The novel mTORC1/2 dual inhibitor INK128 enhances radiosensitivity of breast cancer cell line MCF-7

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Abstract. mTOR, a member of the PIKK family, is crucial for cell growth, survival, motility, proliferation, protein synthesis and DNA transcription. Many studies have demonstrated that mTOR inhibitor could enhance radiosensitivity. However, the effect of the novel mTORC1/2 dual inhibitor, INK128, on the radiosensitivity of breast cancer and the underlying mechanisms are still vague. In the present study, the cell viability was estimated using CCK-8 assay, and the dose-survival relationship was analyzed using a clonogenic survival assay. Cell cycle was evaluated by flow cytometry. The staining of γH2AX foci was assessed by immunofluorescence. In addition, we used western blots to verify the downregulating signal protein and to detect the potential related pathway. We found that the exposure of MCF-7 cells to INK128 decreased the cell viability. Exposure of MCF-7 cells to INK128 and combined ionizing radiation greatly reduced the survival rate. INK128 combined radiotherapy significantly induced G2/M arrest, double strand breaks and inhibited its repair. Furthermore, INK128 plus radiation downregulated p-Chk2, p21 and upregulated cleaved PARP, LC3B expression. These findings suggest that mTOR inhibitor could be used as a novel radiosensitizing target for breast cancer patients.

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Introduction

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females worldwide. There were estimated 1.7 million cases and 521,900 deaths in 2012, which accounted for 25% of all cancer cases and 15% of all cancer deaths. In more developed countries, it even accounts for approximately one-half of all breast cancer cases and 38% of deaths (1). It demonstrated that one in eight women will develop breast cancer during their lifetime (2). Also, owing to the multimodality treatment for breast cancer, the 5-year or 10-year survival rate was obviously improved. Additionally, radiotherapy has been considered as a key component of local treatment to reduce the risks of local recurrence (3). However, we cannot ignore the fact that up to 30% of node-negative and up to 70% of node-positive breast cancers will relapse (4) and heterogeneity of radiation responses, such as severe acute dermatitis, pneumonitis, cough and dysphagia (5). This may contribute to the diversity of radiosensitivity; therefore, many studies have been conducted to improve the radiosensitivity of patients with breast cancer.

The mammalian target of rapamycin (mTOR), which belongs to the phosphoinositide 3-kinase (PI3K), protein kinase B (Akt/PKB) and mTOR signaling pathway, is crucial for cell growth, survival, motility, proliferation, protein synthesis and transcription. The mTOR exists in two different complexes in cells, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). The mTORC1 has functions of nutrient-energy-redox sensor. It controls protein synthesis and stimulates cell growth and proliferation and its activity is mediated by its three major downstream targets: p70S6 ribosomal kinase 1 (p70S6K1), eukaryotic initiation factor 4E binding protein 1 (4EBP1) that regulated protein synthesis and cyclins that mediated cap-dependent translation initiation (6). It showed that 4EBP1 deficiency could drive lymphoma cell resistance to active-site mTOR inhibitors (7). The mTORC2 showed to be involved in the regulation of cytoskeleton functions, by stimulating of actin fibres, paxillin, RhoA, Rac1 and protein kinase C (PKC). Importantly, mTORC2 could activate Akt. The latter is upstream in the mTORC1 pathway and downstream in the mTORC2 pathway. The study also

suggested that mTORC2 plays an important role in the regulation of lipid synthesis (8).

INK128 is an orally-available, potent and selective ATP-competitive mTOR inhibitor; it showed an IC_{50} value of 1 nM against mTOR and more than 100-fold selectivity to PI3K kinases. It even displayed anti-proliferative activity in cell lines resistant to rapamycin (6). Oral administration of INK128 inhibited angiogenesis and tumor growth in multiple preclinical models (6), including diffuse large B-cell lymphoma (9), neuroblastoma (10), thyroid cancer cells (11), prostate cancer metastasis (12), B-cell acute lymphoblastic leukemia (13) and breast cancer (14). Furthermore, in lipopolysaccharide activated RAW264.7 cells, INK128 showed anti-inflammatory activity (15). In addition to *in vitro* condition, it was discovered that INK128 could overcome the resistance of anti-HER2 therapies in three different animal models (16). In the human breast cancer xenograft model of athymic nude mice, INK128 showed potently cell proliferation inhibition and also reduced VEGF-induced lung metastasis (17). Also, a study reported encouraging results that INK128 enhanced *in vitro* and *in vivo* radiosensitivity of pancreatic carcinoma cells (18). Recently, it showed that the activation of the PI3K-AKT-mTOR pathway is considered clinically relevant for tamoxifen resistance and contribute to the poor outcome in breast cancer (19,20), which provides evidence and great expectation for the application of PI3K/AKT/mTOR inhibitors.

Due to the strong rational and these promising effects of INK128, we hypothesized that INK128 combined radiotherapy could induce synergistic effect on breast cancer cells. In the present study, we tested this hypothesis by evaluating the effects of INK128 with clonogenic survival assay, and exploring its potential mechanisms of radiosensitization.

Materials and methods

Cell lines and treatments. Human breast cancer cell line MCF-7 was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and incubated in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT, USA), penicillin (100 U/ml), and streptomycin (100 U/ml) at 37˚C in humidified 5% CO_2 incubator. INK128 (Chemietek, Indianapolis, IN, USA) was dissolved in dimethyl sulfoxide (DMSO). Cells were irradiated using 210 kV X-ray source at 2.16 Gy/min (RS 2000 Biological irradiator; Rad Source Technologies, Suwanee, GA, USA).

Cell viability assay. We analyzed the effect of INK128 as a single agent treatment on clonogenic survival of the breast cancer cell lines in our test. Breast cancer cell viability was measured by the Cell Counting kit (CCK-8) assay. Cells were plated and grown in 96-well clear-bottom plates at 10^4 cells/well. One day after the cells were settled, media was changed and drugs or vehicle was added at the graded concentrations in sextuplicate wells and the cells were then incubated at 37˚C for 1-5 days. Then, a mixture of 10 μl CCK-8 (Dojindo Laboratories) and 190 μl of RPMI-1640 with 10% FBS was added into each well. After 2 h of incubation, luminescence was measured at 450 nm using a microplate reader. Background reading of the media was subtracted from each well to standardize the results. Optical density (OD) was utilized as the indicator of cell survival.

Clonogenic survival assay. Clonogenic survival assays were performed as previously described (21). Cells (250, 500, 1,000, 2,000 and 4,000) were seeded into 6-well tissue culture plates and allowed to settle for 24 h. Then, the cells were exposed to 25 nM of INK128 or vehicle. Similarly, cells were irradiated with different doses of ionizing radiation (0, 2, 4, 6 and 8 Gy) then exposed to the INK128 (25 nM) or same amount of drug-free culture medium for another 24 h. Subsequently, the treated cells were cultured in a 37˚C, 5% CO_2 incubator for 10-14 days. Individual colonies (>50 cells per colony) were fixed with methanol for 10 min, stained with 0.05% crystal violet dye for 10 min, washed twice with tap water, and air dried overnight. The plates were then photographed and the colonies were counted. Each result was performed in triplicate. Plating efficiency (PE) and survival fractions (SF) were calculated. Survival curves were fitted and analyzed using linear-quadratic model [S=exp (-αD-βD2)] by GraphPad Prism software (version 4.0; GraphPad Prism Software, San Diego, CA, USA). The radiation sensitizing enhancement ratio (SER) by INK128 was calculated using the following formula: SER= (SF2 of MCF-7 control)/(SF2 of INK128 MCF-7). SER=1 suggests an additive radiation effect and SER >1, a supra-additive effect as against a sub-additive effect in the case of SER <1.

Fluorescence activating cell sorter (FACS) analysis of cell cycle distribution. The cell suspension was prepared by trypsinization, and $1x10^6$ cells/ml were washed twice with PBS. The cells were resuspended with 10 ml of 70% ethanol (-20˚C), incubated at 4˚C for 4 h, washed twice in cold PBS, incubated with RNase (Sigma-Aldrich) at a concentration of 0.25 mg/ml at 37˚C for 15 min, followed by treatment with PI (10 μl/ml), and incubated for 15 min at 4˚C in the dark. DNA histograms were analyzed using same FACS machine to evaluate the cell cycle distribution.

Immunofluorescent analysis of γH2AX foci. Cells grown in chamber slides, were fixed, permeabilized and blocked as described as follows. The slides were incubated with antibody to γH2AX (Cell Signaling Technology, Danvers, MA, USA) followed by goat anti-mouse Alexa 546 (Thermo Fisher Scientific) and mounted with ProLong Gold anti-fade reagent containing 4'-6-diamidino-2-phenylindole (DAPI; Invitrogen) to visualize nuclei. Slides mounted with ProLong antifade reagent with DAPI (Molecular Probes) and examined by fluorescence microscopy (Carl Zeiss Axioskop 2; Carl Zeiss Microscopy LLC, Thornwood, NY, USA). Cells were judged as 'positive' for γH2AX foci when they displayed 10 or more discrete dots of brightness.

Immunoblotting and antibodies. Cells were grown in 60-mm dishes, and treated with radiation, INK128 or a combination of both INK128 and radiation for the indicated concentrations and times. Cells were washed with ice-cold PBS and scraped into ice-cold lysis buffer. Lysates were cleared by centrifugation at 13,000 rpm for 10 min at 4˚C, and supernatants removed and

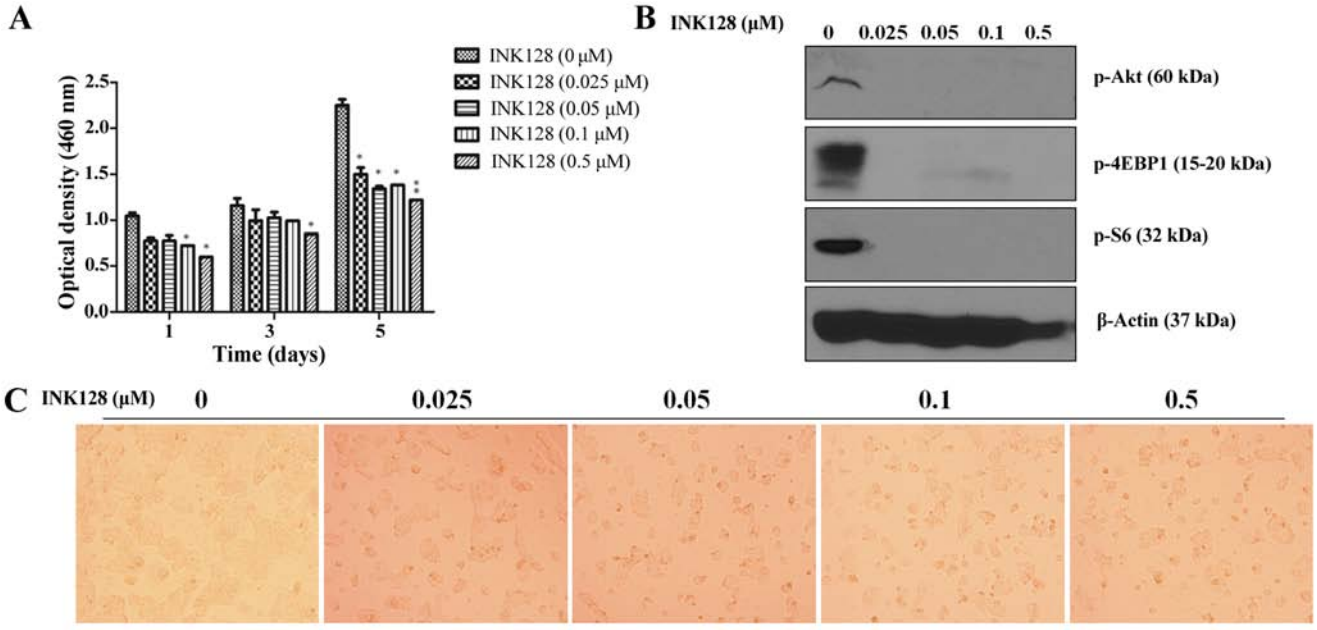

Figure 1. (A) Cell viability was estimated using CCK-8 assay by measuring the absorbance at 460 nm after the cells were exposed to INK-128 ranging from 0 to 0.5 μM for 1-5 days. Values shown represent the means ± SEM for 3 independent experiments, *P<0.05, **P<0.1 according to the Student's t-test. (B) Lysates were subjected to western blot analysis with the labeled antibodies. (C) Clonogenic survival with cells treated with different concentration of INK128, ranging from 0, 0.025, 0.05, 0.1 and 0.5 μM.

assayed for protein concentration using the Pierce BCA bovine serum albumin. Protein was quantified using BCA protein assay (Thermo Fisher Scientific), separated by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF; Bio-Rad Laboratories) and probed with the indicated antibodies. Bands were visualized using Pierce ECL western blotting substrate (Thermo Fisher Scientific). Anti-phospho-Akt, anti-Akt, anti-4EBP1, anti-phospho-4EBP1, anti-phospho-S6, anti-S6, anti-cleaved PARP, anti-LC3B-II, anti-phospho-Chk2, anti-total-Chk2 and anti-p21, γH2AX were purchased from Cell Signaling Technology. Anti-β-actin was obtained from Sigma-Aldrich. Donkey anti-rabbit and sheep anti-mouse horseradish peroxidase-conjugated secondary antibodies were purchased from GE Healthcare. Images were captured with a Fujifilm LASS-3000 camera system.

Statistical analysis. For all experiments, the time-point was chosen based on pre-experiment results where the most significant effect was detected. The data were expressed as means ± SD. Statistical differences were analyzed by one-way ANOVA followed by multiple comparisons performed with post hoc Bonferroni test (SPSS version 16). Values of P<0.05 were considered statistically significant. All experiments were repeated at least three times.

Results

INK128 inhibits breast cancer cell MCF-7 viability and downstream protein. We firstly studied whether INK128 exposure was correlated with cell viability of MCF-7 cells. We further explored the relationship between different concentration with diverse cell viability with the intent to find an optimal concentration. For this purpose, we incubated MCF-7 cells with an indicated concentration. CCK-8 cell viability assay results (Fig. 1A) demonstrated that INK128 dose- and time-dependently inhibited MCF-7 cell viability. In addition, we noted that treatment with INK128 alone reduced cell viability reaching a maximum inhibition by approximately day 5 as indicated by the decrease in optical density levels at the concentration of 25 nM (Fig. 1A). It is consistent with the clonogenic survival assay shown in Fig. 2B. Furthermore, INK128 induced decrease of downstream signal chemicals, such as p-Akt, p-4EBP1 and p-S6, even though at a very low concentration of 25 nM (Fig. 1B). Thus, for the following experiment we chose INK128 at 25 nM as the experiment concentration.

INK128 increases MCF-7 cell radiosensitivity. Next, we asked whether INK128 exposure was able to increase the sensitivity of MCF-7 cells to irradiation. For this purpose, treatment protocol was based upon a previous study (18). INK128 exposure significantly enhanced radiosensitivity compared with control cells (both INK 128 alone and radiation alone) (Fig. 2), INK128 enhanced radiosensitivity by SER=5.2 (SF2=0.47 for control cells; SF2=0.086 for INK128 exposure cells) (Fig. 2A). According to the curve of surviving fraction we adopted radiation dose of 3 Gy to perform the next study, these data suggested that mTOR might be a critical regulator in radiation response in MCF-7 cells.

INK128 decreased the radioresistant S-phase cells and increased G2/M blocks. It has been reported that mTORC1 is involved in the regulation of different cyclins. To the best of our knowledge, the different cycle distribution stands for different radiosensitivity. Thus, we investigated the cell cycle distribu-

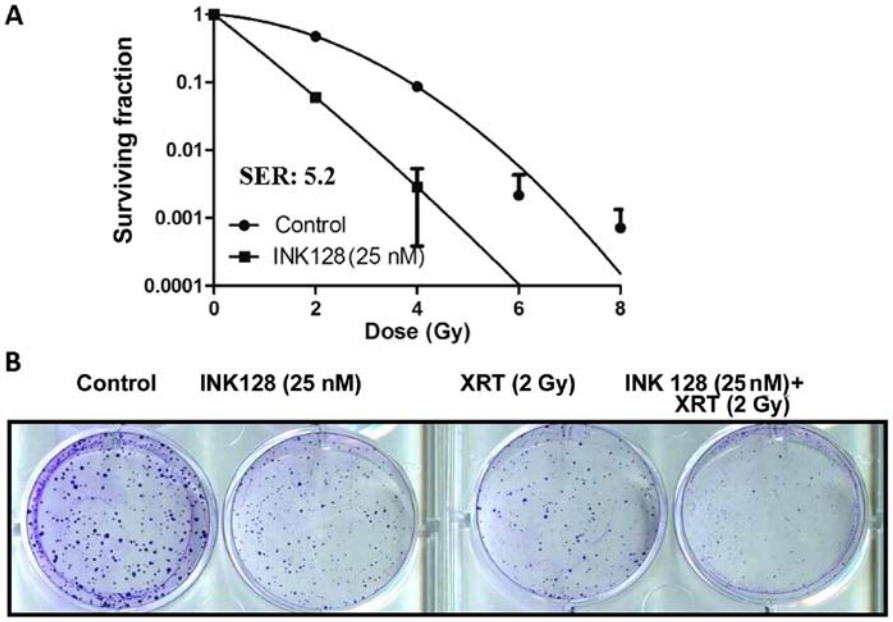

Figure 2. Effect of INK128 on radiosensitivity in MCF-7. (A) INK-128 changed the radiosensitivity. (B) Clonogenic survival with cells treated as indicated. MCF7 cells were plated, attached overnight, irradiated and the indicated concentration of inhibitor was added immediately after radiation. Twenty-four hours after radiation, cell culture media was removed and fresh drug-free media was added. Survival curves were generated after normalizing for cell killing from drug alone 10-14 days later. Values shown represent the means ± SEM for 3 independent experiments, $^{*}P<0.05$, $^{**}P<0.1$ according to the Student's t-test.

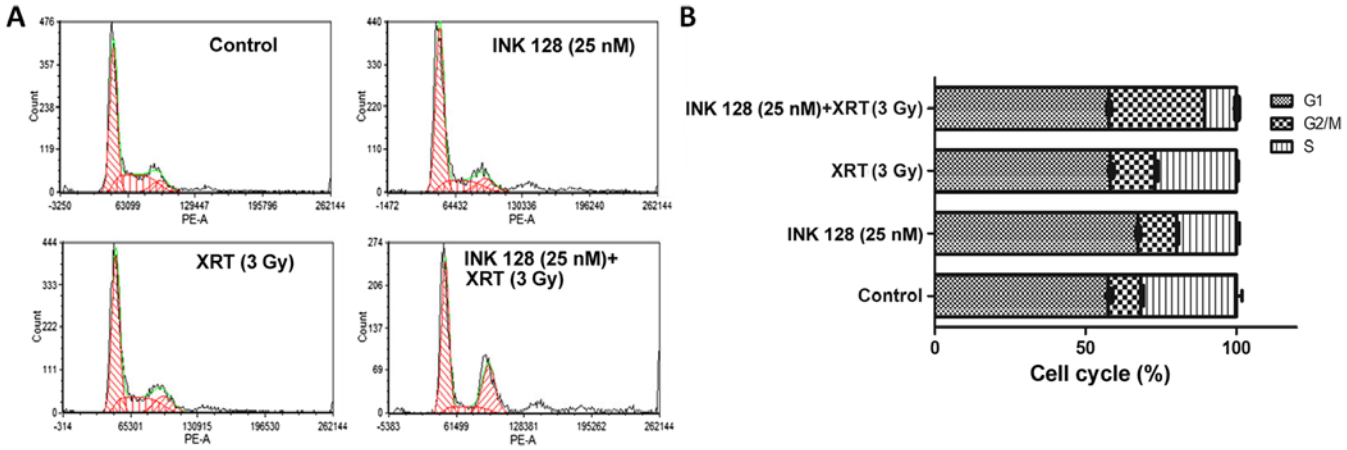

Figure 3. (A) Analysis of cell cycle distribution by flow cytometry. (B) INK128 combined with radiotherapy decreased the radioresistant S-phase cells and increased G2/M arrest.

tion in MCF-7 cells exposed to indicated elements (vehicle, 25 nM INK128, 3-Gy irradiation and its combination) by FACS. The data by flow cytometry showed 3-Gy radiation has slight effect on S-phase cell decrease. Whereby, INK128 could obviously cause radioresistant S-phase cell downregulation with low concentration of 25 nM. When combining radiation and INK128, it further reduced radioresistant S-phase cell population to 10.6±0.25 than the 31.70±1.91% of the control (Fig. 3B). In addition, INK128 combined radiation could obviously induce G2/M arrest as indicated by the maximum percentage of the G2/M (31.66±0.07%).

INK128 enhances radiation induced double strand break (DSB) and suppresses its repair in MCF-7 cells. It is well known that γH2AX is a marker of DNA double strand breaking in cells treated with radiation (22). Next, we began to investigate the mechanisms mediating INK128-induced radiosensitization according to the theory that γH2AX foci corresponds to radiation-induced DSB. The treatment protocol was as follows: vehicle, 25 nM INK128, 3-Gy irradiation, INK128 plus radiation, respectively. Then γH2AX nuclear foci were detected in different time-points (0.5, 3 and 24 h). As shown in Fig. 4A, no difference in foci levels was detected between control (vehicle) and INK128 treated alone cells at 0.5 h after irradiation, suggesting that INK128 had no effect on the initial levels of DSBs. When combining INK128 and radiation at 0.5 h, it was significantly higher than the other three groups. However, after 6 and 24 h of irradiation, the residual numbers of γH2AX foci were significantly greater in the INK128 treated cells as compared to control cells. The

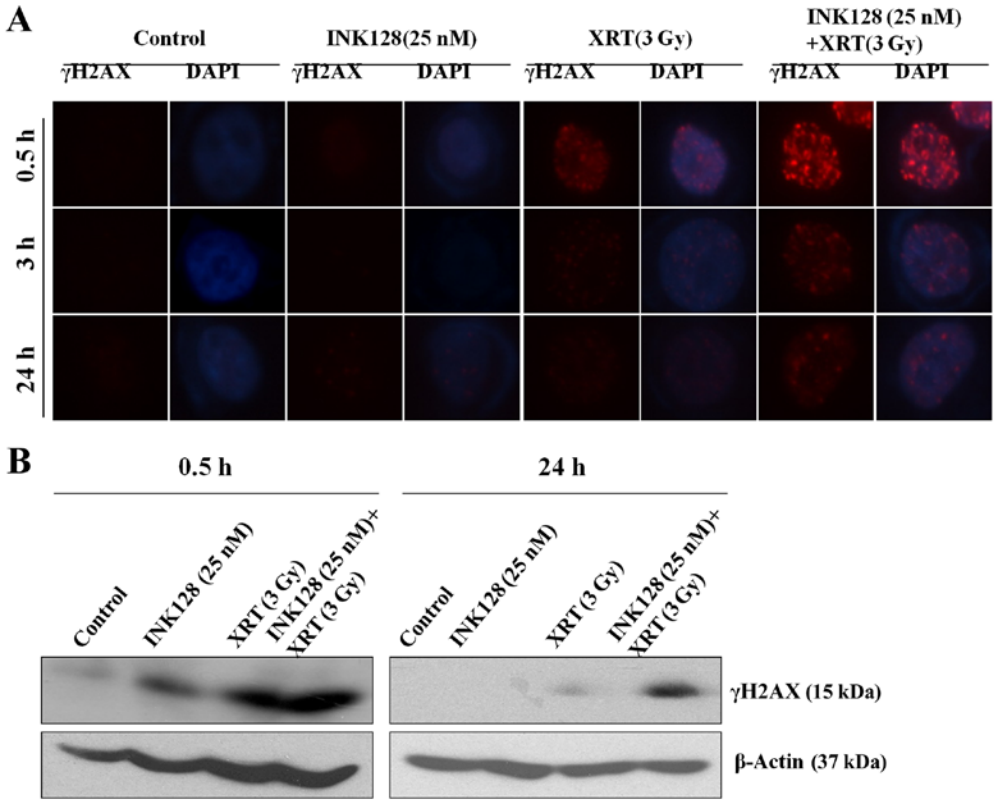

Figure 4. (A) INK-128 suppressed radiation induced DNA DSB damage repair, γH2AX foci were detected in different time-points (0.5, 3 and 24 h). (B) γH2AX expression were detected after 0.5 and 24 h by western blot analysis.

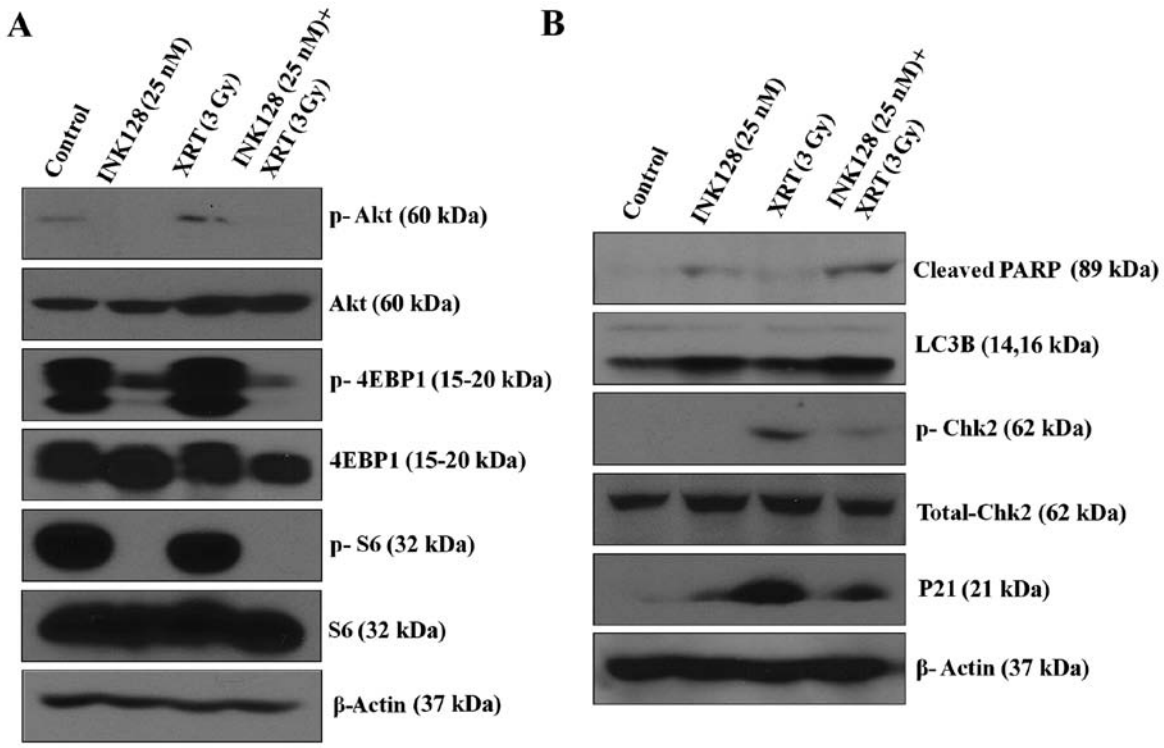

Figure 5. Western blot analysis to verify the downregulating signal protein (A) and to detect the potential related protein (B).

bands of γH2AX from Fig. 4B showed consistent results. It indicated that INK128 enhanced radiation induced DNA DSB and suppressed its repair.

INK128 downregulates signaling pathway protein expression. INK128 is a novel mTORC1 and 2-dual inhibitor, thus, we used western blots to verify the downregulating signal protein and

detect the potential related protein. As indicated in Fig. 5A, INK128 alone or in combination with ionizing radiation could obviously reduce phosphorylation of Akt (the mTORC2 activation indicator), phosphorylation of S6 (mTORC1 activation indicators) and phosphorylation of 4EBP1 (mTORC1 activation indicators).

Next, we tested the probable protein expression, western blot analysis was performed to analyzed the cleaved-PARP, which is the final product of apoptotic state, that is to say increased cleaved-PARP levels indicate apoptosis (23). As shown in Fig. 5B, INK128 alone could promote the cleaved-PARP. When combined with radiation, cleaved-PARP was obviously increased. Similarly, we detected the autophagy related protein, such as LC3B-II. In MCF-7 cells, single treatments (INK128 alone) were able to increase LC3B. LC3B-II was further enhanced in combined therapy group (Fig. 5B). Also, we found that radiation alone could apparently increase expression of phosphorylation of Chk2 and p21. INK128 combined with radiation could decrease expression of phosphorylation of Chk2, an indicator of cell cycle checkpoint kinase 2 (p-Chk2) activation and p21.

Discussion

Breast cancer treatment includes surgery, radiotherapy, chemotherapy and endocrine therapy. Radiotherapy is one of most effective and critical components for local control (24), reduced risk of local recurrence in chest wall and regional lymph nodes (25). However, the heterogeneity of radiation responses among breast cancer patients limits clinical applications of radiotherapy (26). The present study focused on the correlation of mTOR inhibition by a novel mTORC1/2 dual inhibitor INK128 and radiosensitivity of patients with breast cancer. We established the radiosentization effect of INK128 with a SER of 5.2 at 2 Gy. It suggested that mTOR pathway might play an important role in the regulation of cellular response to radiation in MCF-7 cells. Also, the results underscore the importance of mTOR targeting in combination with irradiation in tumor therapy.

In order to investigate the mechanism mediating the INK128-induced radiosensitization, the following steps were taken. The results showed that the combination with INK128 and radiation could cause G2/M block with low concentration of 25 nM, that is to say, enhance the percentage of G2 phase cells, decreased the S phase cells. As is known, S phase cells are relatively resistant to radiation, while G2 cell phase are relatively sensitive. Thus, our results showed that the change of cell cycle distribution maybe the reason to mediate INK128-induced radiosentization. A little different to our result, another study showed that INK128 could enhance the percentage of S and G2 phase cells, decrease the G1 phase cells by downregulating of cyclin D1 with a single agent treatment of INK128 at the concentration of 50 nM for 48 h for human pancreatic cancer cells (27). According to the small difference described above, the other reason may be that single treatment or combined strategy may hold different mechanism that regulate the cell cycle. In terms of p21, it is well known that p21 is a potent inhibitor of cyclin-dependent kinases capable of arresting cell cycle progression (28), which is the key mechanism to prevent apoptosis by promoting cell repair (29). Our results demonstrated that INK128 treatment could decrease the p21 expression, which means increased cell apoptosis. It may be another cause to mediate radiosensitivity.

It is well known that DSBs are suggestive of critical lesions in DNA caused by ionizing radiation. DSB is the main mechanism of tumor cell death after irradiation. The major cause of radiotherapy failure is the success of DSB repair, which could lead to prolonged tumor cell survival. If completion of DNA damage repair fails, apoptosis and autophagy will be triggered for the elimination of damaged cells (21). Our results demonstrated that INK128 exposure markedly increased DSB and significantly decreased the rate of DNA DSB recovery. In the present study, at 0.5 h after irradiation, the number of γH2AX foci remaining was significantly greater when INK128 combined with radiation. Up to 24 h, the content of γH2AX foci was gradually reduced, but it was still more than radiation alone group. It was consistent with another study, at 6 and 24 h after irradiation, the number of γH2AX foci remaining was significantly greater in the INK128-treated cells than in control cells. So the author conclude that INK128 inhibited a later stage of DNA DSB repair (18). There are some other reasons to explain the damaged repair ability. Firstly, Chk2 is a kind of DNA repair proteins (30). Our results from western blot analysis showed that combination of INK128 and radiation could apparently decrease expression of phosphorylation of Chk2. Secondly, a study demonstrated that another PI3K/mTOR inhibitor, BEZ235, could block double strand break repair through attenuating the activation of radiation-activated phosphorylation of ATM and DNA-PKcs, the former are two major kinases for non-homologous end joining and homologous recombination in DNA-DSB repair (22). This may be another mechanism for INK128. Also, it showed that increased p-Akt had been linked to decreased radiation responsiveness; therefore, inhibition of p-Akt has radiosensitizing effect (21). Thus, the present study showed that INK128 treatment could decrease DNA DSB repair, p-Akt and p-Chk2, which might be important factors to mediate INK128-induced radiosensitization.

In conclusion, we proposed that mTOR confers radiation resistance and the strong potential of the novel mTORC1/2 dual inhibitor INK128 to enhance ionizing radiation in breast cancer cells. Thus, effectively inhibition of mTOR by INK128 is a potential therapeutic strategy for sensitizing resistant breast cancer cells to radiation. Next, we need phase I and phase II clinical trials.

Acknowledgements

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