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Overexpression of SMAR1 Enhances Radiosensitivity in Human Breast Cancer Cell Line MCF7 via Activation of p53 Signaling Pathway

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This study sought to investigate the effect of overexpression of SMAR1 (scaffold/matrix-associated region-binding protein 1) on cell radiosensitivity in breast cancer, as well as elucidate its regulatory mechanism. We constructed a lentiviral expression system to successfully overexpress SMAR1 in human breast cancer cell line MCF7. In addition, overexpression of SMAR1 in MCF7 cells enhanced the radiosensitivity to ⁸⁹SrCl₂. Moreover, overexpression of SMAR1 significantly induced cell apoptosis rate and G₂/M phase arrest under the irradiation of ⁸⁹SrCl₂. In addition, Western blot analysis showed that overexpression of SMAR1 in MCF cells significantly increased the expression levels of pP53 (ser15), pP53 (ser20), acP53, and p21 and obviously decreased the expression of MDM2 under the irradiation of ⁸⁹SrCl₂. Notably, these expression changes could be neutralized by PFTα, an inhibitor of p53 signaling pathway that could inhibit p53-dependent transactivation of p53-responsive genes. Therefore, overexpression of SMAR1 may increase radiosensitivity to ⁸⁹SrCl₂ in breast cancer cell line MCF7 by p53-dependent G₂/M checkpoint arrest and apoptosis. Enhanced expression of SMAR1 in tumors will help to improve the clinical efficiency of radiation therapy.

Key words: Breast cancer; Scaffold/matrix-associated region-binding protein 1 (SMAR1); Radiosensitivity; p53; G₂/M checkpoint arrest; Apoptosis

INTRODUCTION

Breast cancer is one of the most common malignant tumors in women, accounting for approximately 29% of all new cancer cases annually among women (1,2). Radiation therapy is an important modality in the management of breast cancers. Several meta-analyses of individual patient data from large-scale randomized trials have shown that radiotherapy after surgery can reduce the risk of recurrence and breast cancer death (3,4). However, the development of radioresistance presents a significant problem over prolonged courses of treatment (5). Therefore, a better understanding of the molecular mechanisms behind radioresistance in breast cancer cells will help to greatly improve clinical outcomes.

Matrix-associated region-binding proteins (MARBPs) are shown to be implicated in regulation of various physiological processes, such as cell cycle progression, DNA damage repair, and apoptosis (6). SMAR1 (scaffold/matrix-associated region-binding protein 1) is a recently identified MARBP (7), which shows 99% homology with BANP

(mapped to the 16q24 locus) in humans (8). It has been suggested that SMAR1 can function as a potent tumor suppressor via interaction with and activation of p53 and subsequently induce G₂/M arrest and delay tumor growth in mice (9). Singh et al. also demonstrated that SMAR1 was downregulated in human breast cancers and could cross talk between p53 and TGF-β signaling pathways to regulate tumor growth and metastases (10). Besides tumor-suppressor function, SMAR1 can also function as a transcriptional repressor to repress the expression of cyclin D1, whose higher expression is a hallmark in breast cancer (11). Malonia et al. also demonstrated that SMAR1 could regulate NF-κB-dependent interleukin-8 transcription, which is important for the metastasis and angiogenesis of breast tumors (12). Despite these, the crucial role of SMAR1 in regulation of radiosensitivity or radioresistance in breast cancer has not been fully investigated.

In the present study, we constructed a lentiviral expression system to overexpress SMAR1 in human breast cancer cell line MCF7. MCF7 cells were then treated with different

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concentrations of strontium-89 chloride ($^{89}\text{SrCl}_2$). Clone formation assay, cell apoptosis, and cell cycle analyses were performed to detect whether overexpression of SMAR1 in MCF7 cells could influence radiosensitivity to $^{89}\text{SrCl}_2$. Finally, the expression levels of p53 signaling pathway-related proteins were determined by Western blot analysis. Our study sought to investigate the effect of overexpression of SMAR1 on cell radiosensitivity in breast cancer, as well as elucidate its regulatory mechanism. A combination of radiation therapy and gene therapy may provide a new insight for improving the treatment outcomes of this disease.

MATERIALS AND METHODS

Cell Culture

Human breast cancer cell line MCF7 was obtained from the experimental center of clinical laboratory diagnostics at Bengbu Medical College (Anhui, China). Human embryonic kidney (HEK) 293FT cells were purchased from Invitrogen (Carlsbad, CA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) in a 5% CO_2 atmosphere at 37°C.

Lentiviral Production and Infection of Human Breast Cancer Cell Line MCF7

Recombinant expression plasmid pEGFP-SMAR1 and blank vector pEGFP were provided by Beijing Genomics Institute (BGI, Shenzhen, Guangdong, China). To establish MCF7 cells that could express pEGFP-SMAR1 and a control pEGFP, we used a lentiviral expression system. In brief, the lentivirus packaging vectors pMD2.G (encoding VSV-G), pMDLg/pRRE (encoding gag/pol), and pRSV-Rev (encoding rev) were obtained from Addgene (<http://www.addgene.org/>). Then the lentivirus packaging vectors pMD2.G, pMDLg/pRRE, pRSV-Rev, and recombinant expression plasmid pEGFP-SMAR1 or blank vector pEGFP were cotransfected into 293FT cells using the Lipofectamine2000 (Invitrogen)-mediated transfection method. Forty-eight hours after transfection, the culture supernatants were collected and filtered through 0.22- μm pore-size filters, and viral particles were concentrated by ultracentrifugation. After the titer of virus was tested using large-scale real-time titration (LaSRT), a multiplicity of infection (MOI) 10 of virus was applied to infect MCF7 cells. Forty-eight hours after infection, the detection rate of GFP⁺ cells was measured by fluorescence microscope and flow cytometry. Meanwhile, the total protein was extracted from these infected MCF7 cells to determine whether pEGFP-SMAR1 or pEGFP was successfully expressed using Western blot. MCF7 cells that can successfully express pEGFP-SMAR1 were defined as the MCF7-SMAR1 group, and MCF7 cells that can successfully express pEGFP were the MCF7-c group. Notably, MCF7 cells without any treatment were defined as the control group.

Clone Formation Assay

A specified number of cells ($n=200$ cells/each well) of each group were seeded into 24-well culture plates and cultured in DMEM medium with different concentrations (0.01, 0.1, 1, 10 $\mu\text{Ci/ml}$, respectively) of Metastron ($^{89}\text{SrCl}_2$), which was obtained from Chengdu Gaotong Isotope Co., Ltd, China. Cells continued to be incubated for colony formation for 14–21 days. Colonies were then fixed with methanol and stained with 0.5% crystal violet. The number of colonies containing at least 50 cells was determined to calculate surviving fractions, and survival curves were then drawn using Kaleidagraph version 3.51 (Synergy Software, Reading, PA, USA).

Cell Apoptosis Analysis

Flow cytometry is an effective method to detect cell apoptosis after drug treatment (13). A specified number of cells of each group were seeded into six-well culture plates. Then cells were treated with 10 $\mu\text{Ci/ml}$ of $^{89}\text{SrCl}_2$ for 24 h. Cells were collected and then resuspended in 1 \times binding buffer at a concentration of 1×10^6 cells/ml. Cell apoptosis analysis was then performed using Annexin-V Apoptosis Detection Kit APC (eBioscience, San Diego, CA, USA) according to the instructions of the manufacturer. Finally, apoptotic cells were analyzed at 488 nm by flow cytometry on a Beckman Coulter FC200 (Fullerton, CA, USA).

Cell Cycle Analysis

A specified number of cells of each group were seeded into six-well culture plates. Then cells were treated with 10 $\mu\text{Ci/ml}$ of $^{89}\text{SrCl}_2$ for 24 h. Cells (1×10^6 cells/ml) were harvested by trypsinization, washed by ice-cold PBS, and fixed in ice-cold 70% ethanol. Cells were then incubated with bovine pancreatic RNAase A (500 U/ml; Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 30 min, and then 0.25% propidium iodide (PI; Sigma-Aldrich) was added to stain cells for 30 min at room temperature. Finally, cells in each group were analyzed at 488 nm by flow cytometry on a Beckman Coulter FC200.

p53 Signaling Pathway Analysis

Cells in a different group were treated with 10 $\mu\text{Ci/ml}$ of $^{89}\text{SrCl}_2$ for 24 h, and cells without treatment with $^{89}\text{SrCl}_2$ were set as control. Moreover, cells that were exposed with 10 $\mu\text{Ci/ml}$ of $^{89}\text{SrCl}_2$ were further treated with 10 μM of pifithrin- α (PFT α), an inhibitor of p53 signaling pathway that could inhibit p53-dependent transactivation of p53-responsive genes (14). Therefore, to further explore the regulatory mechanism involved in SMAR1-enhanced radiosensitivity to $^{89}\text{SrCl}_2$, the expression levels of related proteins in p53 signaling pathway, such as phospho-P53 (pP53, Ser15), pP53 (Ser20), acetylated-P53 (acP53), total P53 (tP53), P21, and MDM2

were determined by Western blot. In addition, clone formation assay was performed to determine the effect of inhibition of p53 signaling pathway.

Western Blot Analysis

Cells in each group were washed three times with ice-cold PBS and resuspended in lysis buffer (iNtRON Biotechnology, Seoul, Korea). Proteins were collected by centrifugation, and the protein concentration was measured using bicinchoninic acid assay (BCA). Equal amounts of protein were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA, USA). After blocked, membranes were probed with primary antibodies [phospho-P53 (Ser15), phospho-P53 (Ser20), acetylated-P53, total P53, P21, and MDM2, respectively]. Membranes were washed and incubated with secondary antibody conjugated with horseradish peroxidase (HRP) at 1:2,000 dilution for 1 h. Antibody binding was detected using an ECL detection kit (Amersham Biosciences, Piscataway, NJ, USA). Notably, the expression level of these proteins was normalized to β -actin.

Statistics Analyses

All the measurement data are presented as the mean \pm SD, and the statistical analyses were analyzed with SPSS 20.0 (SPSS Inc., Chicago, IL, USA). A *t*-test or one-way ANOVA as appropriate was performed for comparisons between groups. A value of $p \leq 0.05$ represents statistical significance.

RESULTS

SMAR1 Was Successfully Overexpressed in MCF7 Cells

A lentivirus system could be used for the overexpression of SMAR1 in human breast cancer cell line MCF7. The results of fluorescence microscope and flow cytometry analysis showed that the detection rate of GFP⁺ cells in both MCF7-c group and MCF7-SMAR1 group were higher than 85% (Fig. 1A, B). Meanwhile, Western blot analysis displayed that SMAR1 was not expressed in MCF7 group, and the lentivirus was able to express GFP in both MCF7-c group and MCF7-SMAR1 group (Fig. 1C). Notably, SMAR1 was successfully overexpressed in MCF7-SMAR1 group (Fig. 1C).

Overexpression of SMAR1 in MCF7 Cells Enhanced the Radiosensitivity to $^{89}\text{SrCl}_2$

As shown in Figure 2, the survival curves of different groups were fitted using shoe model lining. The results showed that the survival curves of MCF7 group and MCF7-c group almost overlapped, indicating that the lentivirus blank vector had no obvious toxicity to cells.

In addition, compared with the survival curve of MCF7 group or MCF7-c group, the shoulder area of the survival curve of MCF7-SMAR1 group was increased and broadened, indicating that overexpression of SMAR1 in MCF7 cells enhanced the radiosensitivity to $^{89}\text{SrCl}_2$.

Overexpression of SMAR1 Induced Cell Apoptosis After Exposure to $^{89}\text{SrCl}_2$

Flow cytometry analysis displayed cell apoptosis of different groups after exposure to 10 $\mu\text{Ci/ml}$ of $^{89}\text{SrCl}_2$ (Fig. 3). Compared with no exposure to $^{89}\text{SrCl}_2$, 10 $\mu\text{Ci/ml}$ of $^{89}\text{SrCl}_2$ could markedly induce cell apoptosis no matter which MCF7 group they were in, the MCF7-c group or the MCF7-SMAR1 group. In addition, under the same dose irradiation, the apoptosis rate of the MCF7-SMAR1 group was higher than in the MCF7 group or the MCF7-c group, indicating that overexpression of SMAR1 enhanced cell radiosensitivity to $^{89}\text{SrCl}_2$ via inducing cell apoptosis.

Overexpression of SMAR1 Induced G_2/M Phase Arrest After Exposure to $^{89}\text{SrCl}_2$

Flow cytometry analysis also displayed the cell cycle of different groups after exposure to 10 $\mu\text{Ci/ml}$ of $^{89}\text{SrCl}_2$ (Fig. 4). The results showed that cells at G_2/M phase transition in MCF7-SMAR1 group were significantly increased when compared to the MCF7 group or the MCF7-c group, while cells at S phase in the MCF7-SMAR1 group were obviously decreased, indicating that overexpression of SMAR1 induced G_2/M phase arrest after exposure to $^{89}\text{SrCl}_2$.

Overexpression of SMAR1 May Enhance the Radiosensitivity to $^{89}\text{SrCl}_2$ via Activation of p53 Signaling Pathway

Western blot analysis showed whether p53 signaling pathway was a key regulatory mechanism involved in SMAR1-enhanced radiosensitivity to $^{89}\text{SrCl}_2$ (Fig. 5A). The results showed that under normal condition (cells were not treated with $^{89}\text{SrCl}_2$ or PFT α), the expression levels of p53 signaling pathway-related proteins in MCF7-SMAR1 group had no change compared with that in the MCF7 group or MCF7-c group. However, under the irradiation of $^{89}\text{SrCl}_2$, the expression level of p53 signaling pathway-related proteins, such as pP53 (ser15), pP53 (ser20), acP53, and p21 in the MCF7-SMAR1 group were significantly increased, while the expression level of MDM2 was decreased compared with that in the MCF7 group or the MCF7-c group, suggesting that activation of p53 signaling pathway might be a key mechanism contributing to SMAR1-enhanced radiosensitivity to $^{89}\text{SrCl}_2$. Notably, after cells were treated with both $^{89}\text{SrCl}_2$ and PFT α (an inhibitor of p53 signaling pathway that could inhibit p53-dependent transactivation of p53-responsive

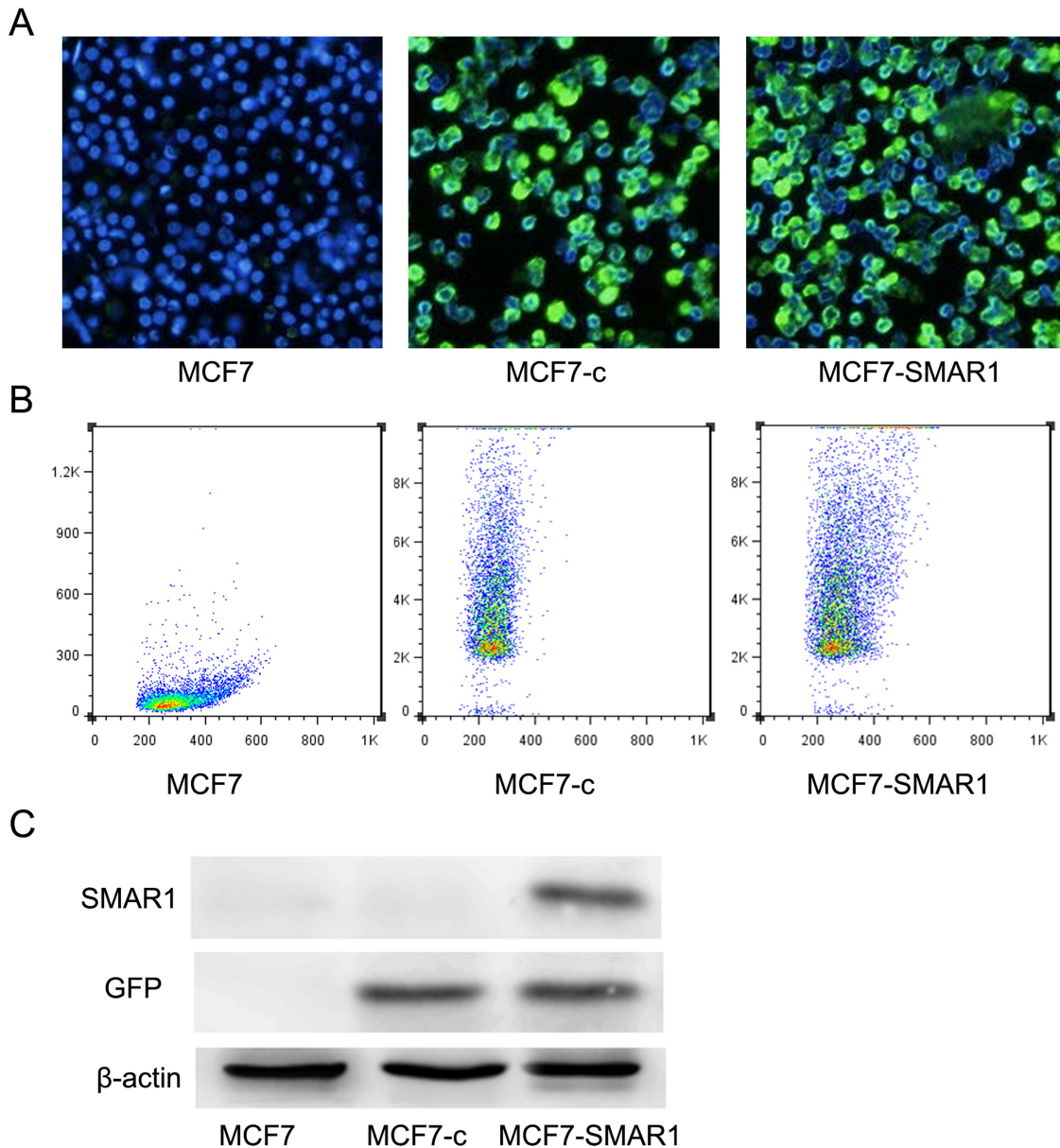


Figure 1. SMAR1 was successfully overexpressed in human breast cancer cell line MCF7. Fluorescence microscope (A) and flow cytometry analysis (B) showed that the detection rate of GFP⁺ cells in both MCF7-c group and MCF7-SMAR1 group were higher than 85%. Western blot analysis (C) displayed that SMAR1 was successfully overexpressed only in MCF7-SMAR1 group.

genes), the expression levels of p53 signaling pathway-related proteins in the MCF7-SMAR1 group had no change compared with that in the MCF7 group or the MCF7-c group, indicating that PFT α could effectively inhibit the radiation-activated p53 signaling pathway in the MCF7-SMAR1 group (Fig. 5A).

Furthermore, the results of clone formation assay showed that after cells were treated with both $^{89}\text{SrCl}_2$ and PFT α , PFT α could reverse the SMAR1-enhanced radiosensitivity to $^{89}\text{SrCl}_2$ in the MCF7-SMAR1 group

(Fig. 5B), implying that SMAR1-enhanced radiosensitivity to $^{89}\text{SrCl}_2$ may be associated with activation of the p53 signaling pathway.

DISCUSSION

Breast cancer in women is commonly associated with bone metastases (15). Systemic radionuclide therapy with few side effects has drawn much attention in the treatment of bone metastasis. Currently, $^{89}\text{SrCl}_2$ has been widely and successfully used for the treatment of bone

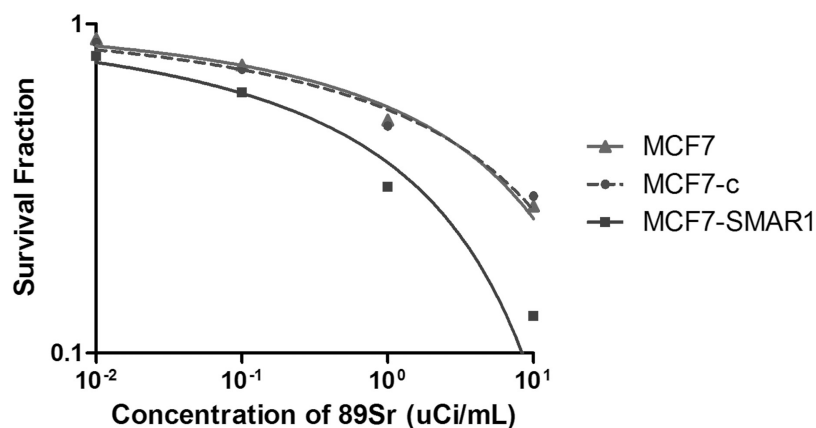


Figure 2. Clone formation assay showed the survival curves of MCF7 group, MCF7-c group, and MCF7-SMAR1 group under the treatment of a different concentration of ⁸⁹SrCl₂.

metastases (16,17). To improve the clinical efficiency of radiation therapy, the molecular mechanism underlying radioresistance was explored in our study. Recently, MARBPs are reported to play a significant role in tumor-specific metabolism. In the present study, SMAR1 was

successfully overexpressed in human breast cancer cell line MCF7. Moreover, we found that overexpression of SMAR1 in MCF7 cells enhanced the radiosensitivity to ⁸⁹SrCl₂ via inducing cell apoptosis and G₂/M phase arrest. In addition, the changes of p53 signaling pathway-related

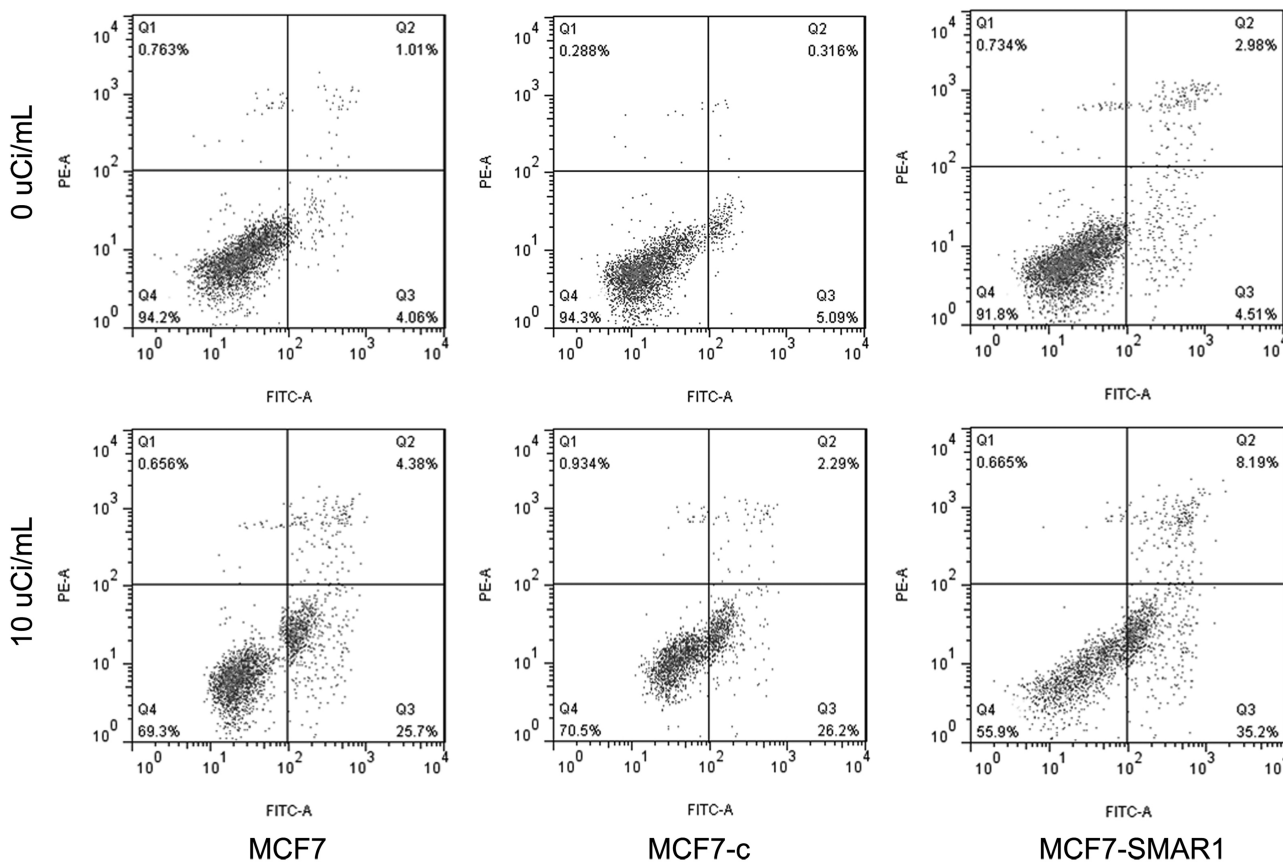


Figure 3. Flow cytometry analysis displayed cell apoptosis of a different group after exposure to 10 μ Ci/ml of ⁸⁹SrCl₂. Under the same dose irradiation of ⁸⁹SrCl₂, the apoptosis rate of MCF7-SMAR1 group was higher than MCF7 group or MCF7-c group.

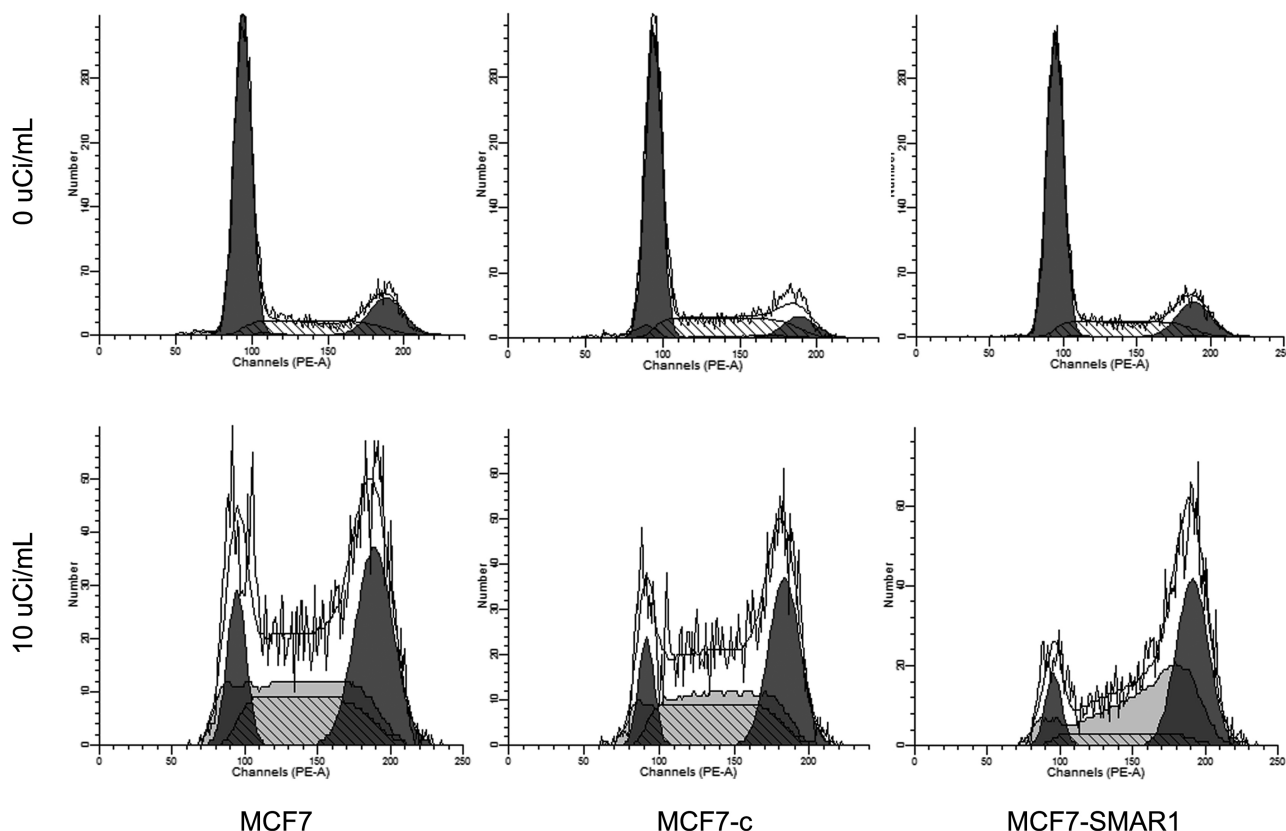


Figure 4. Flow cytometry analysis displayed a cell cycle of different group after exposure to 10 $\mu\text{Ci/ml}$ of $^{89}\text{SrCl}_2$. The results showed that cells at G_2/M phase transition in the MCF7-SMAR1 group were significantly increased than that in the MCF7 group or the MCF7-c group, while cells at S phase in MCF7-SMAR1 group were obviously decreased.

proteins expression implied that activation of the p53 signaling pathway might be a key mechanism involved in SMAR1-enhanced radiosensitivity to $^{89}\text{SrCl}_2$.

In a previous study, SMAR1 was shown to inhibit p53-dependent apoptosis in response to DNA damage (18). Also, Sykes et al. suggested that acetylation of p53 was indispensable for p53-dependent activation of apoptotic targets, such as BAX and PUMA (19). In addition, Jalota et al. confirmed that SMAR1 can interact with p53 and stabilize it through displacing its negative regulator MDM2 (20). p53 can modulate both apoptosis and radiosensitivity, thus to regulate radiotherapy response (21). It has also been confirmed that p53 contributes to the radiosensitivity of lung cancer cells through regulating autophagy and apoptosis (22). In our study, the apoptosis rate of MCF7-SMAR1 group was higher than in the MCF7 group or the MCF7-c group under the irradiation of $^{89}\text{SrCl}_2$, suggesting that SMAR1 may increase radiosensitivity to $^{89}\text{SrCl}_2$ via inducing the apoptosis of breast cancer cells. Furthermore, Western blot analysis showed that the expression level of p53 signaling pathway-related proteins, such as pP53 (ser15), pP53 (ser20), acP53, and p21 in the MCF7-SMAR1 group were significantly

increased, while the expression of MDM2 was obviously decreased compared with that in the MCF7 group or the MCF7-c group under the irradiation of $^{89}\text{SrCl}_2$. Moreover, these expression changes could be neutralized by PFT α , an inhibitor of the p53 signaling pathway. Therefore, our results are in line with previous findings and strongly suggest that p53 signaling pathway-related proteins may provoke apoptosis in response to DNA damage after irradiation in breast cancer, and SMAR1 may responsible for the enhanced radiosensitivity to $^{89}\text{SrCl}_2$ in breast cancer cells by p53-dependent cell apoptosis.

Furthermore, it has been suggested that SMAR1 can positively regulate the onset of ionizing radiation-induced G_2/M checkpoint (23). Kaul et al. suggested that SMAR1 could cause G_2/M delay to retard cell growth via activating p53 through direct interaction (9). In addition, p53 can activate p21 in response to DNA damage, and p21 expression can augment G_2/M arrest in human breast cancer cells via a p53-independent mechanism (24). Also, inhibition of MDM2-p53 interaction is reported to augment radiation response in human tumors (25). The modulation of cell cycle progression is shown to be one possibility to improve therapeutic strategy. It is a fact

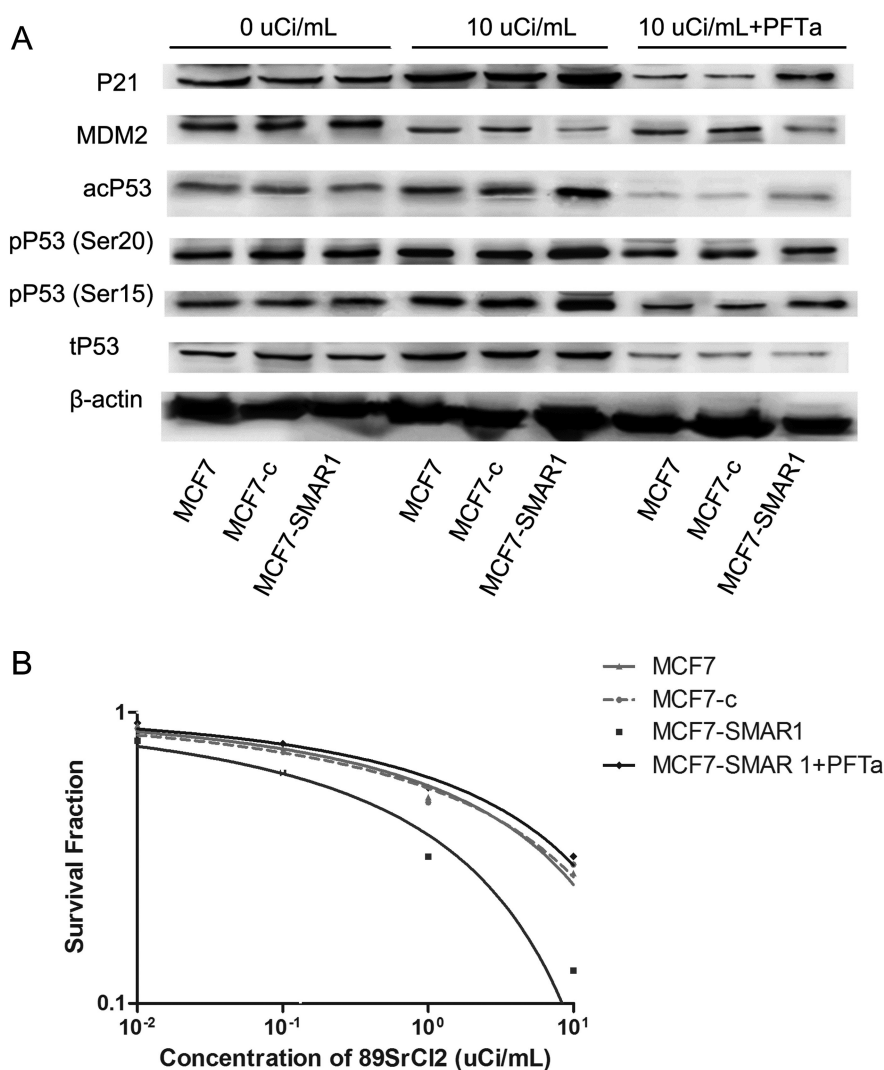


Figure 5. p53 signaling pathway analysis by Western blot analysis (A) and clone formation assay (B). (A) Under the irradiation of $^{89}\text{SrCl}_2$, the expression levels of pP53 (ser15), pP53 (ser20), acP53, and p21 in the MCF7-SMAR1 group were significantly increased, while the expression level of MDM2 was decreased compared with that in the MCF7 group or the MCF7-c group. However, after cells were treated with both $^{89}\text{SrCl}_2$ and PFT α , the expression levels of p53 signaling pathway-related proteins in MCF7-SMAR1 group had no change compared with that in the MCF7 group or the MCF7-c group. (B) After cells were treated with both $^{89}\text{SrCl}_2$ and PFT α , PFT α could reverse the SMAR1-enhanced radiosensitivity to $^{89}\text{SrCl}_2$ in the MCF7-SMAR1 group.

that the radiation-induced G_2 phase block is a universal event in cancer cells, rendering the application prospects of G_2/M checkpoint arrest in breast cancer cells for improved efficacy of radiation therapy (26). Anastasov et al. also confirmed that the extent of the G_2/M arrest following irradiation is associated with the survival of tumor cells, and a potent G_2/M checkpoint inhibitor may help to overcome radiation resistance of breast tumors (27). In our study, overexpression of SMAR1 induced G_2/M phase arrest after exposure to $^{89}\text{SrCl}_2$. In addition to the changes of p53 signaling pathway-related protein expression, it is thus intriguing to speculate that SMAR1 may

induce p53-dependent G_2/M checkpoint arrest to increase radiosensitivity to $^{89}\text{SrCl}_2$ and could function as a potent G_2/M checkpoint inhibitor to overcome radiation resistance of breast tumors.

In conclusion, our findings indicate that SMAR1 may increase radiosensitivity to $^{89}\text{SrCl}_2$ in breast cancer cell line MCF7 by p53-dependent G_2/M checkpoint arrest and apoptosis. Enhanced expression of SMAR1 in tumors will help to improve the clinical efficiency of radiation therapy.

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