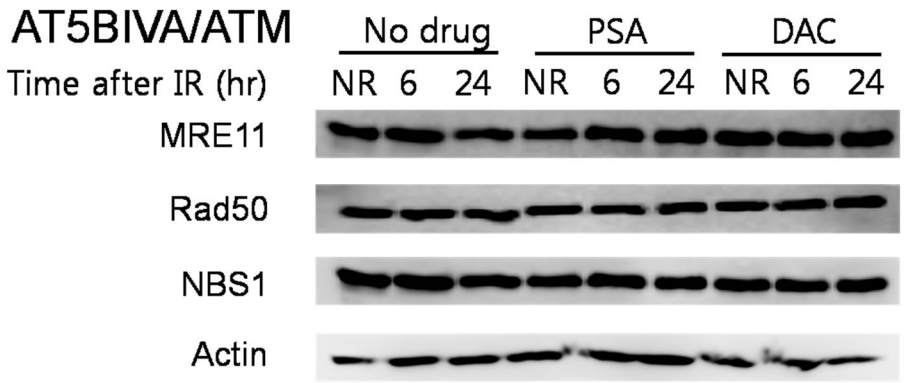
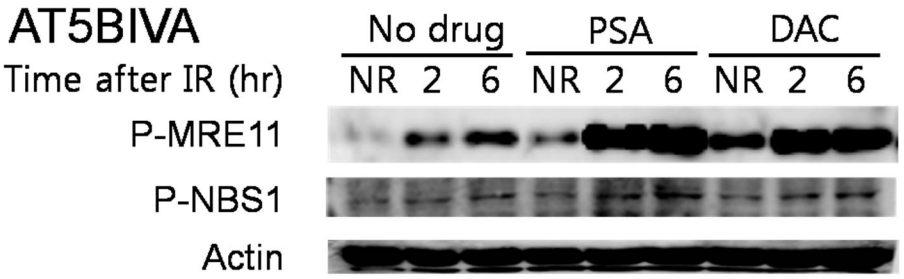


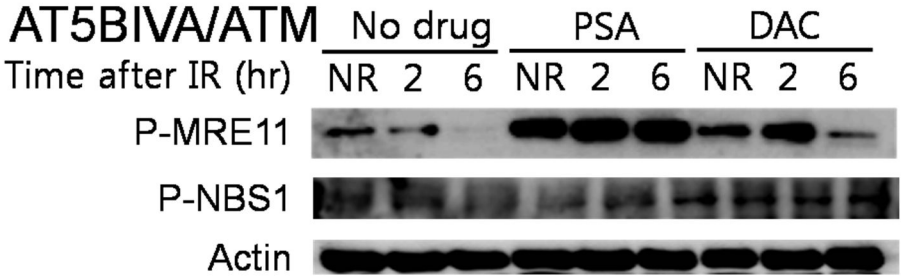
Figure 9. Expression of MRN complex following DNMT inhibition and IR.

AT5BIVA/ATM cells were treated with DNMT inhibitors for 18 hr, and then irradiated in the absence of DNMT inhibition. At specified times, cells were fixed and analyzed with Western blot for each component of MRN complex.

(A)



(B)



(C)

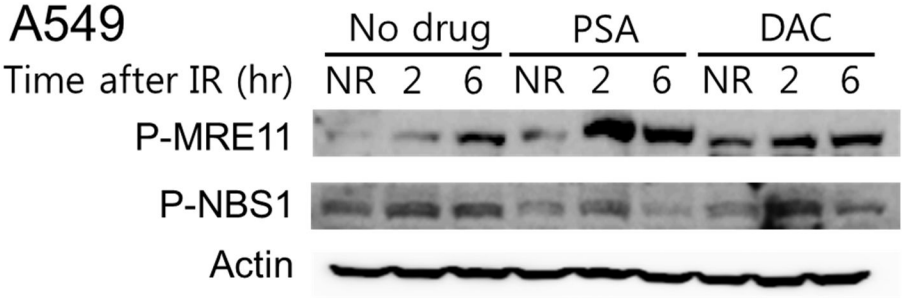


Figure 10. Phosphorylation of MRE11 and NBS1.

Cells were treated with DNMT inhibitors for 18 hr, and then irradiated in the absence of DNMT inhibition. At specified times, cells were fixed and analyzed with Western blot. (A) AT5BIVA. (B) AT5BIVA/ATM. (C) A549.

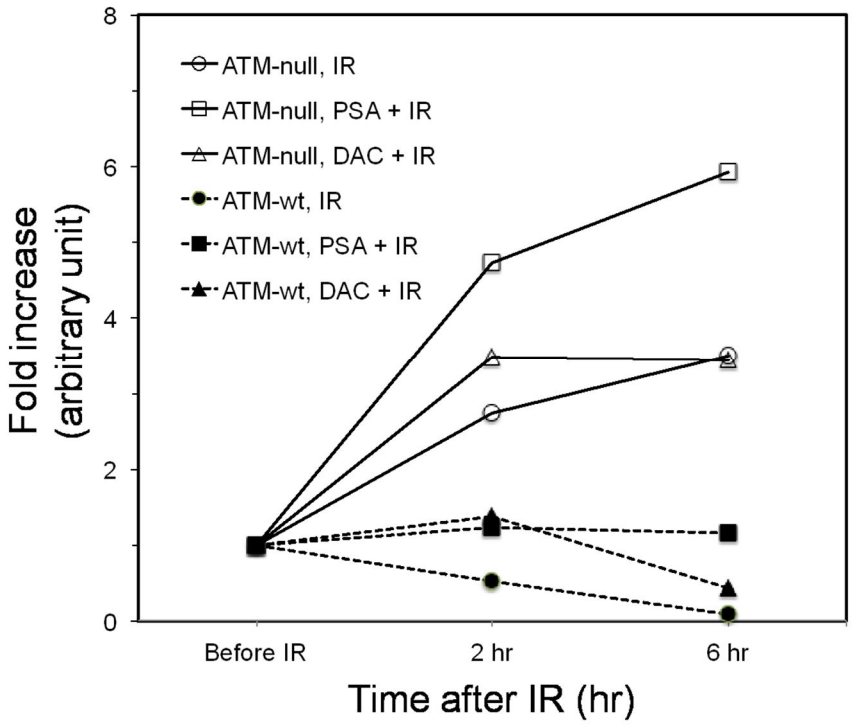


Figure 11. Relative increase in phospho-MRE11 following IR.

Cells were treated with DNMT inhibitors for 18 hr, and then irradiated in the absence of DNMT inhibition. At specified times, cells were fixed and analyzed with Western blot for phosphor-MRE11. OD for each band was measured and normalized by that from unirradiated cells.

DISCUSSION

The current study shows that radiosensitization by DNMT inhibition is dependent on ATM expression status. DNMT inhibitors enhance radiosensitivity only in cells expressing functional ATM. Delayed DNA DSB repair is a major mechanism underlying radiosensitization by DNMT inhibition. Our findings also demonstrate DNA repair process is inhibited only ATM-expressing cells.

It might be argued that ATM-null cells are intrinsically as sensitive as human cells can be that further sensitization is not feasible by any means. However, it has been reported that pharmacological agents sensitize ATM-negative cells to IR. Wang et al have reported that nonspecific inhibitors of ATR and CHK1 enhanced radiosensitivity in AT cells (18). Kim et al have also demonstrated that trichostatin A, histone deacetylase inhibitor, sensitized ATM-null cells (19). Given these studies, our observations that AT5BIVA radiosensitivity was not altered by DNMT inhibitors suggest ATM-dependency of DNMT inhibition-induced radiosensitization. This speculation is consistent with our finding that isogenic AT5BIVA/ATM is sensitized by DNMT inhibitors. The current study demonstrated the direct relationship between DNS DSB repair and radiosensitization: functional ATM is required for DNMT inhibitors to delay γH2AX clearance and enhance radiation cell killing. Our findings corroborate the previous reports that

DNA repair inhibition is a key mechanism underling radiosensitization by DNMT inhibitors (8,9).

The initial formation of DNS DSBs after IR seems independent of ATM status. Our observations show that that the number of γH2AXfoci immediately following IR was not changed by ATM expression status. DNMT inhibition had no apparent effect on initial foci formation, but only delayed foci clearance only in AT5BIVA/ATM cells. This further implies that DNA damage repair inhibition is a key mechanism underlying radiosensitization.

Given that ATM and other proteins colocalizes around DNA DSB sites (14), it is plausible that DNMT inhibition influence the function of proteins interacting with ATM to induce sensitization. We observed that DNMT inhibition delayed phospho-BRCA1 clearance after IR in ATM-expressing cells, but had no apparent effect in ATM-null cell, which coincides with our observations of relationship of ATM status with γH2AX. Persistent phospho-BRCA1, like γH2AX, might be a marker of delayed DNA repair process.

The current study show that MRE11 phosphorylation is ATM-independent and induced by either IR or DNMT inhibition. IR-induced MRE11 phosphorylation was more prominent in ATM-null AT6BIVA. MRN complex plays a crucial role in detecting DNA damage and triggering downstream cascades (20). Uzielet al have suggested that MRN complex is more upstream than ATM and functional MRN

complex is required for ATM to properly mobilize downstream effectors (21). If MRN complex is defunct, ATM-dependent downstream machinery is compromised. Although subunits of MRN complex are phosphorylated by DNA-damaging insults (22), the significance of MRE11 phosphorylation is yet elusive.

Di Virgilio and et al have reported that MRE11 phosphorylation induces MRN complex to dissociate from DNA by reducing MRE11 affinity to DNA (23). They suggested that abrogation of MRE11 dephosphorylation leads to impairing ATM signaling. Thus, it might be speculated that MRE11 phosphorylation prior to IR impairs ATM-mediated activation of DNA damage responses, leading to enhanced cellular radiosensitivity. MRE11 phosphorylation following DNA DSBs might be a negative feedback loop to downregulate ATM-mediated signal cascades. The current study raises an interesting conjecture that loss of functional ATM might lead to unchecked MRE11 phosphorylation. Our observations of more abundant IR-induced MRE11 phosphorylation in AT5BIVA than in AT5BIVA/ATM implies that functional ATM might inhibit excessive MRE11 phosphorylation in AT5BIVA/ATM.

The current study reports the differential modulation of cellular radiosensitivity by DNMT inhibition depending on ATM status. We observed that DNA damage repair inhibition is, at least partly, a mechanism how DNMT inhibitors enhance radiation cell killing. MRE11

phosphorylation is induced by DNMT inhibition independently of ATM status. This phosphorylation of MRE11 might play a role in DNMT inhibition-induced radiosensitization by disrupting coordinated ATM-MRN complex interaction, leading to impaired ATM-dependent activation of DNA damage responses. Further study is needed to elucidate fully the interdependency of ATM and MRE11 in DNMT inhibitor-mediated radiosensitization.

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국문초록

배경: DNA 메틸전달효소(DNMT) 억제제가 인간 암세포주의 방사선 민감도를 증강시킴이 알려져 있다. 본 연구의 목적은 DNMT 억제제의 방사선 감작 현상에 미치는 ATM 단백질의 영향을 규명하는 것이다.

방법: AT 섬유아세포로부터 한 쌍의 동형 세포주를 제작하였다 (ATM 결핍 세포주와 ATM 발현 세포주). 이 세포주에서 Psammaplin A (PSA)와 5-aza-2'-deoxycytidine (DAC)의 2 가지 DNMT 억제제의 방사선 감작 효과를 측정하였다. γH2AX 를측정하여 DNA 손상 수복을 평가하였다. 웨스턴 블롯을 이용하여 DNA 손상부위에 모집되는 단백질의 발현을 측정하였다.

결과: DNMT 억제제는 ATM 발현세포에서 방사선 감작을 일으켰으나, ATM 결핍 세포의 방사선 민감도에 영향이 없었다. DNMT 억제는 ATM 발현 세포에서 방사선 조사 후 γH2AX 와 인산화 BRCA1 의 발현 시간을 증가시켰으나, ATM 결핍 세포에서 이런 현상은 관찰되지 않았다. DNMT 억제제 처리와 방사선 조사 후 MRN 복합체의 구성 단백질(MRE11, Rad50, NBS1)의 발현은 변하지 않았다. DNMT 억제 또는 방사선 조사 후 세포의 ATM 발현 여부에 관계없이 MRE11 의 인산화가 관찰되었다.

결론: DNMT 억제제는 DNA 손상 수복을 지연시킴으로써 방사선 민감도를 증가시킨다. DNMT 억제에 의한 방사선 감작에는 정상

ATM 의 발현이 필요하다.

주요어: DNMT 억제제, 방사선 감작, ATM, BRCA1, MRN 복합체

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