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ATM-deficient human fibroblast cells are resistant to low levels of DNA double-strand break induced apoptosis and subsequently undergo drug-induced premature senescence

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ABSTRACT

DNA DSBs are induced by IR or radiomimetic drugs such as doxorubicin. It has been indicated that cells from ataxia-telangiectasia patients are highly sensitive to radiation due to defects in DNA repair, but whether they have impairment in apoptosis has not been fully elucidated. A-T cells showed increased sensitivity to high levels of DNA damage, however, they were more resistant to low doses. Normal cells treated with combination of KU55933, a specific ATM kinase inhibitor, and doxorubicin showed increased resistance as they do in a similar manner to A-T cells. A-T cells have higher viability but more DNA breaks, in addition, the activations of p53 and apoptotic proteins (Bax and caspase-3) were deficient, but Akt expression was enhanced. A-T cells subsequently underwent premature senescence after treatment with a low dose of doxorubicin, which was confirmed by G2 accumulation, senescent morphology, and SA-β-gal positive until 15 days repair incubation. Finally, A-T cells are radio-resistant at low doses due to its defectiveness in detecting DNA damage and apoptosis, but the accumulation of DNA damage leads cells to premature senescence.

1. Introduction

Ataxia-telangiectasia mutated (ATM) is derived from ataxia-telangiectasia (A-T) in which the responsible gene is mutated. A-T cells exhibit genomic instability, cancer predispositions, premature aging, and hypersensitivity to DNA double-strand breaks (DSBs) [1–3]. ATM is a serine-threonine kinase that belongs to the phosphatidylinositol 3-kinase (PI3K)-related protein kinase (PIKK) family, which also includes ATR and DNA-PKcs [4,5]. ATM activation has been demonstrated to be part of the early event in the cellular response to ionizing radiation [3,5]. Upon sensing DSBs, the ATM is immediately activated through autophosphorylation at serine 1981 and subsequently phosphorylates a multitude of DNA damage response related proteins which initiate activation of DNA damage checkpoints, leading to cell cycle arrest, DNA repair or apoptosis [5–7]. Despite the importance of the DNA damage response, disruption of the ATM pathway, for example, through loss of ATM itself or a downstream effector protein p53, has been observed in up to 70% of tumors [8,9]. These defects enable the proliferation of incipient cancer cells with DNA lesions.

Premature senescence is a major cellular response to chemotherapy in solid tumors [10]. Cells could be induced to become senescent by exposure to anticancer agents [11,12]. A-T cells generally undergo accelerated aging through an ATM-independent pathway because they are associated with DNA damage response (DDR) defects [3,13]. However, the relations between premature aging and sensitivity to DNA damage in A-T cells are poorly understood.

Since most researchers have studied DSB repair defects at high levels of DNA damage, not much research has been conducted regarding mechanisms of lower levels of DNA damage induced by chemotherapeutic drugs. In fact, in the present study, A-T cells showed increased sensitivity to high levels of DSBs, however, they were more resistant to low levels of DNA damage.

Therefore, we investigated the changing level of DNA damage accumulation, DNA repair, premature senescence, and cell death with the course of repair incubation time after DNA DSBs induced by doxorubicin in normal and ATM-deficient fibroblast cells.

2. Materials and methods

2.1. Cell culture and treatments

ATM-defective SV40-transformed human fibroblast cells (GM09607) and normal control cells (GM00637) (Coriell Institute) were grown in minimum essential medium (HyClone) containing 10% FBS (Sigma). Cells were treated with either doxorubicin

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(SIGMA) or X-ray irradiation. For repair incubation, cells were replenished with fresh medium and incubated for additional 0, 24, or 48 h. Prior to inducing DNA DSBs, cells were incubated for 1 h with KU55933 (TOCRIS).

2.2. MTT assay

Cells were incubated with either different concentrations of doxorubicin for 24 h in the presence and absence of the ATM inhibitor or increasing doses of X-irradiation. Before MTT assay was performed, the cells were replenished with fresh medium and incubated for 24 or 48 h. Fifty microliters of MTT solution was added to each well and incubated for 30 min at 37 °C. After incubation, MTT solution and medium were aspirated and 150 μl of DMSO per well was added. The optical densities were measured at 540 nm by an ELISA reader (Multiskan EX).

2.3. Alkaline comet assay

Cells were treated with 50 nM doxorubicin for 24 h and underwent repair incubation in fresh medium for 0, 24, or 48 h. Positive control was prepared with 100 μM hydrogen peroxide for 20 min at 4 °C. Comet Assay kit (Trevigen) was used according to the manufacturer's instructions. The cells were visualized by a Zeiss LSM 700 confocal microscope. The lengths of the comet tails were analyzed using Comet scoring Freeware version 1.5 (©2006 TriTek Corp.).

2.4. Western blotting analysis

Whole cell extracts were prepared in RIPA buffer (10 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2% Triton X-100, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 μg/ml aprotinin and 1 mM benzamide). The cell lysates were electrophoresed through 6–12% SDS–PAGE gels, and the proteins were transferred to nitrocellulose membranes (Pall Corporation). The membranes were blocked with blocking buffer (TBS-T containing 5% skim milk) then incubated with specific primary antibodies against ATM, p53, Bax (SantaCruz), and p-ATM (Ser1981), p-ATR (Ser428), p-p53 (Ser15), Akt, p-Akt (Thr308), Caspase-3 (Cell Signaling). As loading controls, alpha-actinin and beta-actin (SantaCruz) were used. Immunoblots were visualized by enhanced chemiluminescence (SantaCruz).

2.5. Immunofluorescence assay

For dual fluorescence labeling, fixed cells were incubated at 36 °C for 2 h with the following rabbit or mouse antibody: rabbit polyclonal to active anti-Caspase-3 (1:100; Abcam), mouse monoclonal anti-PCNA (1:100; SantaCruz). After washing with PBS, both cell types were incubated at RT for 1 h with Alexa Fluor 488 goat anti-mouse IgG and Texas Red goat anti-rabbit IgG (1:1000; Invitrogen). PBS contained 1% normal horse serum and Triton X-100 for double localization. Slides were coverslipped with vectashield medium (H-1200, Vector). Images were collected on a Zeiss LSM 700 confocal microscope.

2.6. Analysis of cell cycle and apoptosis

For cell cycle analysis, cells were collected and fixed with chilled 70% ethanol at 4 °C for 16 h. The fixed cells were treated with propidium iodine (PI) solution (100 μg/ml RNase and 5 μg/ml in PBS) for 30 min at 37 °C in the dark. Apoptosis was detected using Annexin V-FITC apoptosis detection kit (BD biosciences). The cells were trypsinized and collected by centrifugation. After resuspension in annexin V-FITC binding buffer, the cells were incubated with 1 μg/ml annexin V-FITC and 10 μg/ml PI at RT in the dark for 15 min. The samples were analyzed with Kaluza flow cytometry software (Beckman Coulter).

2.7. Senescence-associated β-galactosidase activity

SA-β-gal-positive cells were detected with β-galactosidase staining kit according to the manufacturer's instructions (BioVision).

2.8. Statistical analysis

Statistical comparisons were performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparison (Graphpad Prism, version 5.0). $^{*}P < 0.05$ between untreated and treated cells, $^{\#}P < 0.05$ between WT and A-T cells, *** or $^{\#\#\#}P < 0.0001$.

3. Results

3.1. ATM-deficient cells are less sensitive to low level of DNA DSBs but present repair defectiveness in response to doxorubicin

We first examined viability of A-T and normal control fibroblast cells (W-T) in response to varying concentrations of doxorubicin for 24 h. The cell viability of W-T was decreased in dose-dependent manners as expected, whereas A-T were resistant to relatively low concentrations (~500 nM) but showed sensitivity to high concentrations of doxorubicin (Fig. 1A1). Both cells were incubated for 24 or 48 h in fresh medium to have sufficient time to be repaired after 24 h treatment with doxorubicin. A-T were still resistant to doxorubicin compared to W-T but highly chemo-sensitive compared to the cells without repair incubation (Fig. 1A2 and A3). To verify that doxorubicin worked well as a radiomimetic drug, varying doses of X-ray were exposed to the cells, and cell viability was confirmed 24 or 48 h after irradiation. The results showed similar cell viability of IR with those of doxorubicin (Supple. Data-1).

A-T displayed no ATM and p-$ATM_{Ser1981}$ expression, and $ATM_{Ser1981}$ phosphorylation was inhibited by Ku55933 in doxorubicin treated W-T (Fig. 1B). Cells were exposed to increasing concentrations of Ku55933 to determine the intrinsic cytotoxicity, and 1 and 5 μM concentrations seemed to cause small and similar cytotoxicity to W-T and A-T, respectively (Fig. 1C). These two concentrations were used in subsequent experiments. Treatment of W-T with doxorubicin following 1 h of incubation with Ku55933 presented an increase in cell viability (at 100–500 nM) compared to only doxorubicin treatment but not in A-T. (Fig. 1D) Ku55933 did not sensitize both cell lines to doxorubicin, but rather it eliminated the effect of doxorubicin-induced cell death in W-T. In order to exclude the cell death effect of Ku55933 itself, the values of Ku55933 and doxorubicin combination-treated cells were divided by those of only KU55933 treated cells (Fig. 1E). These data suggest that cells with the loss of ATM are resistant to low levels of DNA DSBs but show sensitivity to high levels of DSBs or after long incubation time following low levels of DNA damage.

Next, we investigated the association between the resistance to DNA DSBs and repair function in A-T. Cells were treated with 50 nM doxorubicin for 24 h, and then underwent repair incubation for 0, 24, or 48 h. The damaged DNA fragments were increased until 24 h but decreased between 24 and 48 h repair incubation in W-T (Fig. 1F and H). In contrast, those of A-T continued to increase in repair incubation-dependent manners (Fig. 1G and H). This result indicates that A-T present enhanced cellular survival in response to low levels of DNA damage, but they accumulate DNA breaks due to repair defectiveness.

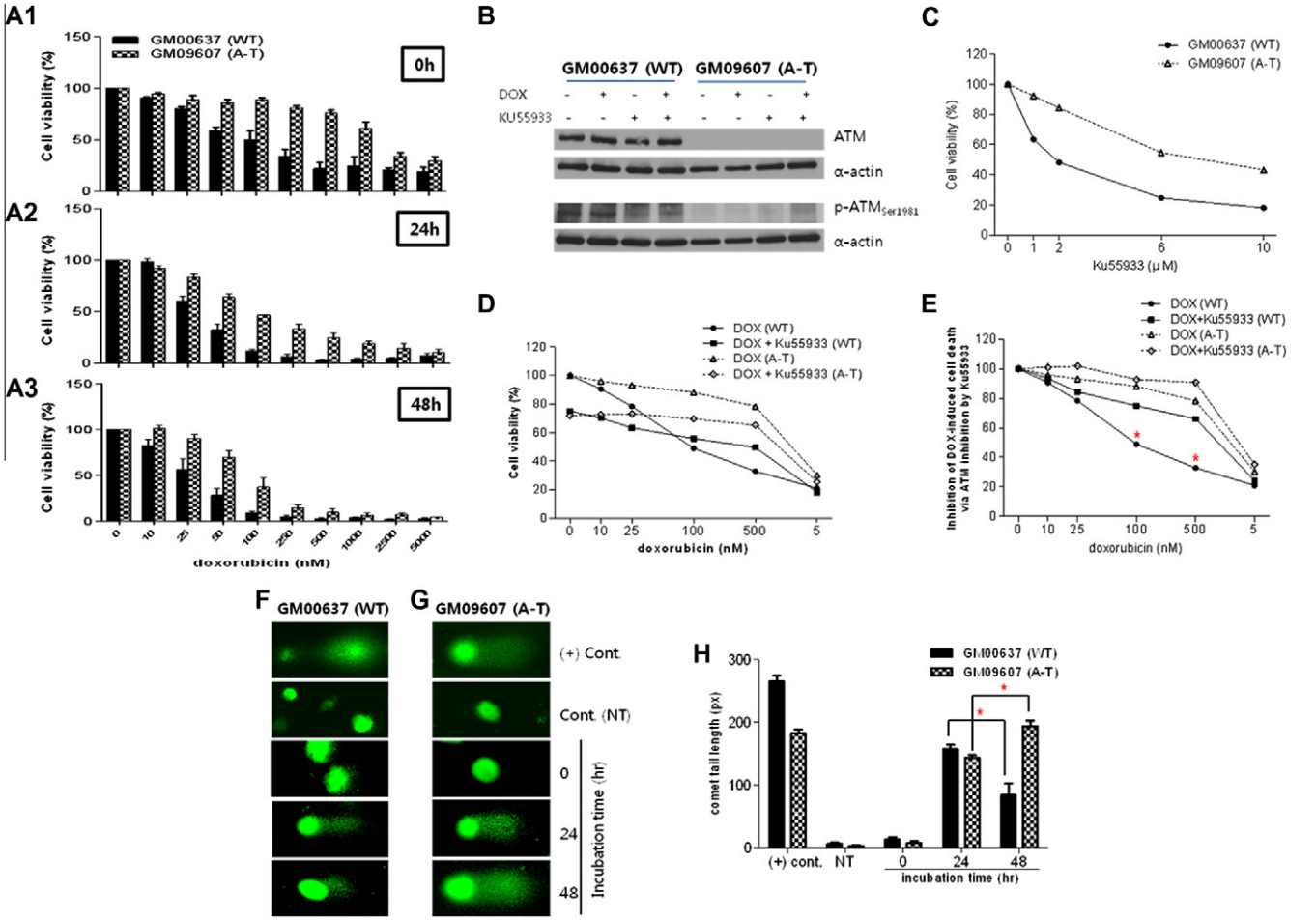


Fig. 1. A-T cells showed a low cell death rate at all time points in response to doxorubicin but underwent DNA damage accumulation. (A) W-T or A-T were incubated with varying concentrations of doxorubicin for 24 h and replenished with fresh medium except (A1) 0 h incubation samples and incubated for (A2) 24 or (A3) 48 h. The cell viability was determined by MTT assay. (B) W-T or A-T were treated with 5 μM KU55933 1 h before treatment of 50 nM doxorubicin for 24 h. Protein expression levels of ATM and p-ATM were measured by western blotting. (C) W-T or A-T were incubated for 1 h with varying concentrations of Ku55933 prior to treatment of 10 nM doxorubicin for 24 h. (D) W-T or A-T were incubated for 1 h with 1 or 5 μM Ku55933, respectively, prior to treatment with varying doxorubicin concentrations for 24 h. (E) The effect of Ku55933 to inhibit doxorubicin-induced cell death to the exclusion of the cytotoxicity of Ku55933 itself was calculated using the equation: $Absorbance_{DOX+Ku}/Absorbance_{Ku}$. (F) W-T or (G) A-T were treated with 50 nM doxorubicin for 24 h. Before the Comet assay was performed, cells were washed with PBS and replenished with fresh medium except for 0 h incubation samples and were incubated for 24 or 48 h. (H) Length of the comet tail was measured in at least 50 cells per sample.

3.2. ATM-deficient cells were defective in p53 activation and apoptotic cell death in response to low dose doxorubicin

$ATM_{Ser1981}$ phosphorylation was significantly induced 24 h after 50 nM doxorubicin (0 h incubation) whereas this was not observed at 24 and 48 h of repair incubation in W-T. In A-T, $p\text{-}ATM_{Ser1981}$ was not expressed at all conditions. Phosphorylation of ATR was similarly induced in both cell lines except in 48 h repair incubation (Fig. 2A). We determined that p53 and p-p53 expressions of W-T were decreased by time lapse of repair incubation except for 0 h, whereas those of A-T were reduced only at 0 h and increased in accordance with the accumulation of DNA damage (Fig. 2B). These data suggest that A-T are defective in the activation of p53 at low levels of DNA damage, but the p53 and p-p53 expressions were induced upon the accumulation of DNA damage.

We next demonstrated whether the resistance to DSBs in A-T was associated with Akt activation. The elevated Akt level was maintained for 24 or 48 h incubation in A-T but not in W-T. In addition, enhanced phosphorylation level of Akt also persisted through repair incubation in only A-T. We also evaluated whether A-T had defectiveness in apoptosis. BAX expression was enhanced and cleaved caspase-3 occurred after doxorubicin treatment in W-T, however, BAX was not increased and cleaved caspase-3 was not detected in A-T in every condition (Fig. 2B). PCNA expression was higher in A-T compared to W-T, and cleaved caspase-3 was not detected by 24 h incubation and only a small amount of cleaved caspase-3 was showed at 48 h incubation in A-T (Fig. 2C and D). Further assessment of apoptosis status using Annexin V/PI-double staining supported the conclusion for minimal/no induction of apoptotic cell death in A-T (Supple. Data-2).

3.3. ATM-deficient cells implemented prolonged G2 accumulation and underwent premature senescence by lapse of incubation time

We determined whether the increased resistance of A-T to low levels of DSBs is accompanied by changes in the cell cycle profile. 50 nM of doxorubicin caused minimal G2/M arrest in W-T (Fig. 3B) compared to a repair incubation-dependent increase in G2 accumulation in A-T (Fig. 3D). In particular, between 24 and 48 h of repair incubation after doxorubicin treatment, the percentage of G2/M phase cells with normal ATM function returned to baseline (Fig. 3B). In contrast, A-T exhibited prolonged G2/M accumulation by 48 h of repair incubation after doxorubicin treatment (Fig. 3D). W-T showed increased number of cell death in a

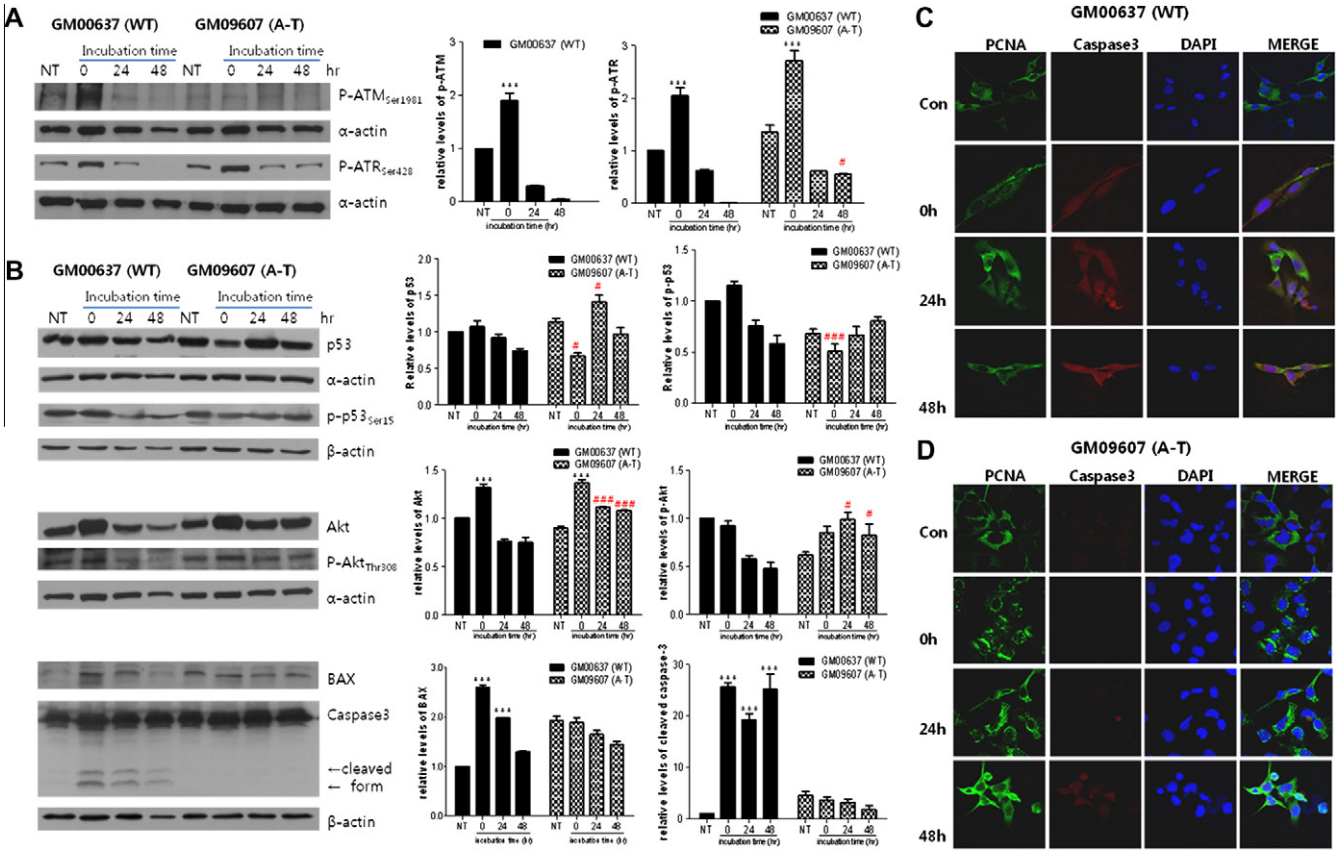


Fig. 2. The basal level of p53 was similar in both W-T and A-T cells while the activation of p53 and apoptosis were defective in A-T cells. Whole cell extracts of W-T and A-T were prepared (A, B) 24 h after 50 nM doxorubicin followed by 0, 24, and 48 h repair incubation in fresh medium. The extracts were assayed by western blotting. Bar charts are respectively the relative density from a representative blot normalized to actin co-loading obtained by densitometry. (C) W-T or (D) A-T were immunostained with anti-PCNA and anti-cleaved caspase-3 antibodies followed by secondary fluorescein conjugate antibodies. Fluorescence images were collected under a confocal microscope.

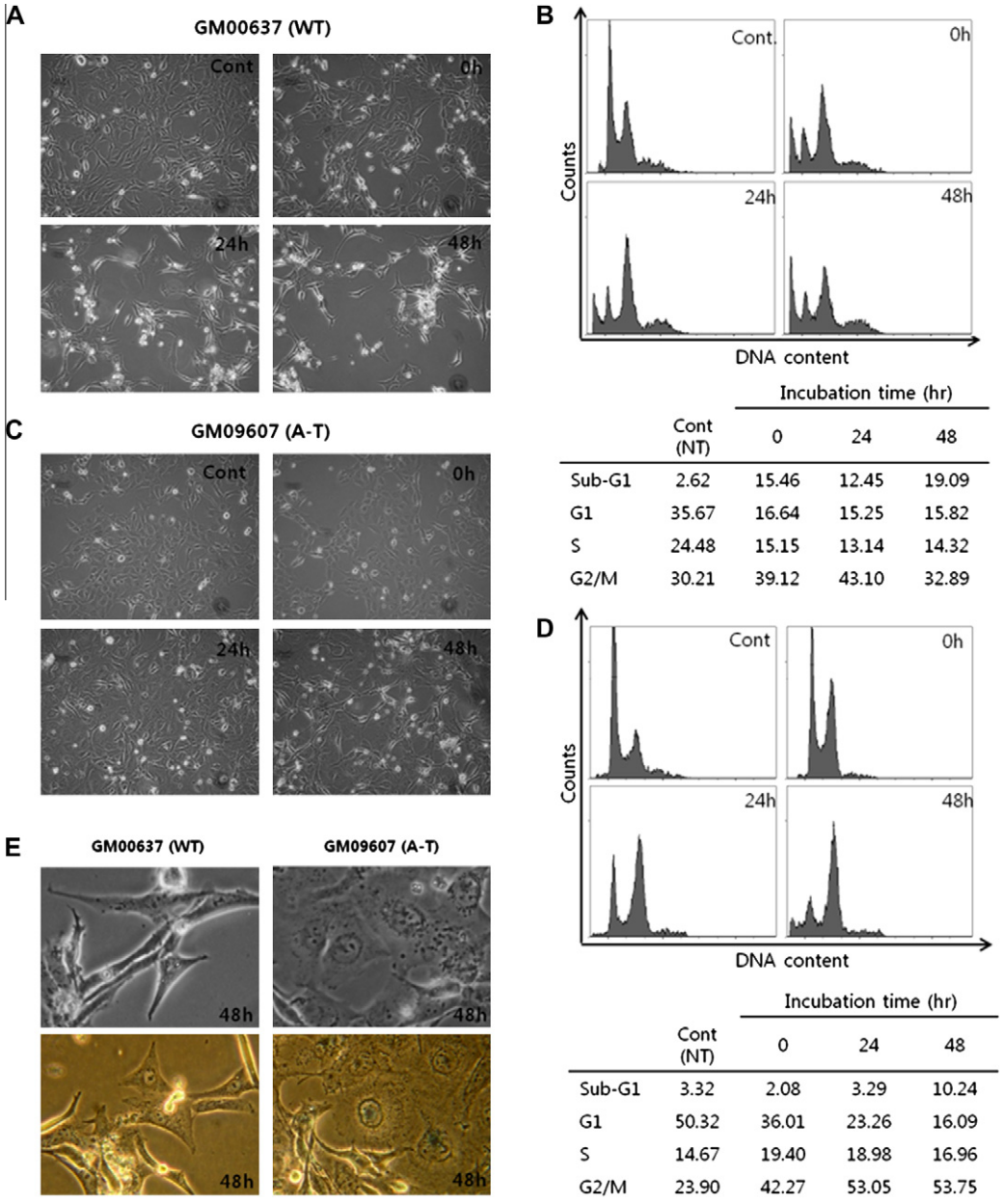
time-dependent manner (Fig. 3A) whereas A-T presented minimal/no change until 24 h and cell death began to be observed at 48 h of repair incubation (Fig. 3C). This results are consistent with the cell cycle profile which showed a similar increase in sub-G1 at every incubation time in W-T whereas a striking increase in sub-G1 at 48 h repair incubation in A-T (Fig. 3B and D). Interestingly, quite a number of A-T showed a senescent morphology and SA-β-gal positive at 48 h repair incubation (Fig. 3E). To confirm whether A-T underwent a senescence pathway in response to low doses of doxorubicin, we observed the cells until 15 days after 50 nM doxorubicin treatment for 24 h. Only repaired cells were remained at day 15 in W-T, whereas more than 50% of senescent morphology was observed from day 3 and almost every cell showed a senescent morphology (flat and enlarged) at day 15 in A-T. SA-β-galactosidase positive cells were also increased in incubation-dependent manners in damaged A-T (Fig. 4A and B).

4. Discussion

This study was designed to investigate different DNA damage responses of ATM-deficient cells in accordance with different levels of DNA DSBs. It has been a widely-accepted theory in the academic world that A-T cells are hypersensitive to irradiation or radiomimetic drugs [1,2]. However, many previous reports revealed that A-T cells showed variations in cellular responses to DNA damage. For example, neurons in $ATM^{-/-}$ mice were resistant to DNA damage-induced apoptosis, whereas other tissues, such as the intestines, were hypersensitive, and other cell types, such as lymphocytes, showed intermediated radiation sensitivity [14]. Some researchers speculate that these varying DNA damage responses may be induced depending on the degree to which PIKK family members compensate for the lack of ATM [15]. Moreover, recently, a report that A-T cells are not radiosensitive for simple chromosomal exchanges at low doses was published [16]. However, more research is still needed to investigate which A-T cells are resistant to DNA damage. In the present study, we confirmed that ATM-deficient cells are not hypersensitive to DNA damage after low doses treatment, and ATM defects are only effective after high levels of DNA damage.

ATM/ATR is implicated in three pivotal roles: regulation and stimulation of DSB repair, activation of cell cycle checkpoints, and signaling to apoptosis. Therefore, they are the key regulators in the decision making between survival and death following DNA damage [7,17]. In this study, ATM was significantly phosphorylated in response to doxorubicin only in 0 h repair incubation cells, but ATR phosphorylation was expressed until 24 h in W-T cells and until 48 h of repair incubation in A-T cells. Taken together, we assume that ATM was activated early in the DDR as a sensor of DNA damage, and this might be the main reason of radio-resistance of ATM-deficient cells to low levels of DNA DSBs.

p53 promotes either cell survival or death depending on the extent of DNA damage. Early in the DNA damage response, p53 promotes cell survival by regulating cell cycle arrest, DNA repair, and other cell survival pathways [18]. On the other hand, upon accumulation of excessive DNA damage, p53 encourages cell death by promoting apoptosis and potentially by inducing senescence and differentiation [19,20]. In fact, p53 phosphorylation of A-T cells in



	Cont (NT)	Incubation time (hr) 0	24	48
Sub-G1	2.62	15.46	12.45	19.09
G1	35.67	16.64	15.25	15.82
S	24.48	15.15	13.14	14.32
G2/M	30.21	39.12	43.10	32.89

	Cont (NT)	Incubation time (hr) 0	24	48
Sub-G1	3.32	2.08	3.29	10.24
G1	50.32	36.01	23.26	16.09
S	14.67	19.40	18.98	16.96
G2/M	23.90	42.27	53.05	53.75

Fig. 3. W-T or A-T cells preferred G1 or G2/M checkpoint arrest, respectively. (A, B) W-T or (C, D) A-T were treated with 50 nM doxorubicin for 24 h, and then cells were washed with PBS and replenished with fresh medium except for 0 h incubation samples and incubated for 24 or 48 h. (A, C) Cell death and morphologic changes in both cells were pictured under a light microscope at the indicated incubation conditions. (B, D) The cell cycle was analyzed by flow cytometry using PI staining at the indicated incubation conditions. (E) W-T and A-T were treated with 50 nM doxorubicin for 24 h and replenished with fresh medium and incubated for 48 h. Top panels, morphologic changes in both cells were pictured by under a light microscope. Bottom panels, premature senescence in A-T were detected after SA-β-galactosidase staining. Representative photomicrographs at the same magnification.

response to X-irradiation is down-regulated, since the ATM acts upstream of p53. P53 activation is induced in a large number of X-irradiated A-T cells by delayed kinetics. ATM is not essential for p53 induction, although it can influence the timing of this process [21,22]. From our data, we concluded that in ATM-deficient cells, because of the defectiveness in sensing DNA damage and DNA repair, low levels of DNA damage may be undetected and could be converted into more severe lesions through continued progression of the cell cycle with unrepaired DSBs. Such lesions might induce p53 expression through an ATM-independent mechanism to induce apoptosis, however, this supposition is not yet clearly proven [21].

Here, we also addressed the expression levels of Akt and apoptosis-involved proteins. Activation of the PI3K-Akt signaling pathway is known to be associated with radio-resistance in many cancers [23,24]. IR induces Akt activation in multiple cell types. For example, it has been reported that the PI-3K/Akt activity contributed to the resistance of human breast cancer cells to ionizing radiation [25]. In the present study, Akt was induced by doxorubicin in normal fibroblast cells. In particular, A-T cells showed constitutively enhanced Akt activation compared to normal cells. In addition, a study defined that senescent cells are resistant to cell death and have specific alterations in apoptotic regulatory proteins [26]. In our results, damaged A-T cells underwent premature senescence and did not express BAX and cleaved caspase-3.

A-T is associated with DDR defects and results in accelerated aging of A-T cells [3]. Recent data have proposed that oxidative stress by ATM deficiency leads to alteration of nuclei structure and senescence in A-T cells [27]. In our study, A-T cells were

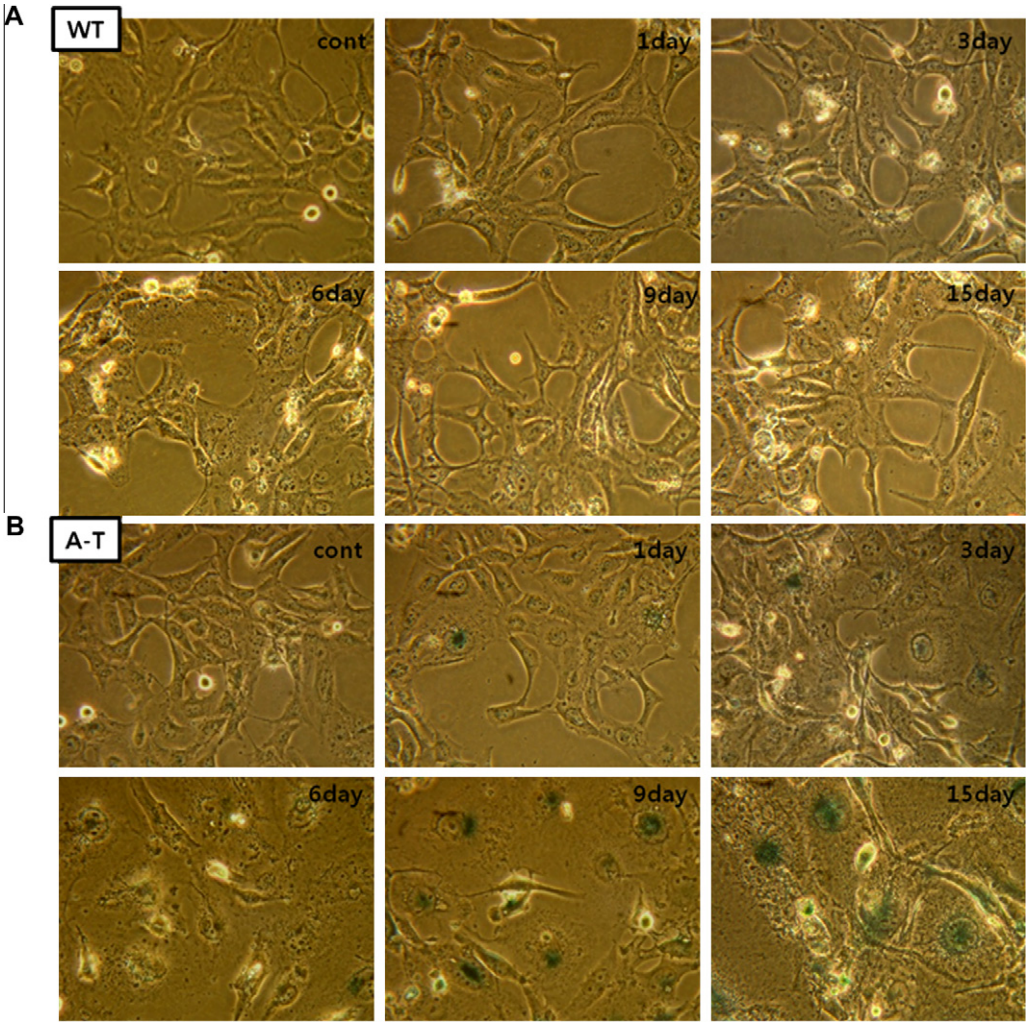


Fig. 4. W-T cells in long term incubation were under repair whereas A-T cells underwent drug-induced premature senescence. 50 nM doxorubicin was treated in (A) W-T or (B) A-T for 24 h. After that, the cells were incubated in fresh media for the indicated days. Cell morphology and SA-β-galactosidase staining were examined under a light microscope. Representative photomicrographs at the same magnification (x200).

induced to undergo premature senescence rather than apoptosis in response to accumulation of DNA damage. These cells showed several markers of premature senescence, for example, flat and enlarged morphology, activated SA-β-galactosidase, and G2 phase accumulation. Both replicative and premature senescent cells acquired similar morphologic and biochemical features, but some differences were found. In particular, premature senescent cells are mainly arrested in the G2 phase whereas replicative senescent cells are arrested in the G1 phase of the cell cycle [28]. Our results show that A-T cells were resistant until 24 h of additional incubation after 50 nM doxorubicin treatment. Only about 10% of A-T cells underwent cell death by apoptosis at 48 h of repair incubation, and at that time most of the A-T cells showed senescent phenotypes. All these results can be evidence to support the data showing resistance and undergoing premature senescence of ATM-deficient cells in response to low levels of DNA damage.

In summary, in the process of finding the reason of radioresistance, we proposed several features of ATM-deficient human fibroblast cells. Briefly, our results suggest that ATM-deficient cells are resistant to low levels of DNA damage first and soon later undergo premature senescence depending on the number of accumulated DNA breaks. Senescent cells are resistant to apoptosis, whereas senescent cells treated with ATM inhibitors undergo cell death [29]. Therefore, the present study provides more understanding that treatment of tumor cells with combination of sub-lethal doses of DNA damaging agents and ATM inhibitor is effective for cancer therapy hence the association with a senescence pathway. Because many tumor cells have loss of ATM, in many cases, anticancer effect could be due to premature senescence rather than direct apoptosis by chemotherapeutic drugs.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.11.040.

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