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53BP1 regulates cell cycle arrest in esophageal cancer model

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Abstract. – OBJECTIVE: This study aims to investigate effects of checkpoint kinase, mediator of DNA damage checkpoint 1 (MDC1) and p53-binding protein 1 (53BP1) silencing on p53, checkpoint kinase 1 and 2 (CHK1 and CHK2), and CHK2-T68 expression.

MATERIALS AND METHODS: Eca109 cells were divided into untransfected Eca109, Blank-vector, MDC1-RNAi transfection, and 53BP1-RNAi transfection group. Streptavidin-peroxidase (SP) immunohistochemical assay was used to examine CHK2-T68 expression. About 4 groups were used to establish esophageal carcinoma nude-mouse models, and assigned as Eca-109 control (or Eca-109 plus 15 Gy γ-rays irradiation, Eca-109+IR), Blank-vector (or Blank-vecor+IR), 53BP1-RNAi (or 53BP1-RNAi+IR), and MDC1-RNAi group (or MDC1-RNAi+IR group) by injecting. The expression of p53, CHK1, CHK2 were evaluated using SP immunohistochemical assay.

RESULTS: 53BP1 and MDC1 down-regulation significantly inhibited expression of CHK2-T68 in Eca-109 cells compared to untreated group ($p<0.05$). There were significant differences for CHK2-T68 expressions in different time and groups ($p<0.05$). 53BP1 down-regulation significantly reduced p53 and enhanced CHK1 and CHK2 expression compared to that of Eca-109 control group ($p<0.05$) in Eca-109 cells. 53BP1 down-regulation significantly regulated CHK1, CHK2, and p53 in xenograft nude mice models exposed to γ-ray irradiation compared to that of untreated group ($p<0.05$). p53 was negatively correlated with CHK1 and CHK2 in xenograft nude mice models.

CONCLUSIONS: 53BP1 regulated the cell cycle arrest by modulating p53, CHK1, and CHK2 expression in both Eca-109 cells and xenograft nude mice models.

Key Words:
Esophageal carcinoma, CHK1, CHK2, p53, CHK2-T68.

Introduction

Esophageal cancer is considered as a most prevalent human cancer worldwide. It shows a poor survival rate and higher aggressive characteristics[1,2]. Esophageal cancer is also the eighth most prevalent cancer and sixth leading risk factor for cancer-associated death in the whole world[3]. In China, esophageal cancer mainly distributes and with a higher incidence in Northern and Western China, and the incidence rates of which are 3-4 folds higher in men compared to that in women[4,5]. The International Agency for Research on Cancer reported that China is the most higher esophageal cancer incidence country, which characterizes with more than 50% morbidity out of the world[6]. Due to histological classification of the cancer tissues, the human esophageal cancer is divided into adenocarcinoma and squamous cell carcinoma (which represents about 90% of cases of esophageal cancer throughout the world)[7]. The 5-year survival rate of esophageal cancer is relatively lower compared to the other cancers, ranging from 15% to 25% in different countries or different populations, because of the advanced-stages of esophageal cancer and the malignant characteristics[8,9]. Therefore, the effective treatments, higher sensitivity to radiotherapeutic or chemotherapeutic drugs, enhanced local control rates of tumor, and reduced recurrence rates for the esophageal cancer are an urgent need. Clinically, the most frequently used method for esophageal cancer is surgery combined with the chemotherapy with or without the radiotherapy for a few serious patients[10]. The cell cycle is closely associated with the sensitivity of radiotherapy, and the cell cycle checkpoint strictly controls the process of the cell cycle[11,12]. The radiotherapy always cau-

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ses the DNA damage and the cells repair the damaged DNA by activating cell cycle checkpoint signaling pathway and stopping the cell cycle to keep genome stability and fidelity of chromosome inheritance[13]. The previous studies[14,15] reported some molecules that participate in the repair processes of DNA damage, among which the p53-binding protein 1 (53BP1) and mediator of DNA damage checkpoint 1 (MDC1) play critical roles in the early stage of esophageal cancer. Both of the cell cycle checkpoint kinase 1 and 2 (CHK1 and CHK2) are the extreme conservative kinases in biological evolution process[16]. The CHK1 and CHK2 play important roles in the DNA damage associated signal transduction, but without regulative effects on the normal cells. After the DNA damage caused by radiotherapy, both MDC1 and 53BP1 could transmit the DNA damage signals to the down-stream molecules, such as CHK1 and CHK2. The activated CHK2 molecule could modulate the cell cycle checkpoints, G1/S, S, G2/M phage, and improve the repair of damaged DNA[17]. Moreover, the p53 gene is also correlated with the process of cell cycle and acts as a functional "checkpoint" in the radiation which caused cell G1-phage delay and DNA repair[18]. Therefore, CHK1, CHK2, and p53 participate in the modulation of the cell cycle and the regulation of the cell cycle checkpoints. The cell cycle arrest is a common and direct response for most tumor cells suffering from radiation[19]. We expected that blocking the cell cycle arrest and inhibiting the repair of damaged DNA could enhance the killing effects of radiation on tumor cells and increase the sensitivity of radiotherapy. The previous studies[20,21] also reported that suppressing the expression of MDC1 and 53BP1 could block the activation of CHK1 and CHK2, and finally inhibit the cell cycle arrest and increase the radiotherapeutic sensitivity. Therefore, we silenced the MDC1 and 53BP1 expression by using the RNA interference technique (RNAi), in the human esophageal cancer cell line, Eca109, and observed expression of CHK2-T68. Meanwhile, we examined the CHK1, CHK2, and p53 expressions in the squamous cell carcinoma Xenografts in nude mice.

Materials and Methods

Cell Culture and Grouping

The Eca109 cells were provided by the Research Center of Forth Clinical Hospital of Hebei Medical University (Shijiazhuang, China). Eca109 cells were cultured in Roswell Park Memorial Institute-1640 (PRMI-1640), Sigma-Aldrich (St. Louis, MO, USA) supplementing with heat-inactivated fetal bovine serum (FBS, 100 ml/l, TBD Biotechnology. Dev., Tianjin, China), 100 U/ml penicillin (North China Pharmaceutical Group, Shijiazhuang, China) and 100 μg/ml streptomycin (North China Pharmaceutical Group) at 37°C in a humidified incubator with 5% CO^2. The above Eca109 cells were divided into 4 groups, including untransfected Eca109 group, Blank vector group (the Eca109 cell were transfected with blank liposome), MDC1 RNAi transfected group (Eca109 cells were transfected with MDC1 RNAi), and 53BP1 RNAi transfected group (Eca109 cells were transfected with 53BP1 RNAi). Then, the Eca109 cells in every group were exposed to 5 Gy γ-ray, which was delivered by a 60Co γ-ray linear-accelerator (Mode: FCC-8000C, Shinva Med. Co. Ltd., Zibo, China) at the final dosage rate of 110 Gy/min. The cells were washed with phosphate-buffered saline (PBS) and treated with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) at 0.5 h, 1 h, 2 h, 4 h, and 12 h after the exposure of γ-ray, respectively. Finally, the Eca109 cells were stored at -80°C for the following experiments.

Mice Model Establishment and Grouping

The 4-6 week-old female BALB/C nude mice (n=48, certificate No. SCXK20050013) were purchased from Institute of Medical Laboratory Animals of Chinese Academy of Medical Sciences, Beijing, China. All of these mice were fed in the specific pathogen free (SPF) conditions and housed at the Fourth Hospital of Hebei Medical University, Shijiazhuang, China. To establish the xenograft nude mice model, a total of 1×106 Eca-109 cells were re-suspended in the sterile PBS (100 μl) and were subcutaneously injected into the left flank of 24 nude mice for Eca-109 control group (injected with Eca-109 cells only), Blank vector group (injected with blank liposome transfected Eca-109 cells), 53BP1 RNAi group (injected with the 53BP1 RNAi transfected Eca-109 cells), and MDC1 RNAi group (injected with the MDC1 RNAi transfected Eca-109 cells), respectively. Other 24 nude mice were randomly divided into Eca-109 plus γ-ray irradiation group (Eca-109 + IR group, injected with Eca-109 cells and exposed toγ-ray irradiation), Blank vector plus γ-ray irradiation group (Blank vector + IR group, injected with blank liposome transfected

Eca-109 cells and exposed to γ-ray irradiation), 53BP1 RNAi plus γ-ray irradiation group (53BP1 + RNAi group, injected with the 53BP1 RNAi transfected Eca-109 cells and exposed to γ-ray irradiation), and MDC1 RNAi plus γ-ray irradiation group (MDC1 RNAi + IR group, injected with the MDC1 RNAi transfected Eca-109 cells and exposed to γ-ray irradiation), respectively. All of the cell experiments and animal studies were approved by the Ethical Committee of the Fourth Hospital of Hebei Medical University (Shijiazhuang, China).

Tumor Growth Observation, Sample Collection and Preparation

The tumor growth was monitored and observed every day. For the mice in the Eca-109 control, Blank vector group, 53BP1 RNAi group, and MDC1 RNAi group, when the tumor diameter of xenografts reached 0.5 cm ×0.5 cm in size, the mice were killed, and the tumor tissues were fixed in the 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA), deparaffinized and cut into sections of 4 mm thickness. For the mice in Eca-109 + IR, Blank vector + IR, 53BP1 RNAi + IR, and MDC1 RNAi + IR group, the mice were exposed to 15 Gy γ-ray for 1 h when the tumor diameter of xenografts reached 0.5 cm ×0.5 cm in size. The tumor tissues were fixed in the 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA), deparaffinized and cut into sections of 4 mm thickness for the following experiments.

Immunohistochemical Staining

The above sections in above groups were incubated with the 3% hydrogen peroxide (Beyotime Biotechnology, Shanghai, China) for 15 min to block the activity of the endogenous antigen, and washed with PBS for 3 times. The sections were treated with the rabbit anti-mouse p53 polyclonal antibody (Cat. No. PR-0256, ZSGB. Bio., Beijing, China), rabbit anti-mouse CHK1 polyclonal antibody (Cat. No. sc-7898, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-mouse CHK2 polyclonal antibody (Cat. No. sc-9064, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-mouse CHK2-T68 monoclonal antibody (Cat. No. #2179, Cell Signaling Technology Inc., Beverly, MA, USA), and then, treated with horseradish peroxidase (HRP)-labeled goat anti-rabbit antibody (Cat. No. ZDR-5306, ZSGB. Biology, Beijing, China). The incubated sections were visualized by using the Biotin-Streptavidin HRP Detection system (Cat. No. SP-9001, ZSGB. Biology, Beijing, China), and counter-stained by using the hematoxylin (ZSGB. Biology, Beijing, China). Finally, the images for the sections were analyzed by using the ImagePro Plus 6.0 software (Media Cybernetics, Inc., Bethesda, MD, USA).

Statistical Analysis

Data were described as mean ± standard deviation (SD) and analyzed with SPSS software 13.0 (SPSS Inc., Chicago, Ull, USA). All of the data were obtained from at least three independent experiments. The Student's *t*-test was used for statistical analysis between the two groups. The Tukey's post-hoc test was employed to validate the analysis of variance (ANOVA) for comparing measurement data among multiple groups. A statistical significance was defined when $p<0.05$.

Results

53BP1 and MDC1 Down-Regulation Inhibited Expression of CHK2-T68 in Eca-109 Cells

At 0.5 h and 1 h after the irradiation, the CHK2-T68 expressions in every group were increased compared to 0 h after the γ-ray irradiation (Table I). Meanwhile, the CHK2-T68 expressions at 2 h and 4 h after the γ-ray irradiation were significantly decreased compared to 1 h post γ-ray irradiation in every group (Table I, $p<0.05$). Therefore, the CHK2-T68 achieved the peak value in every group. The CHK2-T68 expressions in both MDC1 RNAi and 53BP1 RNAi group were significantly decreased compared to the untransfected Eca-109 group at 0.5 h, 1 h, 2 h, and 4 h after the γ-ray irradiation (Table I, $p<0.05$). However, the CHK2-T68 expressions in 53BP1 RNAi group were also significantly decreased compared to that in MDC1 RNAi group from 0.5 h to 4 h after the γ-ray irradiation (Table I, $p<0.05$).

There Were Significant Differences for CHK2-T68 Expressions in Different Time and Groups

Our statistical analysis results showed that the there were significant differences for the CHK2-T68 expressions among the 0.5 h, 1 h, 2 h, and 4 h after the irradiation (Table II, Table III, $p<0.05$). Meanwhile, there were also significant differences for CHK2-T68 expressions among the untransfected Eca-109 group, Blank vector group, MDC1 RNAi transfected group, and 53BP1 RNAi transfected group (Table IV, Table V, $p<0.05$).

Table I. The expression of CHK2-T68 protein after irradiation in each group

Group	0	0.5 h	1 h	2 h	4 h	12 h
Untransfected Eca109 group	7±3.08	72±9.54•	90±11.31	79±12.08•	55±11.27§	6±2.64
Blank vector group	8±3.31	72±10.17•	89±14.40	76±11.76•	53±8.69§	6±2.04
MDC1 RNAi transfected group	6±2.16	55±8.91*•	72±10.78*	61±9.54*•	42±6.63*§	5±1.75
53BP1 RNAi transfected group	6±2.17	40±9.30*°•	49±10.37*°	41±10.80*°•	29±11.20*°§	6±2.16

*p<0.05 vs. control Eca109 at the same time point, °p<0.05 *vs.* MDC1-/-Eca109 at the same time point, •p<0.05 *vs.* 1 h at the same group, §p<0.05 *vs.* 2 h at the same group.

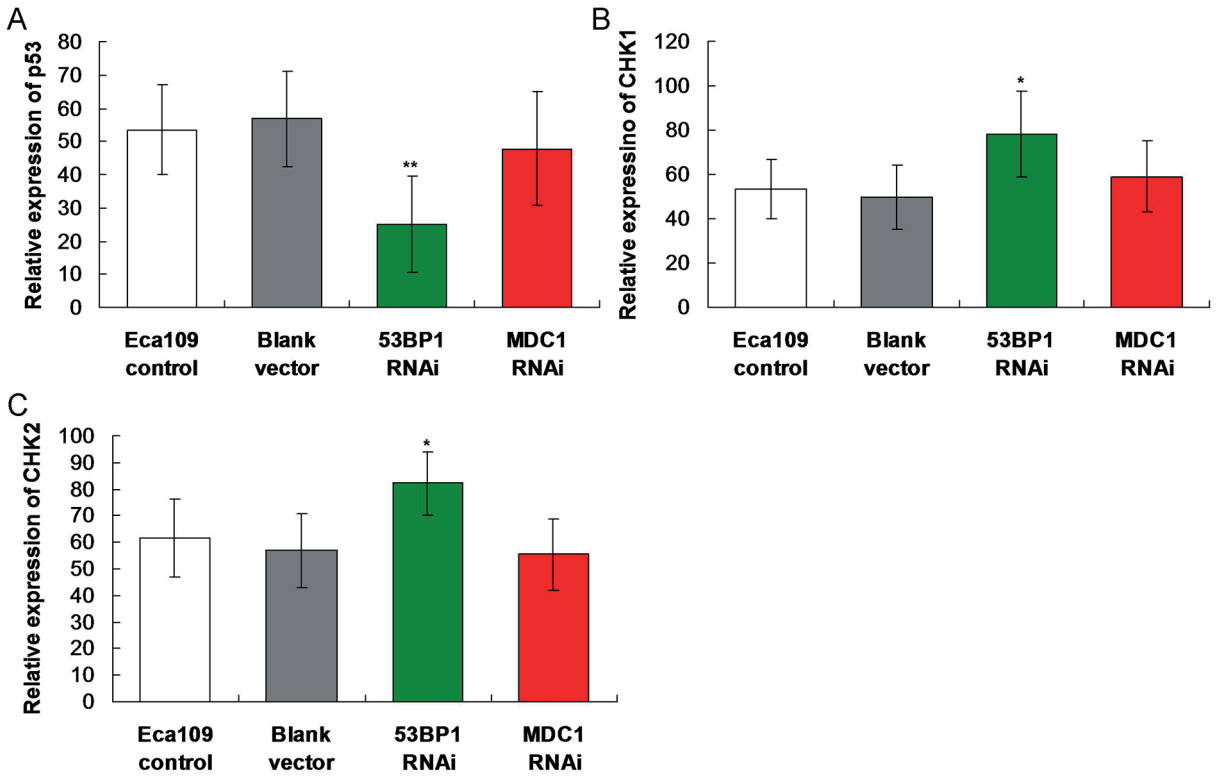

Figure 1. Expression of p53, CHK1, and CHK2 protein in the un-irradiated groups. ***A,*** Statistical analysis for p53 expression in Eca-109 control, Blank vector, 53BP1 RNAi, and MDC1 RNAi group. ***B,*** Statistical analysis for CHK1 expression in Eca-109 control, Blank vector, 53BP1 RNAi, and MDC1 RNAi group. ***C,*** Statistical analysis for CHK2 expression in Eca-109 control, Blank vector, 53BP1 RNAi, and MDC1 RNAi group. *p<0.05, **p<0.01 *vs.* Eca-109 control group.

53BP1 Down-Regulation Reduced p53 and Enhanced CHK1 and CHK2 Expression

To observe the effects of 53BP1 RNAi and MDC1 RNAi treatments on the p53, CHK1, and CHK2, the expression of which were examined in xenograft nude mice models (un-exposed to γ-ray irradiation) by using immunohistochemical staining. The results showed that 53BP1 RNAi injection significantly reduced the expression of p53 compared to that of Eca-109 control group (Figure 1A, p<0.01). The 53BP1 RNAi injection also significantly enhanced the expression of CHK1 (Figure 1B) and CHK2 (Figure 1C) compared to that of Eca-109 control group (p<0.05). However, there were no significant effects of MDC1 RNAi injection on the p53, CHK1, and CHK2 expression in xenograft nude mice models (Figure 1).

53BP1 Down-Regulation Regulated CHK1, CHK2, and p53 in Xenograft Nude Mice Models Exposing to γ-ray Irradiation

In this study, the effects of 53BP1 and MDC1 down-regulation on the γ-ray irradiation sti-

Table II. Main effect of time and crossover effect of time and grouping factor (Greenhouse-Geisser).

Source	df	SS	MS	F	p
Time	1.402	106597.222	76015.531	938.562	0.000
Time*grouping	4.207	6251.994	1486.108	18.349	0.000
Deviation (time)	28.046	2271.500	80.991		

mulating CHK1, CHK2, and p53 expression in xenograft nude mice models were also evaluated. The data indicated that the 53BP1 RNAi injection significantly decreased p53 expression in xenograft nude mice models exposed to γ-ray irradiation, compared to that of Eca-109 control group (Figure 2A, $p<0.05$). Meanwhile, the 53BP1 RNAi injection significantly increased the CHK1 (Figure 2B) and CHK2 (Figure 2C) expressions in xenograft nude mice models exposed to γ-ray irradiation, compared to that of Eca-109 control group ($p<0.05$). However, there were no significant effects of MDC1 RNAi injection on p53, CHK1, and CHK2 expression in xenograft nude mice models exposed to γ-ray irradiation (Figure 2).

p53 Levels Negatively Correlated with CHK1 and CHK2 Levels

The correlations between p53 levels in xenograft nude mice models and CHK1 or CHK2 levels were also evaluated. The results indicated that the p53 levels were negatively correlated with the CHK1 levels in xenograft nude mice models (Figure 3A, $p<0.05$). Meanwhile, the p53 levels were also negatively correlated with levels of CHK2 in xenograft nude mice models (Figure 3B, $p<0.05$).

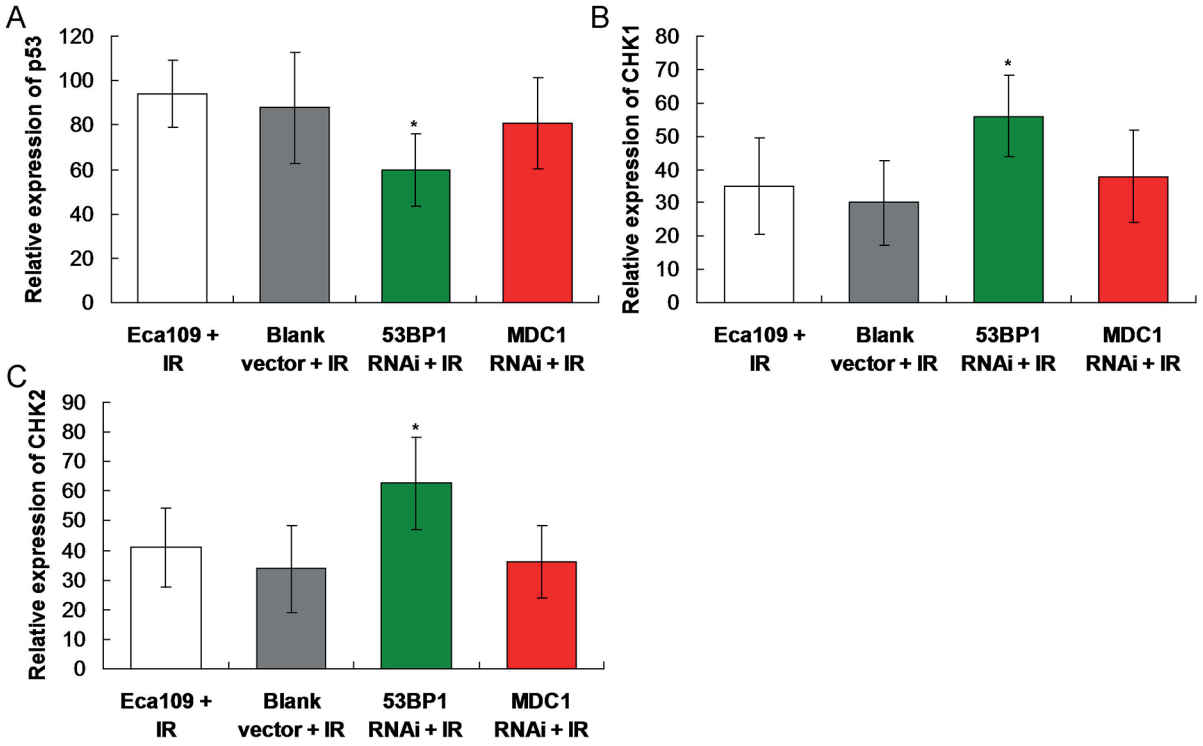

Figure 2. Expression of p53, CHK1, and CHK2 protein in irradiated groups. ***A,*** Statistical analysis for p53 expression in Eca-109 control, Blank vector, 53BP1 RNAi, and MDC1 RNAi group. ***B,*** Statistical analysis for CHK1 expression in Eca-109 control, Blank vector, 53BP1 RNAi, and MDC1 RNAi group. ***C,*** Statistical analysis for CHK2 expression in Eca-109 control, Blank vector, 53BP1 RNAi, and MDC1 RNAi group. $^{*}p<0.05$, $^{**}p<0.01$ *vs.* Eca-109 control group.

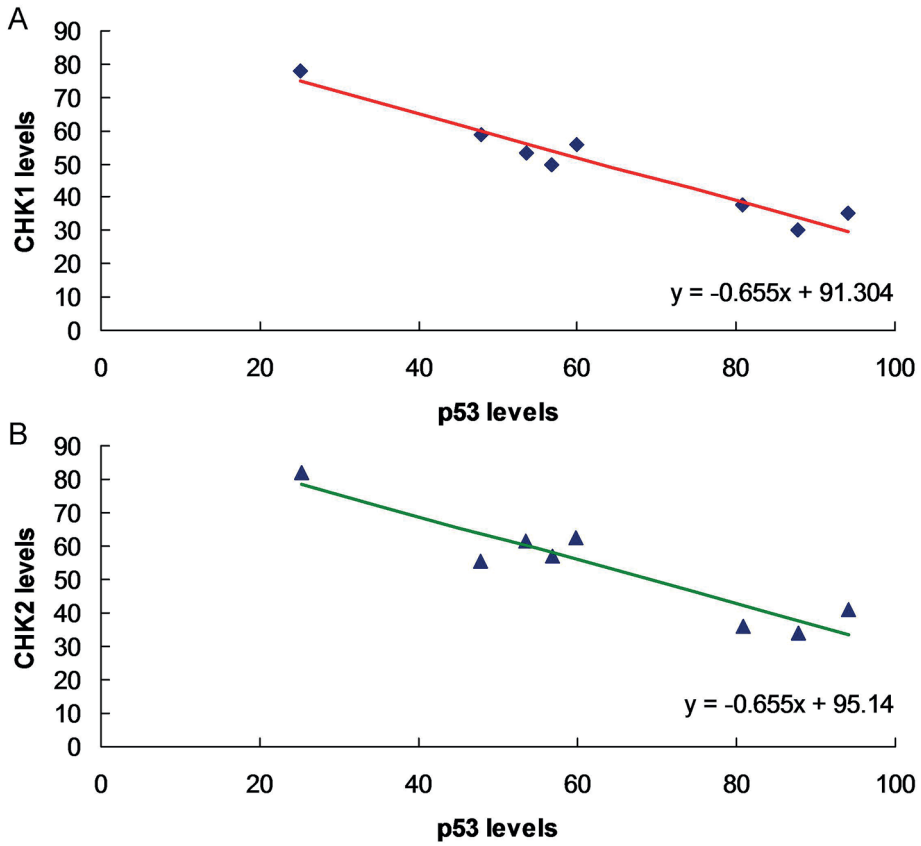

Figure 3. Analysis of the correlations between p53 levels and CHK1 or CHK2 expression. ***A,*** Correlation between p53 and CHK1 expression. ***B,*** Correlation between p53 and CHK2 expression.

Discussion

The MDC1 and 53BP1 are the critical molecules involving in the DNA damage and DNA replication in many cancers, such as esophageal cancer[22]. In the present work, we proved that 53BP1 and MDC1 down-regulation inhibited expression of CHK2-T68 in Eca-109 cells. Meanwhile, 53BP1 down-regulation reduced p53 and enhanced CHK1 and CHK2 expression in xenograft nude mice models treated with or without γ-ray irradiation. Actually, the cell cycle arrest is the most common discipline for most tumor cells suffering from radiation or radiotherapy treatment[19]. In clinic, the cell cycle arrest inhibits or reduces the therapeutic effects of the radiotherapy on the tumor, therefore, suppressing the cell cycle arrest, it could significantly enhance the killing function of the radiation. We employed the RNA interfere

Table III. Multiple comparisons among the levels of the time factor.

(I) Time	(J) Time	Mean difference (I-J)	Std. Error	*p*
0 h	0.5 h	-53.250*	1.473	0.000
	1 h	-68.208*	1.947	0.000
	2 h	-57.542*	1.780	0.000
	4 h	-38.292*	1.515	0.000
	12 h	0.625	0.328	0.071
0.5 h	1 h	-14.958*	0.650	0.000
	2 h	-4.292*	0.532	0.000
	4 h	14.958*	0.821	0.000
	12 h	53.875*	1.604	0.000
1 h	2 h	10.667*	0.599	0.000
	4 h	29.917*	1.066	0.000
	12 h	68.833*	2.071	0.000
2 h	4 h	19.250*	0.939	0.000
	12 h	58.167*	1.926	0.000
4 h	12 h	38.917*	1.592	0.000

*means significant difference, $p<0.05$.

Table IV. Main effect of grouping factor (test of Between-Subjects effect).

Source	df	SS	MS	F	*p*
Intercept	1	260950.694	260950.694	757.458	0.000
Processing	3	12766.472	4255.491	12.352	0.000
Deviation	20	6890.167	344.508		

Table V. Multiple comparisons among the levels of group factor.

(I) Group	(J) Group	Mean difference (I-J)	Std. error	p
Untransfected Eca109 group	Blank vector group	0.889	4.375	0.841
	MDC1 transfected group	11.528*	4.375	0.016
	53BP1 transfected group	23. 194*	4.375	0.000
Blank vector group	MDC1 transfected group	10.639*	4.375	0.025
	53BP1 transfected group	22.306*	4.375	0.000
MDC1 transfected group	53BP1 transfected group	-11.667*	4.375	0.015

*means significant difference, $p<0.05$.

technology to block the expression of MDC1 and 53BP1 in Eca-109 cells or in xenograft nude mice models treated with or without γ-ray irradiation. The RNAi is kind of approach for silencing the post-transcriptional genes and has been discovered in plenty of eukaryotes[23]. The RNAi could always be applied for silencing the specific genes, inducing the apoptosis, suppressing the tumor cell proliferation, and enhancing the sensitivity to chemotherapy or radiotherapy[24]. In this study, we synthesized the combined double-stranded RNA targeting the specific MDC1 and 53BP1 gene sequence, which were transfected into the Eca-109 cells or injected to xenograft nude mice models.

Bartkova et al[25] reported that the phosphorylation of CHK2-T68 occurred in the tissues of invasive breast cancer, colon carcinoma, and lung cancer. Our results showed that the 53BP1 and MDC1 down-regulation inhibited expression of CHK2-T68 in Eca-109 cells. Also, the effects of 53BP1 silencing were significant than the MDC1 silencing, which suggests that the mechanisms are different and needed to be clarified in the future research. Therefore, inhibiting the expression of 53BP1 and MDC1 could cause the G2/M checkpoint deficiency and enhance the sensitivity to the radiotherapy. p53 could always be activated when the cells suffered from cytotoxic stress, such as DNA damage[26]. The activated p53 induces cell cycle arrest, allowing the damaged cells to be eliminated[27]. In this work, we found that the 53BP1 down-regulation significantly reduced the p53 expression in xenograft nude mice models treating with or without γ-ray irradiation. This result suggests that the 53BP1 RNAi treatment could inhibit the cell cycle arrest and decrease the sensitivity to radiotherapy. The CHK1 and CHK2 play important roles in the DNA damage associated signal transduction, but without regulative effects on the normal cells. Scholars[20,28] reported that suppressing the expression of MDC1 and 53BP1 could inhibit cell cycle arrest and increase radiotherapeutic sensitivity. In this study, we examined the CHK1 and CHK2 expressions in MDC1 RNAi or 53BP1 RNAi injected xenograft nude mice models treating with or without γ-ray irradiation. The results showed that 53BP1 down-regulation significantly increased the expression of CHK1 and CHK2, which suggest that the silencing of MDC1 and 53BP1 significantly decreased the sensitivity of radiotherapy. Moreover, our findings also illustrated that there were no effects of MDC1 silencing on the CHK1 and CHK2 expression, which suggest that MDC1 doesn't play the regulative roles in CHK1 and CHK2 expression in the animal models. According to the changes of p53, CHK1, and CHK2, we found that the down-regulation of p53 may induce the expression of both CHK1 and CHK2 in the xenograft nude mice models. Our results are consistent with the previous studies[29,30] that reported the correlation between p53 and the CHK proteins. We also investigated the correlation between p53 and CHK1 or CHK2 expression in xenograft nude mice models. The results showed that p53 levels were negatively correlated with CHK1 and CHK2 levels in xenograft nude mice models, which are consistent with Shigeishi et al work[31].

Conclusions

We found that 53BP1 and MDC1 down-regulation inhibited CHK2-T68 expression in Eca-109 cells. 53BP1 down-regulation reduced p53 and enhanced CHK1 and CHK2 expression in xenograft nude mice models treated with or without γ-ray irradiation. Meanwhile, the p53 was negatively correlated with CHK1 and CHK2 expression. In summary, 53BP1 regulated the cell cycle arrest by modulating p53, CHK1, and CHK2 expression in both Eca-109 cells and xenograft nude mice models.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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