


Knockdown of Ubiquitin-Specific Protease 53 Enhances the Radiosensitivity of Human Cervical Squamous Cell Carcinoma by Regulating DNA Damage-Binding Protein 2

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Abstract

Background: Cervical cancer ranks fourth in incidence and mortality among women. Ubiquitin-specific protein 53 binds to damage-specific DNA binding protein 2 and affects the biological properties of colon cancer. Damage-specific DNA binding protein is involved in nucleotide excision repair, which can repair DNA damage. However, the mechanism by which ubiquitin-specific protein 53 regulates the radiosensitivity of cervical cancer through damage-specific DNA binding protein remains unclear. **Methods:** Tissue samples from 40 patients with cervical squamous cell carcinoma who received radiotherapy were examined by immunohistochemistry to detect the expression of ubiquitin-specific protein 53, and clinical data were collected for statistical analysis. The cell cycle was detected by flow cytometry in Siha cells transfected with Si-USP53 and exposed to 8 Gy irradiation. Cell viability was determined by the CCK8 method in cells transfected with Si-USP53 and exposed to 0, 2, 4, 6, 8, or 10 Gy. The expression of damage-specific DNA binding protein, cyclin-dependent kinase 1, and cell cycle checkpoint kinase 2 was detected in cells transfected with Si-USP53. **Results:** The expression of ubiquitin-specific protein 53 in the tissues of patients with cervical squamous cell carcinoma was correlated with the sensitivity to radiotherapy. Knockdown of ubiquitin-specific protein 53 in Siha cells downregulated damage-specific DNA binding protein and caused G2/M cell cycle arrest and decreased the survival rate of cells in response to radiation. **Conclusion:** Ubiquitin-specific protein 53-induced cell cycle arrest and affected the radiotherapy sensitivity of tumors through damage-specific DNA binding protein.

Keywords

ubiquitin-specific protein 53, DNA binding protein 2, cervical cancer, radiotherapy, sensitivity

Abbreviations

CDK1, cyclin-dependent kinase 1; CHK2, cell cycle checkpoint kinase 2; DDB2, damage-specific DNA binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PI, propidium iodide; siRNA, small interfering RNA; USP53, ubiquitin-specific protein 53

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Introduction

Cervical cancer ranks fourth in both incidence and mortality among women.¹ The NCCN International Treatment Guidelines for cervical cancer indicate that advanced cervical cancer, including Federation International of Gynecology and Obstetrics (FIGO) II and above, should be treated with radiotherapy alone or neoadjuvant radiotherapy followed by radical hysterectomy.² Radiotherapy induces DNA damage, leading to tumor cell death and cell cycle arrest.³ The induction of apoptosis and inhibition of proliferation increase the sensitivity to radiation.⁴

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However, the resistance of cervical cancer to radiotherapy has become an important obstacle to the clinical treatment of this disease.

Ubiquitin-specific proteases 53 (USP53) belongs to the family of deubiquitinating enzymes, which catalyze the reversible modification of target proteins with ubiquitin and stabilize proteins.⁵ Ubiquitin-specific protein 53 is a nonprotease homolog of the USP family identified in 2003 by human genome database screening and complementary DNA (cDNA) cloning.⁶ Many homologous proteins in the USP family are associated with human diseases, such as USP33, which is involved in the invasion and metastasis of lung cancer,⁷ and USP48, which is related to human glioma cells.⁸ However, there are few reports on the involvement of USP53 in human diseases. Kazmierczak *et al* showed that mutation of the gene encoding USP53 results in progressive hearing loss in the mouse.⁹ Wenbin *et al* indicated that deregulation of USP53 in colorectal cancer is suggestive of poor prognosis.¹⁰

Damage-specific DNA binding protein 2 (DDB2) is involved in nucleotide excision repair, which can repair DNA damage, and prevent gene mutation and tumorigenesis.¹¹ Zou *et al* showed that knockdown of DDB2 expression in human lung cancer cells decreases the G2 phase and the repair efficiency of homologous recombination to increase the sensitivity of lung cancer cells to radiotherapy.¹² Damage-specific DNA binding protein has been shown to interact with USP53, although the physiological relevance of USP53–DDB2 interactions remains unclear.¹³ In this study, we knocked down USP53 to provide evidence that the relationship between USP53 and DDB2 increases the radiosensitivity of cervical squamous cell carcinoma.

Materials and Methods

Reagents and Antibodies

Anti-DDB2, anti-cyclin-dependent kinase 1 (CDK1), anti-cell cycle checkpoint kinase 2 (CHK2), and anti- β actin were purchased from Abcam. Anti-USP53 monoclonal antibody was purchased from NOVUS. Lipo3000 was purchased from Thermo Fisher. Ubiquitin-specific protein 53–small interfering RNA (siRNA) was purchased from Santa Cruz. The cell cycle kit was purchased from Beijing Sizhengbai.

Patient Samples

Follow-up data for 40 patients diagnosed with human cervical squamous cell carcinoma between January 2010 and January 2016 were regularly collected at the same hospital to assess the overall survival rate and monitor cancer metastasis and recurrence. Patient information was extracted from medical records, including age and the following parameters: tumor size, radiotherapy dose, pathological grade, and FIGO stage. Informed consent was obtained from all patients, and the study design was approved by the Research Ethics Committee.

Immunohistochemical Staining

Immunohistochemical staining was performed on 4- μ m tissue microarray sections of formalin-fixed and paraffin-embedded tissue samples, which were incubated with antibodies against USP53 (1:100) followed by biotinylated secondary antibodies for immunostaining assays. The results were scored by 2 experienced pathologists according to the 12-point method.

Cell Culture

Human Siha cells were obtained from the Cell Bank of the Chinese Academy of Sciences and grown in Roswell Park Memorial Institute-1640 supplemented with 10% fetal bovine serum in a 37 °C humidified chamber in the presence of 5% CO₂.

Cell Transfection

When the Siha cells were in the log phase of growth, they were diluted to a density of 1×10^6 cells/mL, and each well was inoculated with 1 mL of the cell suspension. When the cell density reached 40% to 50%, the cell culture medium was changed to 1640 serum-free medium for 12 hours. Then, 2.5 μ g USP53–siRNA and 5 μ L lipo3000 were diluted with 100 μ L 1640, mixed, added to the cells, and shielded from the light for 10 minutes, and the medium was changed to complete medium after 6 hours.

Cell Irradiation

After 24 hours of transfection, the cells were wrapped with a parafilm and subjected to a linear accelerator 6MV X-ray irradiation at a dose rate of 2 Gy/min, an irradiation field of 35×35 cm, a source-target distance of 100 cm, and a total irradiation dose of 8 Gy. After the end of the irradiation, cells were disinfected with alcohol and placed in the incubator to continue the cultivation.

Apoptosis Assay

Cells exposed to 24 hours of irradiation were digested by EDTA-free trypsin, washed twice with precooled PBS, and then resuspended in $1 \times$ binding buffer at a concentration of 1×10^6 cells/mL. A volume of 100 μ L of the solution (1×10^5 cells) was mixed with 5 μ L of FITC Annexin V and 5 μ L of propidium iodide (PI) and incubated for 15 minutes at room temperature in the dark, followed by flow cytometry analysis within 1 hour.

Cell Cycle Assay

Cells exposed to 24 hours of irradiation were digested and fixed with 75% alcohol at -20 °C for 24 hours. According to the number of samples, PI staining solution was prepared and 0.4 mL of staining solution was added to each sample, incubated at 37 °C for 30 minutes in the dark, and analyzed by flow cytometry.

Table 1. The Primer Sequences.

Name of primer	Sequence
USP53-F	5'-ATGGGTGTCAGATGCCAA-3'
USP53-R	5'-CTGTGCTTCGGAAGATGAGA-3'
DDB2-F	5'-TGGAGGGAACAACCTAGGCTG-3'
DDB2-R	5'-ATTCCAAAGCTCTTTGCCGTC-3'
GAPDH-F	5'-GACATCAAGAAGGTGGTGAA-3'
GAPDH-R	5'-TGTCATACCAGGAAATGAGC-3'

Abbreviations: DDB2, damage-specific DNA binding protein; USP, ubiquitin-specific proteases.

CCK8 Assay

After transfection for 24 hours, the cell suspension was diluted to a concentration of 10 cells/ μ L, and 100 μ L of the cell suspension was added to each well. The 2 groups of cells were exposed 0, 2, 4, 6, 8, or 10 Gy radiation, and after including a blank group to each culture dish, each component was divided into 5 replicate wells. At 12 hours after plating, the cells were sealed and irradiated. After treatment for 24 hours, cell viability was measured using the CCK8 kit.

Western Blot Analysis

After protein extraction, Western blotting was performed using standard protocols. The following antibodies and concentrations were used: USP53 (1:1000), DDB2 (1:1000), β -actin (1:2000), CDK1 (1:200000), and CHK2 (1:50000).

Real-Time PCR

Total RNA was extracted and reverse transcribed into cDNA. The sequences of the primers are shown in Table 1. The mRNA expression of USP53 and DDB2 in each group of cells was detected according to the instructions of the Bio-Rad IQ5TM Real-Time PCR system.

Statistical Analysis

Statistical analysis was performed using SPSS 22.0 software and the GraphPad Prism software package. All data are presented as the mean \pm standard deviation. A *t* test, χ^2 test, or Fisher exact test were used to assess the correlations between immunohistochemical expression and the clinical variables. $P < .05$ was considered statistically significant.

Results

The Expression of USP53 Is Related to the Efficacy of Radiotherapy

Immunohistochemical staining of 40 specimens from patients with cervical cancer detected positive USP53 expression in cervical cancer tissues in 10 patients. According to the clinical data of 40 patients, the expression of USP53 was not correlated with age, tumor size, tumor shape, and clinical FIGO stage

($P > .05$), whereas it was related to the response to radiotherapy ($P = .048$). Response time was defined from the start of radiation therapy to the end of radiation therapy. The difference response time with curative effects is not statistically significant. The curative effect was determined jointly by the 2 experienced clinicians according to the radiotherapy curative effect evaluation process based on the results of computed tomography and magnetic resonance imaging examinations after treatment (complete response [CR]) was defined as complete disappearance of the tumor, partial response (PR) was tumor shrinkage to $\geq 50\%$, stable disease (SD) was tumor shrinkage to $< 50\%$). In these 40 patients, there was no correlation between the efficacy of radiotherapy and clinical stage, which may be due to the small sample size, and most of the patients were stage II patients (Figure 1, Table 2).

After a period of regular radiotherapy and chemotherapy, the curative effect (NV, PR, CR) is evaluated by the clinician based on the test results and clinical symptoms.

Knocking Down the Expression of USP53 Increases the Proportion of Siha Cells in the G2/M Phase of the Cell Cycle During Radiotherapy

Because tumor cells are more sensitive to radiation in the G2/M phase, promoting G2/M phase arrest increases the sensitivity of tumor cells to radiation.¹⁴ After knocking down the expression of USP53, cells were exposed to irradiation at 8 Gy, and cell cycle progression was detected by flow cytometry. The results showed that the number of cells in the G2/M phase increased in response to 8 Gy radiation + Si-USP53, whereas the difference between the control group and the 8 Gy group was not statistically significant (Figure 2). These results suggested that USP53 caused Siha cells to accumulate in the G2/M phase postirradiation.

The Survival Rate Was Lower in the Si-USP53 Group Than in the Control Group After Treatment With Different Doses of Radiation

After counting the cells transfected with Si-USP53 and CON, which were inoculated into the 96-well plate at a density of 1×10^3 cells/well, cells were divided into 0, 2, 4, 6, 8, and 10 Gy groups, and each group of cells was plated into 5 duplicate wells. The cells were sealed and treated with radiation after they were attached, and then further cultured for 24 hours. For the CCK8 assay, the survival rate was calculated using the following formula: survival rate = [(experimental well absorbance – blank well absorbance)/(control well absorbance – blank well absorbance)] $\times 100\%$. The cell survival rate of the Si-USP53 group was lower than that of the CON group (Figure 3).

Knockdown of the Expression of USP53 Downregulates DDB2

Ubiquitin-specific protein 53 was shown to bind to DDB2 by immunoprecipitation.¹³ We showed that knockdown of USP53

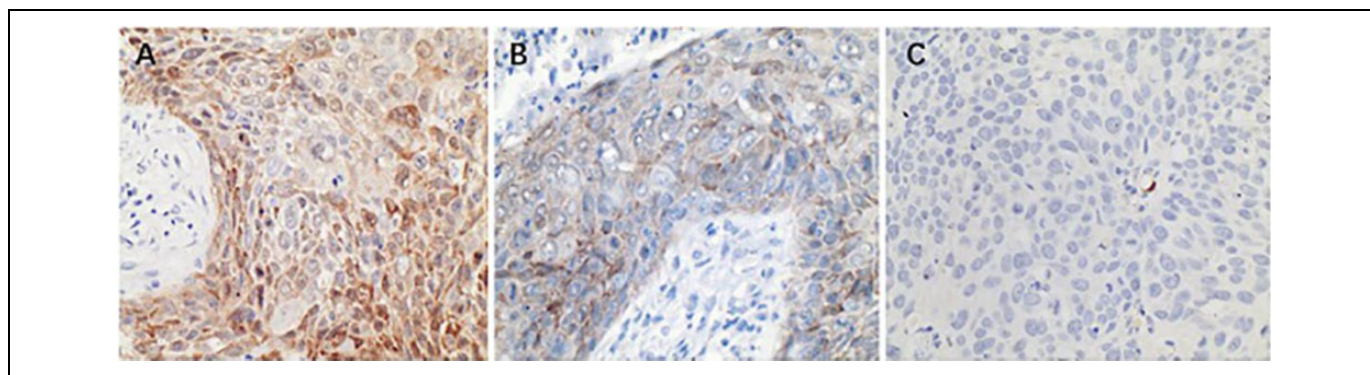


Figure 1. Immunohistochemical micrographs of USP53 expression in cervical cancer ($\times 400$); (A) high, (B) middle, and (C) negative. USP53, ubiquitin-specific protein 53.

Table 2. Relationship Between USP53 Expression, the Response to Radiotherapy, and Clinicopathological Parameters.

Characteristics	Patients (n = 40)	USP53 expression positive (n = 10)	USP53 expression negative (n = 30)	P^a
Age (years)				
≤ 50	22	6	16	.503
50	18	4	14	
Tumor diameter				
<4	9	3	6	.399
≥ 4	31	7	24	
FIGO stage				
II	37	9	28	.589
III + IV	3	1	2	
Tumor shape				
Endogenous type	13	9	4	.414
Exogenous type	27	21	6	
Radiation sensitivity				
NV	4	3	1	.048
PR	32	7	25	
CR	4	0	4	

Abbreviations: CR, complete response; FIGO, Federation International of Gynecology and Obstetrics; PR, partial response; USP53, ubiquitin-specific protein 53.

^a P value was estimated by Fisher exact test.

downregulated the mRNA and protein expression of DDB2 (Figure 4A, Figure 4B). Ubiquitin-specific protein 53 is a member of the ubiquitin-specific protease family, and because of the lack of histidine residues, the members show differences in the catalytic domain.⁶ Therefore, reversing ubiquitination does not stabilize DDB2 but reduces the expression of DDB2.

Knockdown of USP53 Reverses the Upregulation of DDB2 Caused by Radiation, thus Reducing the DNA Repair Ability

After radiation treatment, the expression of DDB2 in the cells of the CON group was increased, and DNA repair was initiated.

In the Si-USP53 group, the expression of DDB2 was inhibited after radiation (Figure 4A), thus reducing the ability to repair DNA and increasing the sensitivity to radiation.

Knockdown of USP53 Expression Reduces the Expression of CDK1 and Increases Cell Cycle-Associated Protein Expression After Radiation

The levels of CDK1 (Figure 5B) and CHK2 (Figure 5A) in the CON +8 Gy group and the Si-USP53+ 8 Gy group increased after radiation treatment, indicating that knockdown of USP53/DDB2 may cause cell cycle arrest at the G2/M phase by regulating G2/M phase -associated proteins, thereby affecting the sensitivity of cells to radiation

Discussion

Radiotherapy is beneficial in the treatment of cervical cancer and is one of the main treatment methods for patients with cervical cancer¹⁵; however, radiotherapy resistance is associated with poor prognosis in cervical cancer. The reasons for radiotherapy resistance include DNA repair enhancement,¹⁶ cell cycle arrest,¹⁷ and gene variation.¹⁸

Ubiquitin-specific proteases have become a hot topic; however, USP53 was identified recently and there are few reports on this protein in the literature. Here, we investigated the biological function and radiotherapy sensitization mechanism of USP53, which belongs to the ubiquitin-specific protease family.

In this study, 40 biopsy specimens from patients with cervical squamous cell carcinoma were collected to prepare tissue microarrays, and the clinical data of patients were collected. The results showed that positive expression of USP53 in tissues was associated with the sensitivity to radiotherapy. In a study of USP24, USP53 was shown to bind to DDB2, although the specific mechanism of action was not elucidated. Damage-specific DNA binding protein is a nucleic acid repair protein that is closely related to radiotherapy resistance.¹⁹ Therefore, we performed cell experiments to verify that the expression of USP53 was positively correlated with the expression of DDB2,

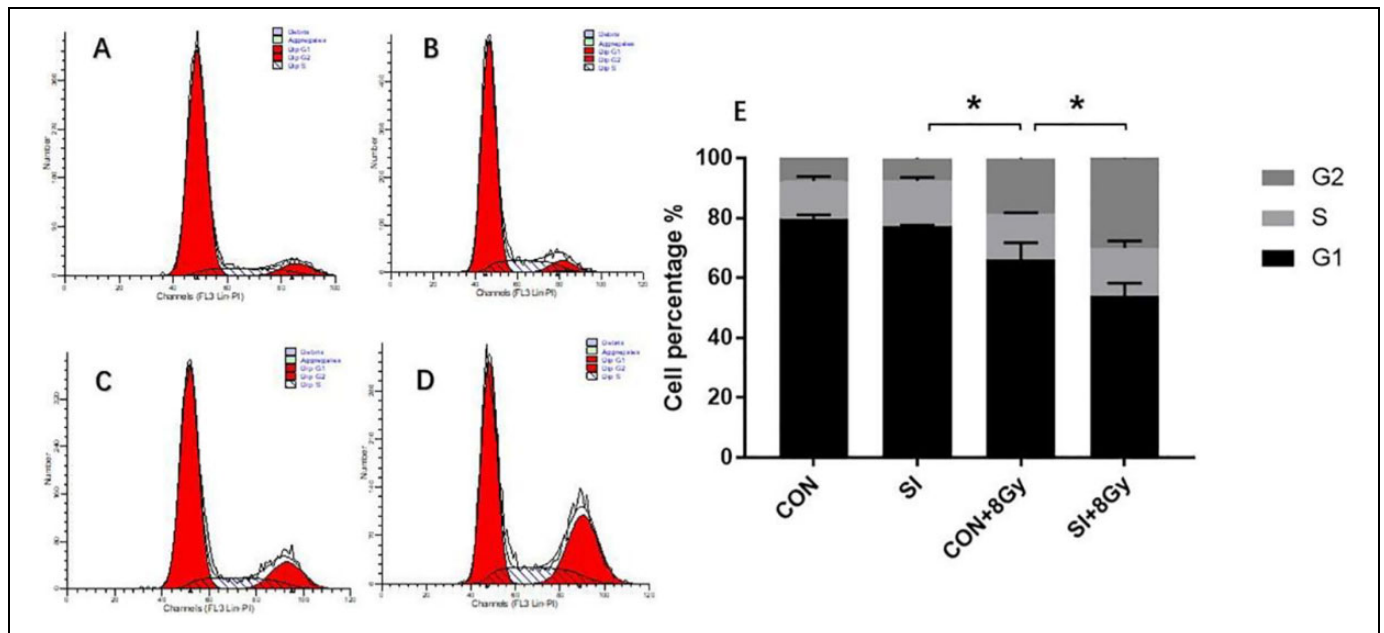


Figure 2. Flow cytometry was performed to detect the effect of knockdown of ubiquitin-specific protein 53 (USP53) in cervical cancer cells on cell cycle progression after radiotherapy. (A) CON group; (B) Si-USP53 group; (C) CON + 8 Gy group; (D) Si-USP53 + 8 Gy group; (E) statistical histogram (* $P > .05$). USP53, ubiquitin-specific protein 53.

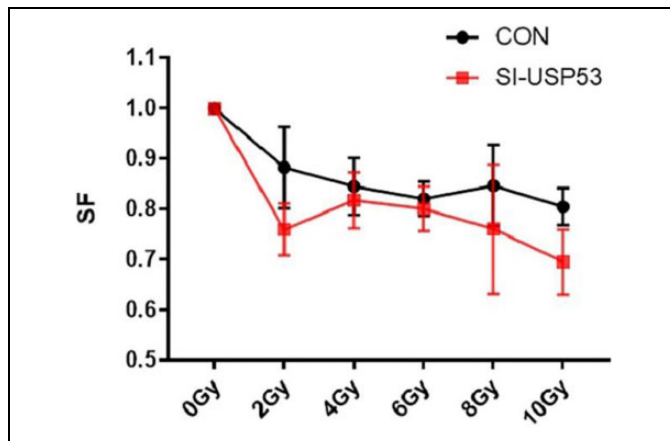


Figure 3. Cell survival rate calculated by CCK8 after 0, 2, 4, 6, 8, 10 Gy of irradiation.

and the results suggested that USP53 regulates the expression of DDB2 and the sensitivity to radiotherapy. Knockdown of USP53 expression induced G2/M phase arrest in tumor cells, which increased the sensitivity of cells to radiotherapy. When the cells are in the G2/M phase, they are close to the mitotic phase, and they are therefore more sensitive to radiation.²⁰ Studies have suggested that when tumor cells undergo cell cycle arrest after irradiation, the checkpoint repair phase of the cell cycle is initiated, and the repair of damaged DNA increases radiation resistance.²¹ However, in the DNA checkpoint repair process, there may be repair failure and cell death.²²

In this study, the CCK8 assay was performed to assess the survival rate of cells treated with different doses of radiation

after USP53 knockdown, and the results showed that the survival rate of cells with USP53 knockdown was lower than that of the control group at different doses. We further investigated the mechanism underlying radiotherapy sensitization induced by USP53 knockdown and found that DDB2 was downregulated by USP53 knockdown, whereas the expression of DDB2 in the CON group increased after radiation treatment, suggesting an increase in intracellular DNA repair. Damage-specific DNA binding protein was not upregulated in the Si-USP53 + 8 Gy group, indicating that knockdown of USP53 not only downregulated DDB2 expression but also reversed the increase in DDB2 caused by radiation and reduced DNA nucleic acid repair. Moreover, changes in the cell cycle-associated proteins CDK1 and CHK2 induced by G2/M phase arrest eventually lead to cell death, increasing the sensitivity of tumor cells to radiotherapy. In studies of the ubiquitin-specific protease family, USP53 is included in the nonprotease homolog class of USPs because of the lack of essential His residues. Ubiquitin-specific protein 53 may have different biological functions than the ubiquitinated protease family and may not only affect the radiosensitivity of tumors by affecting DDB2 but also deubiquitinate proteins with other important functions. Therefore, USP53 may become a new research target for tumor radiosensitization in the future.

Conclusions

Our research confirms that the expression of USP53 in some patients with cervical cancer may be related to its radiotherapy efficacy. Knockdown of USP53/DDB2 in Siha cells caused cell cycle arrest and changes in the cell cycle-associated proteins CDK1 and CHK2, thus affecting the radiosensitivity of tumors.

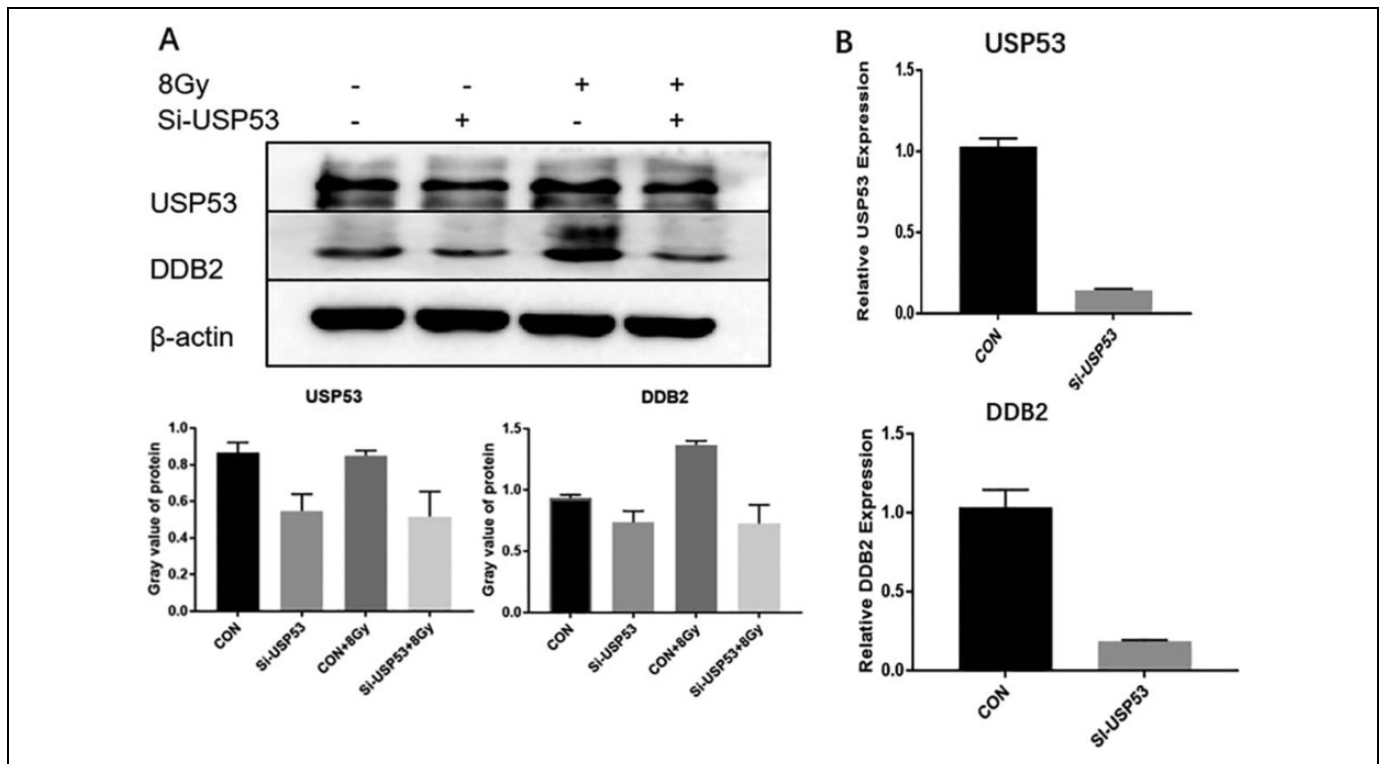


Figure 4. (A) Protein expression of ubiquitin-specific protein 53 (USP53) and damage-specific DNA binding protein (DDB2); (B) mRNA expression of USP53 and DDB2 ($*P > .05$).

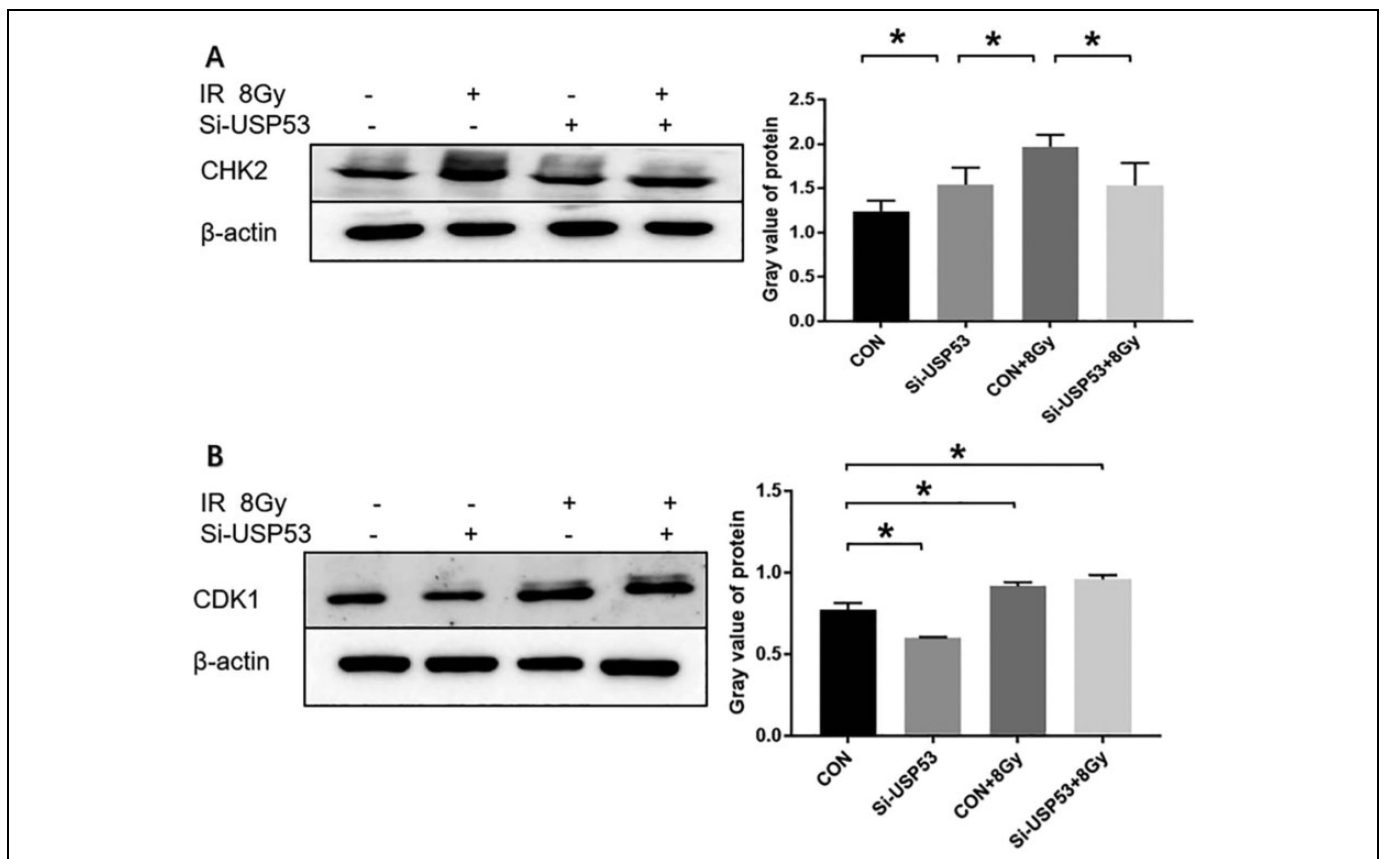


Figure 5. (A) Expression of the cell cycle-related proteins cell cycle checkpoint kinase 2 (CHK2); (B) Expression of the cyclin-dependent kinase 1 (CDK1; $*P > .05$).

Authors' Note

The study design was approved by the Research Ethics Committee of The Second Affiliated Hospital of Fujian Medical University (2019,164), Quanzhou, China.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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