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ATM inhibition induces synthetic lethality and enhances sensitivity of PTEN-deficient breast cancer cells to cisplatin

Ke Li[a,b,1], Huaying Yan[c,1], Wenhao Guo[d], Mei Tang[b], Xinyu Zhao[b], Aiping Tong[b], Yong Peng[b], Qintong Li[e], Zhu Yuan[b,*]

[a] Key Laboratory of Bio-Resource and Eco-Environment of Ministry of Education, College of Life Sciences, Sichuan University, Chengdu 610065, Sichuan, China
[b] Laboratory of Tumor Biotherapy and Cancer Center, West China Hospital, West China Hospital, Sichuan University, 17 People's South Road, Chengdu 610041, China
[c] Department of Functional Imaging, Sichuan Provincial Women's and Children's Hospital, 290# Sha Yan West Two Street, Jinyang Road, Wuhou District, Chengdu 610031, China
[d] Department of Abdominal Oncology, Cancer Center and State Key Laboratory of Biotherapy, West China Hospital, West China Medical School, Sichuan University, No. 37, Guoxue Road, Chengdu 610041, Sichuan Province, China
[e] Department of Pediatrics, West China Second University Hospital, Key Laboratory of Birth Defects and Related Diseases of Women and Children (Sichuan University), Ministry of Education, Sichuan University, Chengdu 610041, China

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ABSTRACT

PTEN deficiency often causes defects in DNA damage repair. Currently, effective therapies for breast cancer are lacking. ATM is an attractive target for cancer treatment. Previous studies suggested a synthetic lethality between PTEN and PARP. However, the synthetically lethal interaction between PTEN and ATM in breast cancer has not been reported. Moreover, the mechanism remains elusive. Here, using KU-60019, an ATM kinase inhibitor, we investigated ATM inhibition as a synthetically lethal strategy to target breast cancer cells with PTEN defects. We found that KU-60019 preferentially sensitizes PTEN-deficient MDA-MB-468 breast cancer cells to cisplatin, though it also slightly enhances sensitivity of PTEN wild-type breast cancer cells. The increased cytotoxic sensitivity is associated with apoptosis, as evidenced by flow cytometry and PARP cleavage. Additionally, the increase of DNA damage accumulation due to the decreased capability of DNA repair, as indicated by γ-H2AX and Rad51 foci, also contributed to this selective cytotoxicity. Mechanistically, compared with PTEN wild-type MDA-MB-231 cells, PTEN-deficient MDA-MB-468 cells have lower level of Rad51, higher ATM kinase activity, and display the elevated level of DNA damage. Moreover, these differences could be further enlarged by cisplatin. Our findings suggest that ATM is a promising target for PTEN-defective breast cancer.

1. Introduction

Breast cancer is one of the most malignant gynecologic cancers, and often causes injury to women worldwide [1–3]. Breast cancer is a highly heterogeneous disease [4], and many breast cancer subtypes have been identified based on their morphological features, response to treatment, and clinical outcome [2,4]. The most common breast cancer subtype is hormone receptor-positive (HR+) disease [5], which constitutes 75% of all breast cancers [6]. Triple-negative breast cancer (TNBC) is another breast cancer subtype with high rate of tumor recurrence and metastasis, which is characterized by lack of estrogen receptor, progesterone receptor and Her-2 receptor [2,4,7]. The major cause of metastasis and recurrence in TNBC may be due to lack of molecular targets for targeted therapy [2]. Therefore, identification of some important molecular targets and development of the corresponding small molecular inhibitors is of significance for treating breast cancer.

PTEN is a tumor suppressor gene located on chromosome 10q23 [2,8]. Loss of PTEN is a frequent event in breast cancer. Notably, PTEN loss in triple-negative breast cancer is higher than other breast cancer subgroups [2,9,10]. Furthermore, Loss of PTEN is associated with aggressive behavior and poor prognosis [2,11]. PTEN is a lipid phosphatase with a canonical role in inhibiting phosphatidylinositol 3-kinase (PI3K)-AKT pathway. Recent reports have shown that PTEN has a new nuclear function, including transcriptional regulation of the RAD51 gene, whose product is essential for homologous recombination

* Corresponding author at: Laboratory of Tumor Biotherapy and Cancer Center, West China Hospital, West China Hospital, Sichuan University, 17 People's South Road, Chengdu 610041, China.
E-mail address: yuanzhu@scu.edu.cn (Z. Yuan).
[1] These authors contributed equally to this work.

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(HR) repair of DNA breaks [12], and regulation of cell cycle checkpoints [13], suggesting that PTEN plays an important role in regulating DNA damage repair during the DNA damage response (DDR).

The DNA damage response (DDR) is essential to maintain genomic stability in the face of ongoing insult from environmental and endogenous DNA damage [14,15]. The DDR transduces DNA damage signals for DNA repair and to cell cycle checkpoints that stalled cell progress until DNA repair is completed [15]. During the DNA damage response, PI3-Kinase related kinases (PIKKs) are responsible for recruiting DNA repair proteins and activate cell cycle checkpoints. These PIKKs mainly includes ATM (ataxia telangiectasia mutated), ATR (ATM and Rad3 related) and DNA-PKcs (DNA-dependent protein kinase catalytic subunit) [16]. The response to DNA double strand breaks (DSBs) and collapsed replication forks is particularly critical for cell survival as these types of damage are difficult to repair [16]. Thus, tumor cells are inherently vulnerable to these exogenous DNA damage and DDR inhibition. This largely explains therapeutic efficacy of radiotherapy and DNA damage-based chemotherapeutics, and also help to develop and explore DDR-enzyme inhibitors for anti-cancer therapy.

ATM functions to regulate DSBs repair directly or indirectly through cell cycle checkpoint control, and inhibition or absence of ATM increases radiosensitivity and chemosensitivity [17–20]. Thus, ATM is an attractive target for tumor radiosensitization or DNA-damaging chemosensitization. Recently, small-molecule inhibitors that disrupt ATM function and sensitize tumor cells to radiation and chemotherapeutic agents has been developed and explored [19]. One of the first specific ATM inhibitors was KU-55933, which binds competitively to the ATP-binding pocket of the ATM kinase [21]. Recently, another ATM inhibitor, CP466722, was also described [22]. KU-60019 is a novel, second-generation, and highly specific ATM kinase inhibitor that blocks radiation-induced DNA damage response [23].

A hallmark of breast cancer is their genomic instability [24], which often leads to accumulation of lethal DNA damage [25]. It is well known that radiotherapy and DNA-damaging chemotherapy for cancer is based on this principle, though it is accompanied by unwanted side effects on normal tissue [25]. Furthermore, genomic instability is often caused by defects in DNA damage repair (DDR) pathways [25]. Therefore, targeted therapy based on inhibiting the DDR in cancers offers the potential for a greater therapeutic window by tailoring treatment to patients with tumors lacking specific DDR functions [25]. That is, it offers the potential for targeted therapies to exploit the concept of synthetic lethality, i.e., loss of one cellular pathway results in high reliance on another pathway that is not essential under normal settings. This principle is best exemplified by the use of PARP inhibitors (olaparib) to target DNA repair-deficient tumors [26,27]. In addition, a previous study showed that fanconi anemia (FA)/BRCA pathway-deficient tumor cells are hypersensitive to ATM inhibitor [28], indicating ATM inhibitor could induce synthetic lethality in fanconi anemia (FA) / BRCA pathway-deficient tumor cells. Recently, ATM inhibition induces synthetic lethality in PTEN-deficient HCT116 colon cancer cells and PC3 prostate carcinoma cells [29]. However, the ability of ATM inhibitor for inducing synthetic lethality in PTEN-deficient breast cancer cells has not yet been reported.

These observations, combined with the previous reports that PTEN deficiency leads to defects in DNA repair, promoted us to hypothesize that ATM inhibitor *KU-60019* induces synthetic lethality and enhances sensitivity of cisplatin to PTEN-deficient breast cancer cells. To test the hypothesis, we first selected several breast cancer cell lines with different PTEN status including MDA-MB-231, T47D, MCF-7 (PTEN wild-type, PTEN WT) as human breast cancer cell models, and MDA-MB-468 (PTEN deletion, PTEN -/-) as human triple-negative breast cancer cell model, respectively. Then we used these cell lines to test the effects of ATM inhibitor *KU-60019* and / or ciplatin on breast cancer proliferation. As expected, we observed that *KU-60019* enhances sensitivity of cisplatin in both PTEN-wild type and PTEN-deficient breast cancer cells. Importantly, we found that *KU-60019* induces synthetic lethality in PTEN-deficient MDA-MB-468 breast cancer cells, and that the cytotoxic activity of the combination of *KU-60019* and cisplatin is higher than that in PTEN-wild type MDA-MB-231 breast cancer cells. We further tried to elucidated the action mechanism for synthetic lethality, and found that increase of ATM kinase activity and decrease of DNA repair ability caused by PTEN-deficiency is the main reason for the synthetically lethal interaction between PTEN and ATM. To our knowledge, we provide novel molecular evidence for synthetic lethal interaction between PTEN and ATM in breast cancer, and a new potentially therapeutic strategy for breast cancer, especially for PTEN-deficient triple-negative breast cancer.

2. Materials and methods

2.1. Reagents and chemicals

MTT, dimethyl sulfoxide (DMSO), PI and DAPI were purchased from Sigma (St Louis, MO). RIPA buffer were purchased from Beyotime Biotechnology, China. The following antibodies were used: anti-ATM, anti-phospho-ATM (Ser-1981), anti-Chk1, anti-Chk2, anti-phospho-Chk1 (Ser-317), anti-phospho-Chk2 (Thr-68), anti-Rad51, anti-γH2AX (Abcam, Cambridge, MA); anti-PARP (46D11) (Cell signaling technology), anti-GAPDH (Abways technology, China). Rhodamine (TRITC) AffiniPure Goat anti-Rabbit IgG was from Santa Cruz Biotechnology; ATM inhibitor KU-60019 was obtained from Selleck (USA). KU-60019 was dissolved in DMSO and diluted to appropriate concentrations with cell culture media.

2.2. Cell culture and treatments

Human MDA-MB-231, T47D, MCF-7 and MDA-MB-468 cells were obtained from the ATCC (Manassas, VA). Cells were cultured in DMEM medium (GIBCO). All culture medium were supplemented with 10% FBS, penicillin (100 units/mL) and streptomycin (100 μg/mL), respectively, at 37 °C in a humidified atmosphere containing 5% CO2. For single treatment, cells were left untreated or treated with KU-60019 or cisplatin for an indicated period of time. For combination treatments, cells were treated with KU-60019 in combination with cisplatin for 48 h. After treatments, cells were harvested and used for the following experiments including MTT assay, Colony formation assays, flow cytometric analysis, immunofluorescence microscopy and western blotting analysis.

2.3. Cell viability and apoptosis assays

Cell viability was assessed by MTT assay [30,31]. Briefly, cells were plated in 96-well plates at 5×10^3 cells /well and incubated at 37 °C in 5% CO2, 95% humidity air for 24 h, and then cells were treated as described above. MTT was added to the medium (0.5 mg/mL) and incubated at 37 °C for 4 h. The resulting insoluble formazan was dissolved with DMSO and measured at 570 nm using a spectrophotometer. Data represent the average of three wells, and the experiment was repeated three times. Apoptosis was assessed by flow cytometry analysis and detecting the cleavages of PARP.

2.4. Flow cytometry analysis

Apoptosis was analyzed by flow cytometry using the apoptosis detection Kit (Tianjin Sungene Biotech Co., Ltd., China) as described previously [31]. Briefly, cells were harvested and pelleted at 4 °C with 1000 rpm for 5 min, then suspended in 200ul binding buffer, added with 5 μl Annexin V-FITC and propidium iodide (PI) solution, treated for 10 min in room temperature without light. The samples were analyzed by the use of a flow cytometer (ESP Elite, Coulter, Miami, FL).

2.5. Colony formation assays

Colony formation assays were conducted as described previously [30,32]. Briefly, MDA-MB-231 and MDA-MB-468 cells treated with *KU-60019* and/or cisplatin for 48 h were harvested and plated in triplicate at 500 cells/well in 6-well plates and cultured for 10 days. Then the treated cells were washed twice in PBS, fixed in cold methanol, and stained with 2% crystal violet. After incubation at room temperature for 20 min, the 6-well plates were washed twice in double-distilled H_2O and dried, and colonies containing more than 50 cells were counted. All of the experiments were repeated three times, and the average values were reported.

2.6. Western blot

Protein samples were mixed with loading buffer (100 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM dithiothreitol, 0.01% bromphenol blue, and 10% glycerol), heated at 100 °C for 5 min, and loaded onto a 12% SDS-PAGE in electrophoresis buffer containing 25 mM Tris-HCl, pH 8.3, 250 mM glycine, and 0.1% SDS. Protein was then transferred to PVDF using the Trans-Blot semidry system (Bio-Rad). The membranes were immunoblotted with primary antibodies as described under "Reagents and chemicals."

2.7. Indirect immunofluorescence microscopy

MDA-MB-231 and MDA-MB-468 cells were left untreated or treated with KU-60019 or cisplatin, and incubated in a 5% CO2 incubator at 37 °C for 24 h. After fixation with methanol at − 20 °C, the cells were immunostained with monoclonal anti-γ-H2AX (Ser-139) or anti-Rad51 antibodies and subsequently with rhodamine (TRITC) affinipure goat anti-rabbit IgG and counterstained with DAPI (Invitrogen). The fluorescence images were visualized with a Zeiss LSM510 Meta upright confocal microscope. Quantification analysis was performed using ImageJ software.

2.8. Statistical methods

The statistical analysis was performed with SPSS software (version 17.0 for Windows). Results are presented as mean ± S.D. Analysis of variance and the Tukey-Kramer multiple-comparison test were used in comparisons. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Verification of ATM inhibitor KU-60019 and PTEN status in breast cancer cells

Previous studies have shown that KU-55933 was first identified as a specific inhibitor of the ATM kinase [21,23]. Compared with KU55933, KU-60019, the structure of which is shown in Fig. 1A, was recently reported to be an improved inhibitor of the ATM kinase. Similarly, we observed that KU-60019 effectively inhibited cisplatin-induced phosphorylation of ATM (S1981) at 5μmol/L (Fig. 1B).

Considering that PTEN deficiency is associated with aggressive behavior and poor prognosis [2,11], we selected several breast cancer cell with different PTEN status, as cell models to investigate the differential effects of KU-60019 plus cisplatin on the cell proliferation. These breast cancer cell lines includes MDA-MB-231 (PTEN WT), T47D (PTEN WT), MCF-7 (PTEN WT) and MDA-MB-468 (PTEN deletion, PTEN -/-), whose PTEN status were confirmed by western blot (Fig. 1C and D).

3.2. PTEN-deficient breast cancer cells display increased cytotoxic sensitivity against KU-60019 and cisplatin

To assess whether KU-60019 inhibits proliferation and enhances the anti-proliferative effects of cisplatin, we first treated MDA-MB-231 and MDA-MB-468 cells with cisplatin for 48 h at different concentration, and found that the dose of IC50 of cisplatin is about 6 μg/mL and 2 μg/mL, respectively (Fig. 2A). Then, in the following in vitro experiments, we treated tumor cells with cisplatin at a suboptimal dose (2 μg/mL or 5 μg/mL), with a 48-h interval. After treatment, viability of cells was detected by MTT assay. As shown in Fig. 2, compared with the control group, either cisplatin or KU-60019 significantly inhibited the cell viability of PTEN wild-type MCF-7, T47D and MBA-MB-231 breast cancer cells and PTEN-deficient MDA-MB-468 breast cancer cells (Fig. 2B, C and D). Treatment with *KU-60019* plus cisplatin further significantly reduced the cell viability. Importantly, the inhibition percentage of cell proliferation in PTEN-deficient MDA-MB-468 was 61.3% ($P < 0.01$), which was higher than that (39.8%) in PTEN wild-type MBA-MB-231 cells (Fig. 2D).

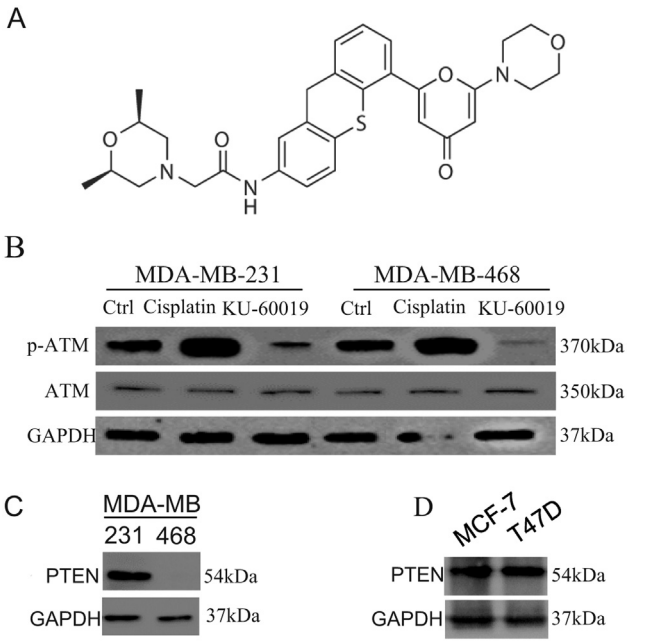

Fig. 1. Confirmation of ATM inhibitor KU-60019 and PTEN status in breast cancer cells **(A)** Structure of KU-60019. **(B)** Inhibition effect of KU-60019 was confirmed by Western blotting. MDA-MB-231 breast cancer cells were untreated or were treated with 5 μg/mL cisplatin in the presence or absence of 5 μM KU-60019 for 48 h, and then the levels of total and phosphorylated ATM were detected by Western blotting. GAPDH was used as a loading control. **(C)** PTEN status of MDA-MB-231 and MDA-MB-468 breast cancer cells was confirmed by Western blotting using anti-PTEN antibody. GAPDH was used as a loading control. **(D)** PTEN status of MCF-7 and T47D breast cancer cells was confirmed by Western blotting using anti-PTEN antibody. GAPDH was used as a loading control.

Based on the observation that PTEN status plays an important role in the cytotoxic activity of cisplatin plus KU-60019, we mainly selected PTEN wild-type MBA-MB-231 and PTEN-deficient MDA-MB-468 cells in the following experiments to elucidate the underlying action mechanism for the effect of ATM inhibition on cisplatin sensitivity.

3.3. PTEN-deficient breast cancer cells displays increased suppression of long-term cell viability by KU-60019 and cisplatin

Considering that MTT assay was used to measure the viability and short-term survival (up to 72 h) of cells, we measured the long-term survival of MBA-MB-231 and MDA-MB-468 cells after treatments with KU-60019 and cisplatin by colony-formation assays. Similar to the results above, the reduced viability of MBA-MB-231 and MDA-MB-468 cells was further confirmed by colony formation assays (Fig. 3). Notably, the colony number of PTEN-deficient MDA-MB-468 cells treated with KU-60019 and cisplatin is significantly less than that of PTEN wild-type MBA-MB-231 cells.

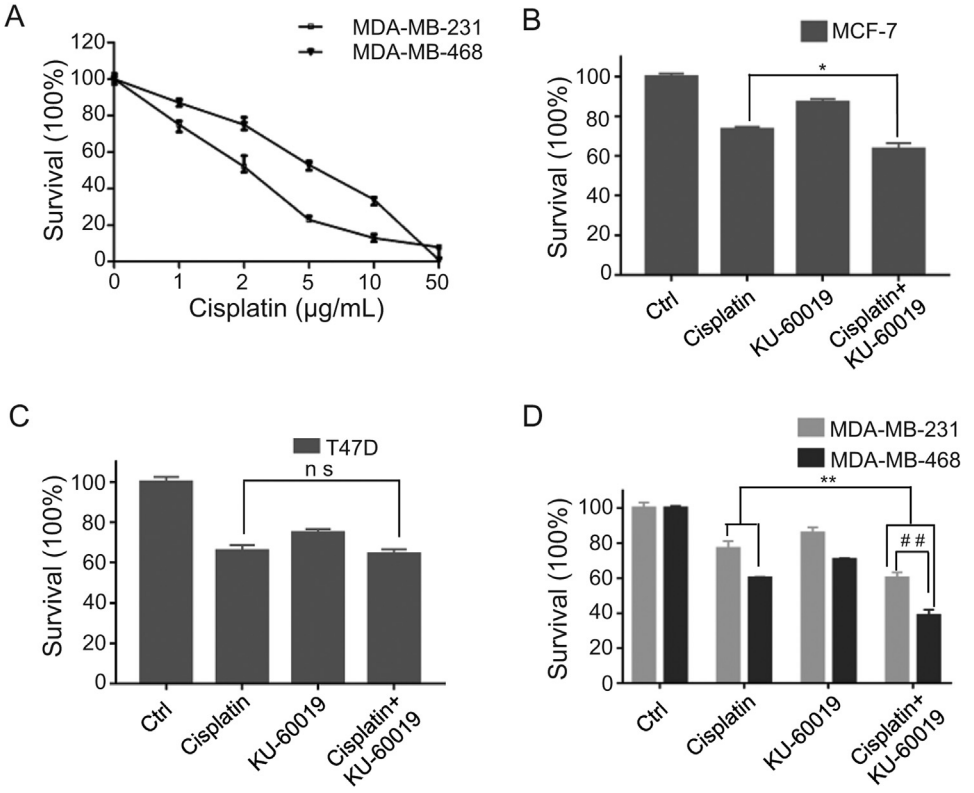

Fig. 2. PTEN-deficient MDA-MB-468 breast cancer cells display increased cytotoxic sensitivity against KU-60019 and cisplatin. MTT was carried out to analyze the survival of breast cancer cells. **(A)** Effect of cisplatin at different concentrations on survival of MDA-MB-231 and MDA-MB-468 cells. Cells were treated with cisplatin at different concentrations for 48 h, then used to analyze the survival by MTT assay. **(B)** Effect of cisplatin /KU-60019 on survival of MCF-7 cells. MCF-7 Cells were treated with cisplatin and/or KU-60019 for 48 h, then used to analyze the survival by MTT assay. *The percentage of survival was calculated. In each experiment, the medium-only treatment (untreated) indicates 100% cell viability. Bars, mean; error bars, S.D. (n = 3; *p < 0.05).* **(C)** Effect of cisplatin /KU-60019 on survival of T47D cells. T47D Cells were treated with cisplatin and/or KU-60019 for 48 h, then used to analyze the survival by MTT assay. *The percentage of survival was calculated. In each experiment, the medium-only treatment (untreated) indicates 100% cell viability. Bars, mean; error bars, S.D. (n = 3; n s, no statistically significant difference).* **(D)** Effect of cisplatin / KU-60019 on cell survival of MDA-MB-231 and MDA-MB-468. The percentage of survival was calculated. In each experiment, the medium-only treatment (untreated) indicates 100% cell viability. *Bars, mean; error bars, S.D. (n = 3; ** p < 0.01 ; ##p < 0.01).*

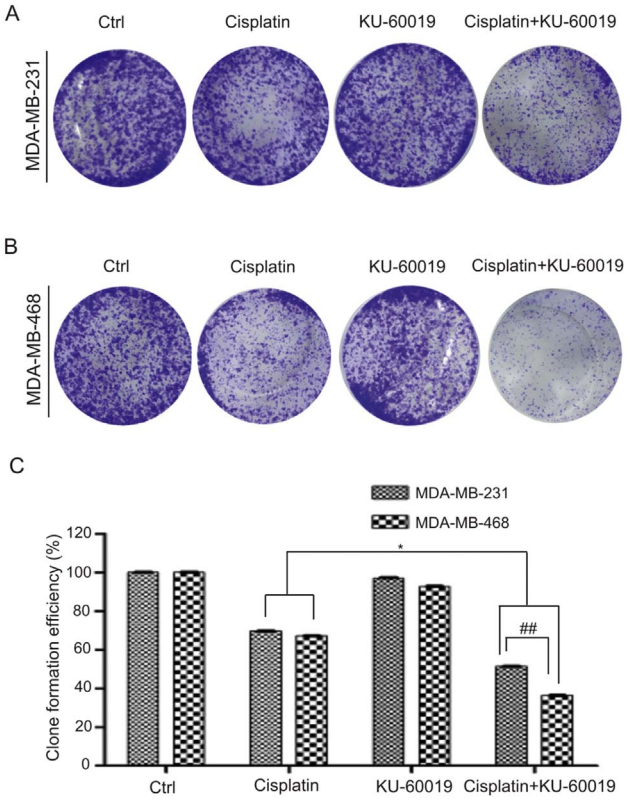

Fig. 3. Colony formation assays were further used to evaluate the viability of MDA-MB-231 and MDA-MB-468 cells. (A) Combination of cisplatin and KU-60019 resulted in significant inhibition of clone formation of MDA-MB-231 cells compared with the cisplatin-treated group. (B) Combination of cisplatin and KU-60019 resulted in significant inhibition of clone formation of MDA-MB-468 cells compared with the cisplatin-treated group. (C) The clones of MDA-MB-231 and MDA-MB-468 cells were further counted. Bars, mean; error bars, S.D. (n = 3; *p < 0.05; ##p < 0.01).

Taken together, these results showed that ATM inhibitor *KU-60019* can enhance the anti-proliferation effects of cisplatin on breast cancer, furthermore, the anti-proliferation effects of cisplatin plus *KU-60019* on PTEN-deficient breast cancer cells is stronger than that on PTEN wild-type breast cancer cells, implying that the survival of PTEN-deficient breast cells rely more heavily on ATM than PTEN wild-type breast cancer cells.

3.4. KU-60019 and cisplatin inhibits cell proliferation by inducing apoptosis

Apoptosis plays an important role in reduction of cell viability by ATM inhibition and/or cisplatin [29,33]. Similarly, we found a significant increase in the percentage of apoptotic MBA-MB-231 cells and MBA-MB-468 breast cancer cells treated with KU-60019 and/or cisplatin for 48 h, as evidenced by the flow cytometry analysis. Furthermore, the apoptotic percentage of PTEN-deficient MDA-MB-468 cells was significantly higher than that of PTEN wild-type MDA-MB-231 cells (Fig. 4A-C), which is consistent with the above results of proliferation inhibition. These results indicated that, compared with PTEN wild-type MDA-MB-468 cells, the survivals of PTEN-deficient breast cells rely more heavily on ATM dependency. Previous studies have shown that an early transient burst of poly(ADP-ribosyl)ation of nuclear proteins is required for apoptosis, followed by cleavage of poly(ADP-ribose) polymerase (PARP), catalyzed by caspase-3 [34,35]. Based on these observations, we detected the cleavage of PARP in both PTEN wild-type MDA-MB-231 cells and PTEN-deficient MDA-MB-468 cells after treatment with KU-60019 and/or cisplatin. As shown in Fig. 4D, cisplatin results in PARP cleavage. However, combination of KU-60019 and cisplatin further caused PARP cleavage. Taken together, these results suggested that caspase-3-mediated PARP cleavage pathway might be associated with ATM inhibition and/or cisplatin-induced apoptosis.

3.5. ATM inhibition promotes cisplatin-induced DNA damage accumulation

Since cisplatin can cause DNA damage [36,37], we investigated if

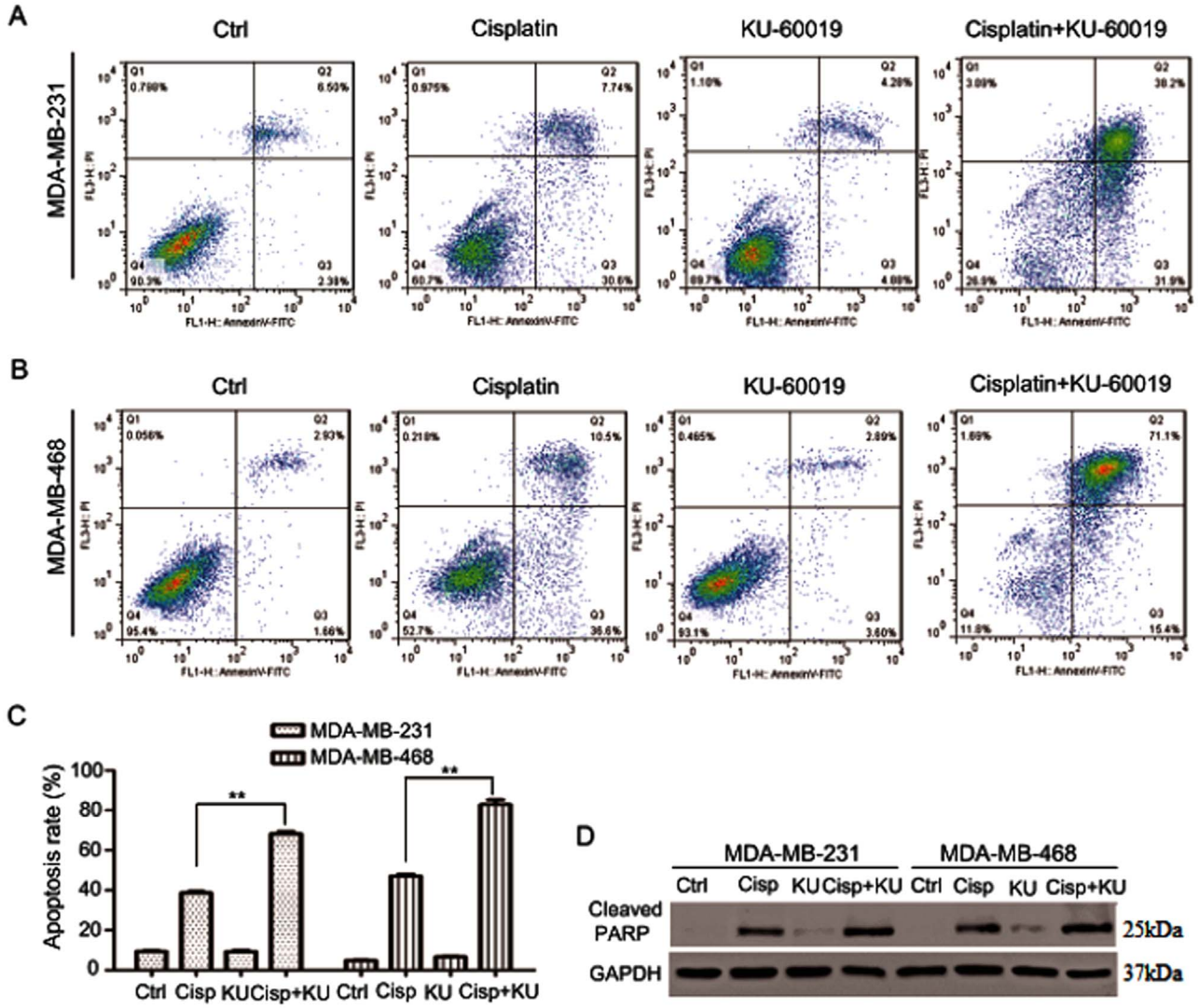

Fig. 4. KU-60019 and cisplatin inhibit cell proliferation by inducing apoptosis **(A)** MDA-MB-231 cells were treated with KU-60019 and/or cisplatin for 48 h, then used to measured apoptosis by flow cytometry analysis. **(B)** MDA-MB-468 cells were treated as described in A, then the treated cells were used for analysis of apoptosis by flow cytometry. **(C)** Apoptotic cells were further counted, and the results were presented as bar graphs. Bars, mean; error bars, S.D. (n = 3; $^{**}p < 0.01$). **(D)** Western blotting was conducted to detect PARP cleavage in MDA-MB-231 and MDA-MB-468 cells following treatment with the KU-60019 and/or cisplatin for 48 h.

ATM inhibition affects cisplatin-induced DNA damage response by γ-H2AX staining. As expected, cisplatin or KU-60019 alone produced some typical γ-H2AX foci in both PTEN wild-type MDA-MB-468 cells and PTEN-deficient MDA-MB-468 cells. In contrast, untreated breast cancer cells display little γ-H2AX foci (Fig. 5). Importantly, KU-60019 significantly augmented cisplatin-induced DNA damage in PTEN-deficient MDA-MB-468 cells, whereas it slightly (but not significantly) increase the DNA damage accumulation in PTEN-WT MDA-MB-231 cells, as evidenced by γ-H2AX staining (Fig. 5B and D). These results indicated that ATM inhibition promoted cisplatin-induced DNA damage accumulation, while PTEN deficiency further potentiates the effect of ATM inhibition on cisplatin-induced DNA damage, finally causing more DNA damage accumulation.

3.6. ATM inhibition impairs DNA damage repair in cisplatin-induced DNA damage response

Previous studies have shown that ATM is responsible for recruiting DNA repair proteins during DDR, contributing to HR-mediated DNA repair [15,20,25,38]. Thus, we hypothesized that ATM inhibition would partly impair DNA damage repair, which will further increase accumulation of cisplatin-induced DNA damage. To confirm if DNA damage accumulation was caused by defective HR repair due to the inhibition of ATM activation, an immunofluorescence assay was conducted to assess RAD51 foci formation, which is indicative of functional HR repair activity. As shown in Fig. 6, cisplatin or KU-60019 alone produced some typical Rad51 foci in both PTEN wild-type MDA-MB-231 cells and PTEN-deficient MDA-MB-468 cells (Fig. 6). Importantly, ATM inhibitor KU-60019 significantly reduced HR-mediated DNA repair efficiency, as indicated by the decreased number of RAD51 foci in cells treated with cisplatin and KU-60019 in both cells (Fig. 6). Notably, numbers of RAD51 foci in PTEN-deficient MDA-MB-468 cells were significantly lower than that in PTEN-WT MDA-MB-231 cells when treated with KU-60019 plus cisplatin (Fig. 6B and D). These observations support our hypothesis that ATM inhibition causes the reduction of HR-mediated DNA repair efficiency, while PTEN deficiency further impairs HR-mediated DNA repair, finally leading to the accumulation of DNA damage.

3.7. Mechanistic rationale to selectively target PTEN-deficient tumor cells with ATM inhibitor KU-60019 and cisplatin

Our data suggest a synthetically lethal interaction between PTEN and ATM in breast cancer cell lines. However, the mechanism for this

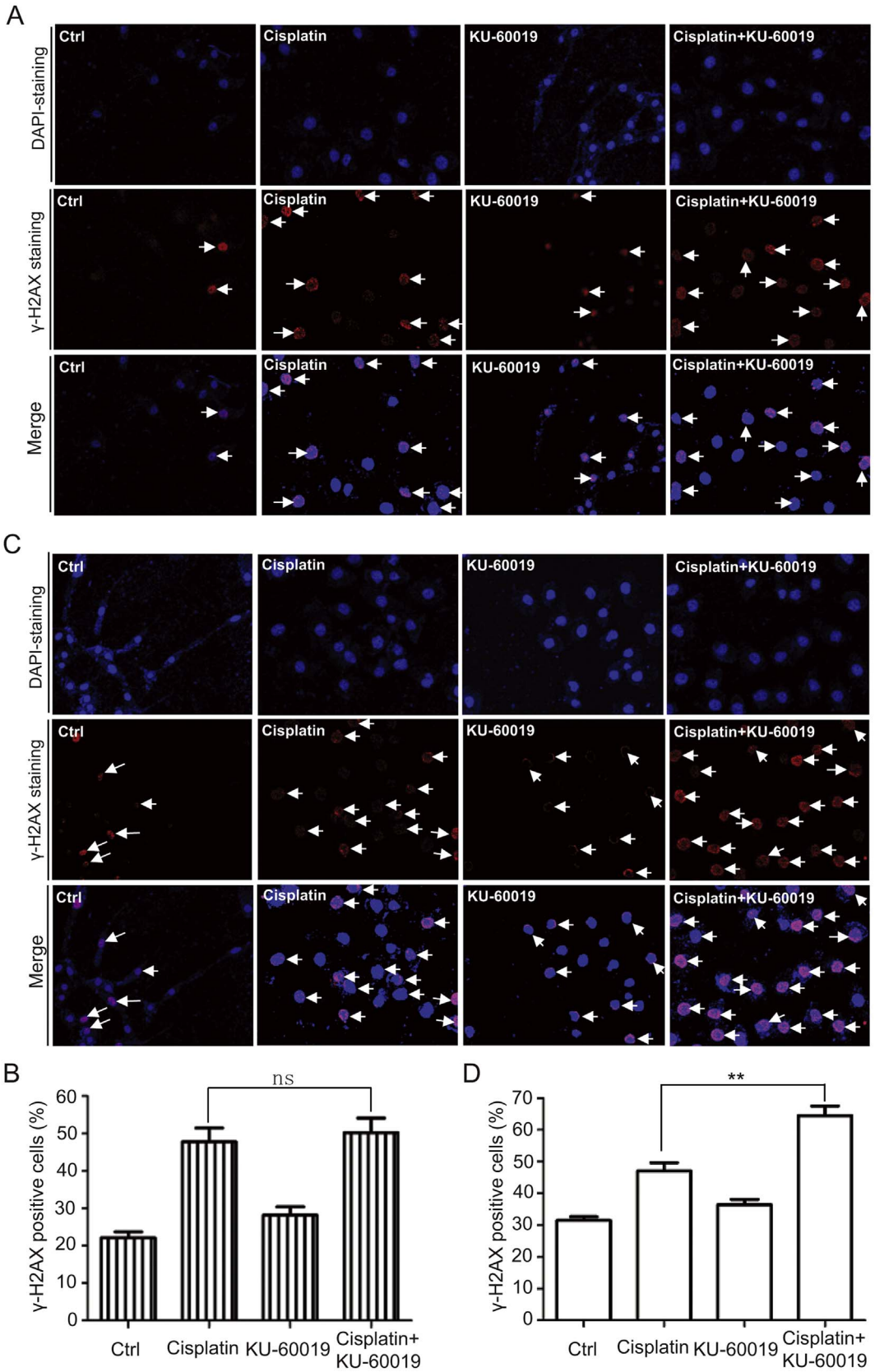

Fig. 5. KU-60019 promotes cisplatin-induced DNA damage accumulation. An immunofluorescence assay was conducted to monitor γ-H2AX damage foci formation. **(A)** KU-60019 and /or cisplatin results in formation of γ-H2AX damage foci in MDA-MB-231 cells. The representative pictures of γ-H2AX foci were shown. Arrows, some typical γ-H2AX foci. **(B)** Quantification of KU-60019/cisplatin-induced γ-H2AX foci in MDA-MB-231 cells. The percentage of γ-H2AX foci-positive cells (at least 100 total cells) was counted. *Bars, mean; error bars, S.D. (n = 3; n s, no statistically significant difference)*. **(C)** KU-60019 and /or cisplatin results in formation of γ-H2AX damage foci in MDA-MB-468 cells. The representative pictures of γ-H2AX foci were shown. Arrows, some typical γ-H2AX foci. **(D)** Quantification of KU-60019/cisplatin-induced γ-H2AX foci in MDA-MB-468 cells. The percentage of γ-H2AX foci-positive cells (at least 100 total cells) was counted. Bars, mean; error bars, S.D. (n = 3; $^{**}p < 0.01$).

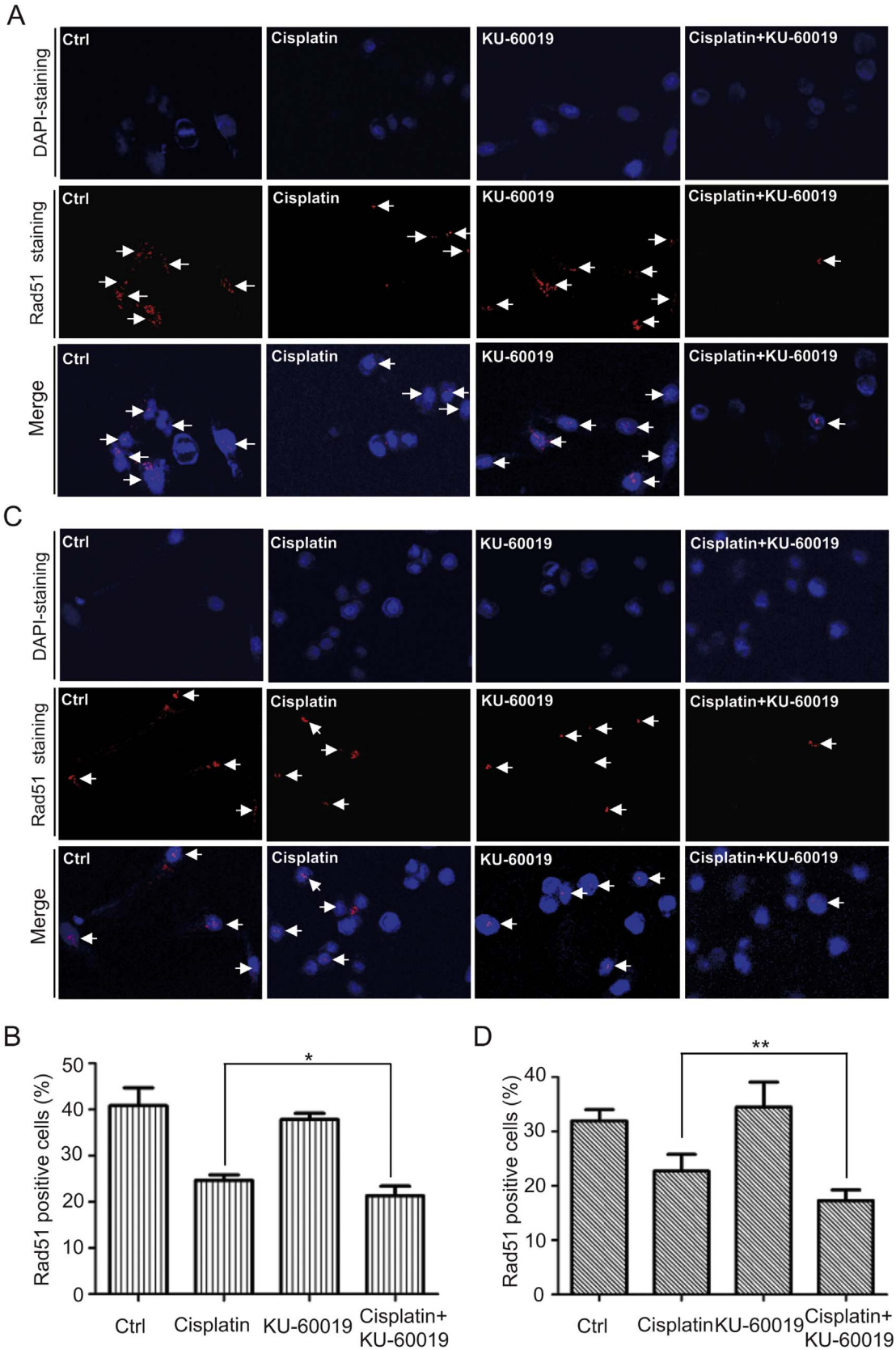

Fig. 6. KU-60019 augments DSB repair defects. An immunofluorescence assay was conducted to monitor RAD51 foci formation and determine whether DNA damage accumulation is due to a decreased HR repair capacity. DAPI (blue) was used for counterstaining. **(A)** KU-60019 reduced cisplatin-induced Rad51 foci in MDA-MB-231 cells. The representative pictures of Rad51 foci were shown. Arrows, some typical Rad51 foci. **(B)** Quantification of KU-60019/Cisplatin-induced Rad51 foci in MDA-MB-231 cells. The percentage of Rad51 foci-positive cells (at least 100 total cells) was counted. Bars, mean; error bars, S.D. ($n = 3$; $^{*}p < 0.05$). **(C)** KU-60019 reduced cisplatin-induced Rad51 foci in MDA-MB-468 cells. The representative pictures of Rad51 foci were shown. Arrows, some typical Rad51 foci. **(D)** Quantification of KU-60019 /Cisplatin-induced Rad51 foci in MDA-MB-468 cells. The percentage of Rad51 foci-positive cells (at least 100 total cells) was counted. Bars, mean; error bars, S.D. ($n = 3$; $^{**}p < 0.01$).

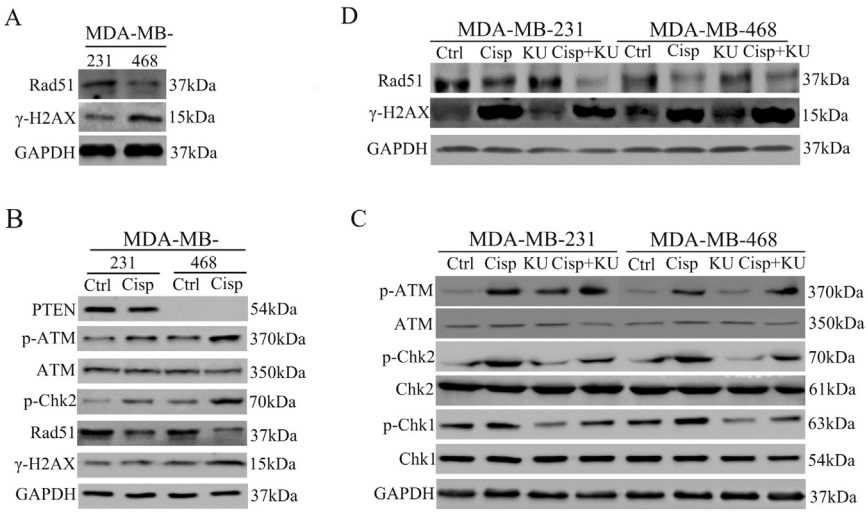

Fig. 7. PTEN-deficient MDA-MB-468 cells have elevated expression levels of DNA damage response-related molecules, reduced levels of RAD51, and activation of ATM. (A) Western blotting for endogenous Rad51 was performed to determine whether there was a difference of HR-mediated DNA repair capacity between PTEN-deficient MDA-MB-468 cell line and PTEN wild-type MDA-MB-231 cell line. GAPDH was used as a loading control. (B) Western blotting for PTEN, Rad51, γ-H2AX, p-Chk2, ATM and p-ATM was performed to determine how cisplatin affects the DDR pathway between PTEN-deficient MDA-MB-468 cell line and PTEN wild-type MDA-MB-231 cell line. MDA-MB-231 and MDA-MB-468 cells were untreated or treated with cisplatin for 48 h, and then used to measure the expression changes of DDR-related molecules. GAPDH was used as a loading control. (C) Western blotting for ATM, p-ATM, p-Chk2, Chk2, p-Chk1 and Chk1 was performed to determine how cisplatin/KU-60019 affect the DDR pathway between PTEN-deficient MDA-MB-468 cell line and PTEN wild-type MDA-MB-231 cell line. MDA-MB-231 and MDA-MB-468 cells were untreated or treated with cisplatin/ KU-60019 for 48 h, and then used to measure the expression changes of DDR-related molecules. GAPDH was used as a loading control. (D) Western blotting for Rad51 and γ-H2AX was performed to determine whether the reduction of DNA damage accumulation in PTEN-deficient MDA-MB-468 cells is due to a decreased HR repair capacity. PTEN-deficient MDA-MB-468 and PTEN wild-type MDA-MB-231 cells were untreated or treated with cisplatin/KU-60019 for 48 h, and then used to measure the expression changes of Rad51 and γ-H2AX. GAPDH was used as a loading control.

interaction remains elusive. Toward this end, we decided to detect the expression change of DNA damage response (DDR)-related molecules between PTEN wild-type and PTEN-deficient breast cancer cells. It has previously been reported that PTEN-deficient cells exhibit abnormal homologous recombination (HR)–mediated DNA repair through loss of expression of Rad51 [12,29,39]. Similarly, we found that the expression level of endogenous Rad51 in PTEN-deficient MDA-MB-468 cell lines was lower than that in PTEN-wild type MDA-MB-231 cell lines (Fig. 7A). By contrast, the level of γ-H2AX, an indicator of DNA damage, in PTEN-deficient MDA-MB-468 cells was higher than that in PTEN wild-type MDA-MB-231 cells (Fig. 7A). We further detect DDR-related molecules between PTEN wild-type MDA-MB-231 and PTEN-deficient MDA-MB-468 breast cancer cells in the absence or presence of cisplatin, and found that, in PTEN-deficient MDA-MB-468 cell lines, the expression levels of phospho-ATM, phospho-Chk2 and phospho-H2AX (γ-H2AX) were higher, while Rad51 was lower, than those in PTEN-wild type MDA-MB-231 cell lines (Fig. 7B). The expression differences of these proteins were further enlarged between the two cells in response to cisplatin (Fig. 7B). Furthermore, we found that, in PTEN wild-type MDA-MB-231 and PTEN-deficient MDA-MB-468 cells treated with KU-60019 and cisplatin, KU-60019 significantly abrogated cisplatin-induced phosphorylation of Chk1 and Chk2 (Fig. 7C), and caused further down-regulation of Rad51 as well as up-regulation of γ-H2AX (Fig. 7D), indicating that KU-60019 plus cisplatin-induced changes of DDR-related molecules, which reflected reduction of DNA repair and increase of DNA damage accumulation, might be the reason for KU-60019 sensitizes breast cancer cells to cisplatin.

4. Discussion

Breast cancer is one of the most malignant gynecologic cancers which often cause cancer mortality [2,3]. Hormone receptor-positive (HR+) cancer is the most common breast cancer subtype [5,6], while triple-negative breast cancer (TNBC) is another breast cancer subtype with high rate of tumor recurrence, metastasis and the poorest clinical outcomes [4,7]. Previous studies have shown that PTEN deletion often occurs in breast cancers, with reduced PTEN protein found in 31–48% of breast cancers [40,41]. Furthermore, PTEN deficiency is associated with aggressive behavior and poor prognosis in triple-negative breast cancers [2,11]. Interestingly, recent reports showed an important nuclear function of PTEN in DNA double strand break repair [12,29,42].

Synthetic lethal approach is a promising therapy strategy for cancer treatment [39,43,44]. Based on the “Synthetic lethal” concept, PARP inhibitor olaparib is approved by FDA to be used to treat BRCA1 or BRCA2 mutation tumors [43]. Previous studies have shown that ATR inhibitor AZD-6738 induces synthetic lethality and overcomes chemoresistance in TP53- or ATM-defective chronic lymphocytic leukemia, colorectal, lung and gastric cancer cells [44–47]. Similarly, ATM inhibitor induces synthetic lethality in fanconi anemia (FA) / BRCA pathway-deficient tumor cells [28]. Recently, McCabe et al. found that ATM inhibition also induces synthetic lethality in PTEN-deficient HCT116 colon cancer cells and PC3 prostate carcinoma cells [29]. However, the synthetic lethal interaction between ATM and PTEN has not been reported in breast cancer cells. The present study was designed to investigate whether an ATM inhibitor KU-60019 induces synthetic lethality and enhances sensitivity of cisplatin to PTEN-deficient breast cancer cells, and to further elucidate the action mechanism for synthetic lethality.

Several observations have been made in this work concerning inhibition of proliferation by KU-60019 and/or cisplatin in breast cancer cells, as evidenced by MTT assay (Fig. 2), colony formation assays (Fig. 3). Interestingly, the anti-proliferation effects of KU-60019 and cisplatin on PTEN-deficient breast cancer cells is significantly stronger than PTEN wild-type breast cancer cells (Figs. 2 and 3), indicating a synthetic lethal interaction between PTEN and ATM in PTEN-deficient breast cancer cells.

The reduction of cell viability by ATM inhibition and/or cisplatin was also associated with induction of apoptosis [29,33]. Similar to these observations, KU-60019 and/or cisplatin resulted in a significant increase in the percentage of apoptotic MBA-MB-231 cells and MBA-MB-468 breast cancer cells, as evidenced by the flow cytometry analysis (Fig. 4-C) and detection of PARP cleavage (Fig. 4D). Collectively, these data showed that apoptosis contributed to the anti-proliferation of breast cancer cells by KU-60019 and/or cisplatin.

Our data that the anti-proliferation efficiency by KU-60019 and/or cisplatin in PTEN-deficient MBA-MB-468 cells is higher than PTEN wild-type MBA-MB-231 cells (Figs. 2–4) promoted us to further explore the action mechanism. We found that the increase of DNA damage accumulation and reduction of DNA damage repair also contributed to the anti-proliferation effects by KU-60019 and/or cisplatin, as indicated by γ-H2AX staining (Fig. 5) and Rad51 foci (Fig. 6). Importantly, the accumulation of DNA damage in PTEN-deficient MBA-MB-468 cells is higher, while the reduction degree of DNA repair is lower, than those in PTEN wild-type MBA-MB-231 cells (Figs. 5 and 6).

Mechanistically, we found that, compared with PTEN wild-type MDA-MB-231 cells, PTEN-deficient MDA-MB-468 cells have lower level

of Rad51 expression (Fig. 7A), which is similar to the previous reports that PTEN-deficient cells exhibit defective DNA repair through loss of RAD51, an indicative of HR-mediated DNA repair [12,29,39]. Furthermore, PTEN-deficient MDA-MB-468 cells were observed to have higher constitutive ATM kinase activity than PTEN wild-type MDA-MB-231 cells, as indicated by the phosphorylation of ATM and its substrate Chk2 (Fig. 7B), indicating that ATM as a target in PTEN-deficient breast cancers is better than PTEN wild-type breast cancers. The differences of ATM kinase activity in the two cell lines were further augmented by cisplatin (Fig. 7B). Similar to this observation, the differences of phosphorylation of H2AX (γ-H2AX), the indicative of DNA damage [29,31], were also observed (Fig. 7B). Furthermore, KU-60019 significantly abrogated cisplatin-induced phosphorylation of Chk2 and Chk1 (Fig. 7C), and further caused down-regulation of Rad51 as well as up-regulation of γ-H2AX (Fig. 7D). Taken together, our data explain at the molecular level why PTEN-deficient MDA-MB-468 cells display the elevated levels of DNA damage, the decreased capability of DNA repair and the increased cytotoxic sensitivity against KU-60019 and cisplatin, compared to PTEN wild-type MDA-MB-231 cells.

In summary, we have shown that ATM inhibitor KU-60019 induces synthetic lethality in PTEN-deficient MDA-MB-468 breast cancer cells, and enhances sensitivity of cisplatin in both PTEN-wild type and PTEN-deficient breast cancer cells. We have further demonstrated a requirement for ATM in the maintenance of cell survival in the presence of increased DNA damage secondary to PTEN deficiency. The preferentially selective sensitivity of PTEN deficient breast cancers to ATM inhibitor KU-60019 and cisplatin suggests that this may represent a novel approach to targeted breast cancer therapy. To our knowledge, we provide a novel therapeutic strategy for breast cancer, especially for PTEN-deficient triple-negative breast cancer.

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Conflict of interest

No potential conflicts of interest were disclosed.

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