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ORIGINAL ARTICLE

Nutlin-3, an Antagonist of MDM2, Enhances the Radiosensitivity of Esophageal Squamous Cancer with Wild-Type p53

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Abstract Murine double minute 2 (MDM2) negatively regulates the activity of the p53 protein and plays a vital role in cell cycle arrest, apoptosis, and senescence mediated by p53. Nutlin-3, an antagonist of MDM2, is frequently used in anticancer studies. In many human tumors, nutlin-3 stabilizes p53 status and enhances p53 expression in cells with wild-type p53. However, the effect of nutlin-3 combined with radiotherapy on esophageal squamous cancer (ESCC) has not been reported. In this study, we examined whether nutlin-3 increases the radiosensitivity of ESCC in vitro and in vivo.

We chose two cell lines, ECA-109 (wild-type p53) and TE-13 (p53 mutated), for the following experiments. Cell proliferation and clonogenic survival experiments showed that nutlin-3 inhibits the cell growth and colony formation of ECA-109 cells in a dose-dependent manner. Flow cytometry analysis showed that the apoptosis rate of ECA-109 cells co-treated with nutlin-3 and irradiation(IR) was significantly increased compared with cells treated with irradiation or nutlin-3 alone. Western blotting detected the expression of apoptosis-associated proteins in ECA-109 cells in response to nutlin-3 and irradiation. These effects were not evident in TE-13 cells. Xenograft mouse models indicated that nutlin-3 suppresses tumor growth and promotes radiosensitivity in the ESCC cell line ECA-109 in vivo. We have demonstrated that co-treatment of nutlin-3 with irradiation can significantly inhibit the growth and improve the radiosensitivity of ESCC cells with wild-type p53. The study suggests that nutlin-3 may be a potent therapeutic agent in conjunction with radiotherapy in ESCC.

Keywords Esophageal squamous cancer · MDM2 · Radiotherapy · Nutlin-3 · p53

Introduction

Esophageal cancer is one of the most common malignant tumors and has two main subtypes. In developing countries, esophageal squamous cell carcinoma (ESCC) is the main histological type of esophageal cancer. Clinically, radiotherapy is the foundation of treatment for esophageal cancer patients with locally advanced disease and contraindications to surgery [1]. However, radioresistance is the main problem for ESCC patients. Accordingly, there is intense interest in developing targeted therapeutic strategies for radio sensitization [2].

Murine double minute 2 (MDM2) is an ubiquitin ligase that was first identified in double-minute chromosomes in transformed mouse fibroblasts [3–5]. MDM2 negatively regulates p53 function in various cellular pathways, such as cell cycle, apoptosis, and senescence, and also suppresses p53 gene transcription and translation [6]. In many human

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tumors, mdm2 gene overexpression occurs via amplification and\or increased transcription and translation [7, 8]. Moreover, mdm2 gene overexpression correlates with poor prognosis in various cancers, such as breast, lung, stomach, and esophageal cancers, liposarcomas, and glioblastomas [7, 9–11]. MDM2 is also associated with the chemora dioresistance of ESCC cells [12].

Many studies indicate that MDM2 plays a central role in oncogenic functions that are dependent on p53, such as proliferation, apoptosis, and metastasis [13, 14]. Mdm2 inhibits cell apoptosis through the E2F1/Rb pathway and its interaction with various apoptosis mediators, such as p73 and FOXO3a [15–17]. MDM2 also inhibits caspase-mediated apoptosis and improves the translation of anti-apoptotic XIAP [18]. In addition, inhibition of MDM2 radiosensitizes cancer cells in many malignant tumors via p53-dependent and -independent mechanisms [19–23].

Nutlins, which are isolated from a class of cis-imidazoline compounds, are promising and effective small-molecule antagonists of MDM2 [24]. Nutlin-3, which is frequently used in anti-cancer studies, stabilizes p53 status and, in many human tumors, improves the expression of p53 in cells with wild-type p53 [25]. By inhibiting the interaction of p53 and mdm2, nutlin-3 allows the signaling function of p53 pathway in cancer cells [26–31]. However, the effect of nutlin-3 combined with radiotherapy on esophageal squamous cancer (ESCC) has not been reported.

The purpose of this study was to determine whether nutlin-3 increases the radiosensitivity of ESCC in vitro and in vivo. Thus, we performed cell proliferation and clonogenic survival experiments. We also detected the apoptosis rate of ESCC cells with or without nutlin-3 and analyzed whether nutlin-3 enhances the radiosensitivity of ESCC cells in a xenograft mouse model.

Materials and Methods

Cell Culture

ECA-109 with wild-type p53 [32] and TE-13 with p53 mutated [33, 34], two esophageal cancer cell lines, were purchased from Shanghai Institutes for Biological Science, China. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Gibco, CA, USA) and 1% penicillin/streptomycin (Sigma, St. Louis, USA). Cells were maintained in an incubator with an atmosphere of 5% CO_2 at 37 °C. Cells and mice were irradiated (IR) as described previously [35]. Cells were given a single dose of 566 cGy/min by an X-ray irradiator (Elekta, Sweden). Animals were received 6 Gy (2.47 cGy/min) for tumor radiotherapy (RS- 200 Pro Biological Irradiator).

Reagents and Antibodies

Nutlin-3 was purchased from Selleckem, dissolved in DMSO as a 10 mM stock solution, and frozen. The CCK8 kit was obtained from Beyotime Institute of Biotechnology. The following primary antibodies were used: mdm2 (2 μg/ml, Abcam), p53, caspase3, cleaved caspase3, bax, bcl-2, and β-actin (1:1000 Cell Signaling Technology, CST). The secondary antibody was goat anti-mouse/rabbit IgG (1:5000 Cell Signaling Technology, CST).

Cell Proliferation Assay

We used the CCK8 assay to detect the proliferation of ESCC cells. We plated ECA-109 and TE-13 cells in 96-well plates at a density of 4×10^4 cells per well and incubated the plate at 37 °C overnight. Then, cells were incubated with different concentrations of nutlin-3 (0, 5, 10, 15, 20, 25,30, 40, 60 μM) for 24 and 48 h. Next, a CCK8 proliferation and cytotoxicity assay kit was employed for an additional 1 h incubation. Cell viability was estimated by detecting the absorbance at 450 nm in a microplate reader (BioTek ELx800, USA).

Clonogenic Survival Assay

A defined number of ECA-109 and TE-13 cells were seeded into 6-well plates. After incubation in an incubator overnight, the cells were pre-treated for 24 h in the absence or presence of nutlin-3. The cells were irradiated with various doses of X-rays (2, 4, 6, 8 Gy) at room temperature. After incubation for an additional 10 to 15 days, the cells were harvested and stained. Colonies (≥50 cells) were counted under a microscope.

Flow Cytometry Analysis

ECA-109 and TE-13 cells were plated in 6-well plates. After incubation at 37 °C overnight, the cells were pre-treated with or without nutlin-3 for 24 h and exposed to X-rays (8 Gy). After incubation for another 24 h, the cells were harvested and stained with an AnnexinV-FITC Apoptosis Detection kit (BD Bioscience, Oxford, UK) according to the manufacturer's instructions. The apoptosis rate was evaluated by flow cytometry.

Western Blot Analysis

The protein concentration in cell lysates in RIPA buffer was determined using a BCA kit (Beyotime). Typically, 50 μg of protein sample was separated as described previously [1]. The membranes were blocked, probed with primary antibodies overnight at 4 °C, and incubated with secondary antibody

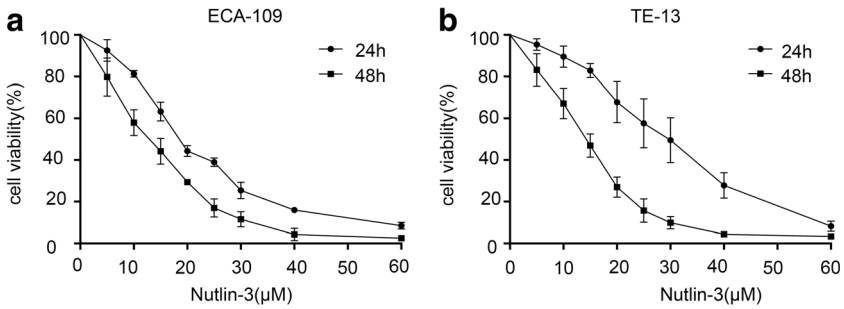

Fig. 1 Nutlin-3 inhibits ESCC cell proliferation in a dose-dependent manner; the IC_{50} values of nutlin-3 in ECA-109 and TE-13 cells were 18.98 and 27.83 μM, respectively

for 1 h at room temperature. Immunoblotted proteins were detected by enhanced chemiluminescence.

Xenograft mouse model.

Male BALB/C nude mice (5–6 weeks of age) were obtained from the Nanjing Medical University Animal Centre. Nude mice were injected with 0.1 mL of PBS containing 1×10^6 Eca109 cells into one site of the right armpit to establish the xenografts. When the tumor size reached 100 to 300 mm^3, the mice were randomly assigned to four groups (n = 5): (1) vehicle (DMSO in PBS), (2) 6 Gy irradiation (6 Gy), (3) 50 mg/kg nutlin-3, and (4) 50 mg/kg nutlin-3 plus 6 Gy irradiation. The nude mice were administered nutlin-3 (50 mg/kg mice, in PBS) or vehicle (DMSO in PBS) via intra-peritoneal (IP) injection daily for one week before radiation and one week after radiation. Tumors were irradiated on day 10. Body weight and tumor volume were measured every other day. Tumor volumes were calculated by caliper measurement of the length[L] and width[W] tumor diameters. The formula tumor volume = (length[L] width[W^2]) /2 was used to calculate the tumor volume. Animal experiments were approved by the Ethics Committee of Nanjing Medical University.

Statistical Analysis

The mean ± standard deviation (SD) was analyzed by GraphPad Prism program version 5.0 (Graph Pad Software, San Diego, CA, USA). Differences between groups were determined using the ANOVA test. A P-value <0.05 was considered statistically significant. All experiments were repeated three times.

Results

Nutlin-3 Improves Radiosensitivity of ESCC Cell Line with Wild-Type p53

CCK8 assays indicated that nutlin-3 inhibits the growth of ESCC cell in a dose- and time-dependent manner (Fig. 1). The IC_{50} (half maximal inhibitory concentration) of nutlin-3 in ECA-109 and TE-13 cells was 18.98 and 27.83 μM, respectively. Thus, nutlin-3 concentrations of 5 μmol/L and 10 μmol/L were employed in the following experiments.

To examine the ability of nutlin-3 to confer radiosensitivity to ESCC cells, we treated ESCC cells with different concentrations of nutlin-3. As shown in Fig. 2a, nutlin-3 suppresses colony formation in ECA-109 cells in a dose-dependent manner. The survival fraction (SF) of ECA-109 at 2 Gy in the presence of nutlin-3 (5 μmol/L and 10 μmol/L) was 0.52 and 0.41, respectively (Table 1). The single-hit multitarget model formula [SF = 1 − (1 − e − D/D0)^n] was used to calculate the SF value. Pre-treatment of TE-13 cells with nutlin-3 had no significant influence on colony formation (Fig. 2b). The SER (sensitization enhancement ratio) in TE-

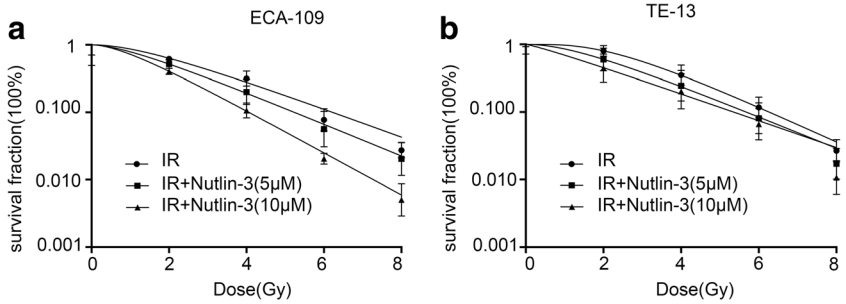

Fig. 2 Clonogenic survival assay of ESCC. **a**, **b** ECA-109 and TE-13 cells exposed to different doses of irradiation and 5 μM, 10 μM, or no nutlin-3 treatment. The SER of TE-13 in the presence of nutlin-3 (5 μmol/L and 10 μmol/L) was 0.9 and 0.76, respectively. Each bar represents the mean ± SE of 3 independent experiments

Table 1 The radiosensitization activity of nutlin-3 in ECA-109 cells

	D0	Dq	SF2	SER
Control	2.06	1.54	0.63	
Nutlin-3 5 μM	1.83	1.07	0.52	1.13
Nutlin-3 10 μM	1.38	0.91	0.41	1.49

13 cells in the presence of nutlin-3 (5 μmol/L and 10 μmol/L) was 0.9 and 0.76, respectively. In addition, the apoptosis rate of ECA-109 cells treated with nutlin-3 combined with radiotherapy was significantly increased compared with cells treated with IR or nutlin-3 alone (Fig. 3a,c). No significant difference in the apoptosis rate of TE-13 cells was noted (Fig. 3b,d). Our data suggest that nutlin-3 promotes the apoptosis rate of ECA-109 cells in a dose-dependent manner and enhances radiosensitivity of ESCC cells harboring wild-type p53.

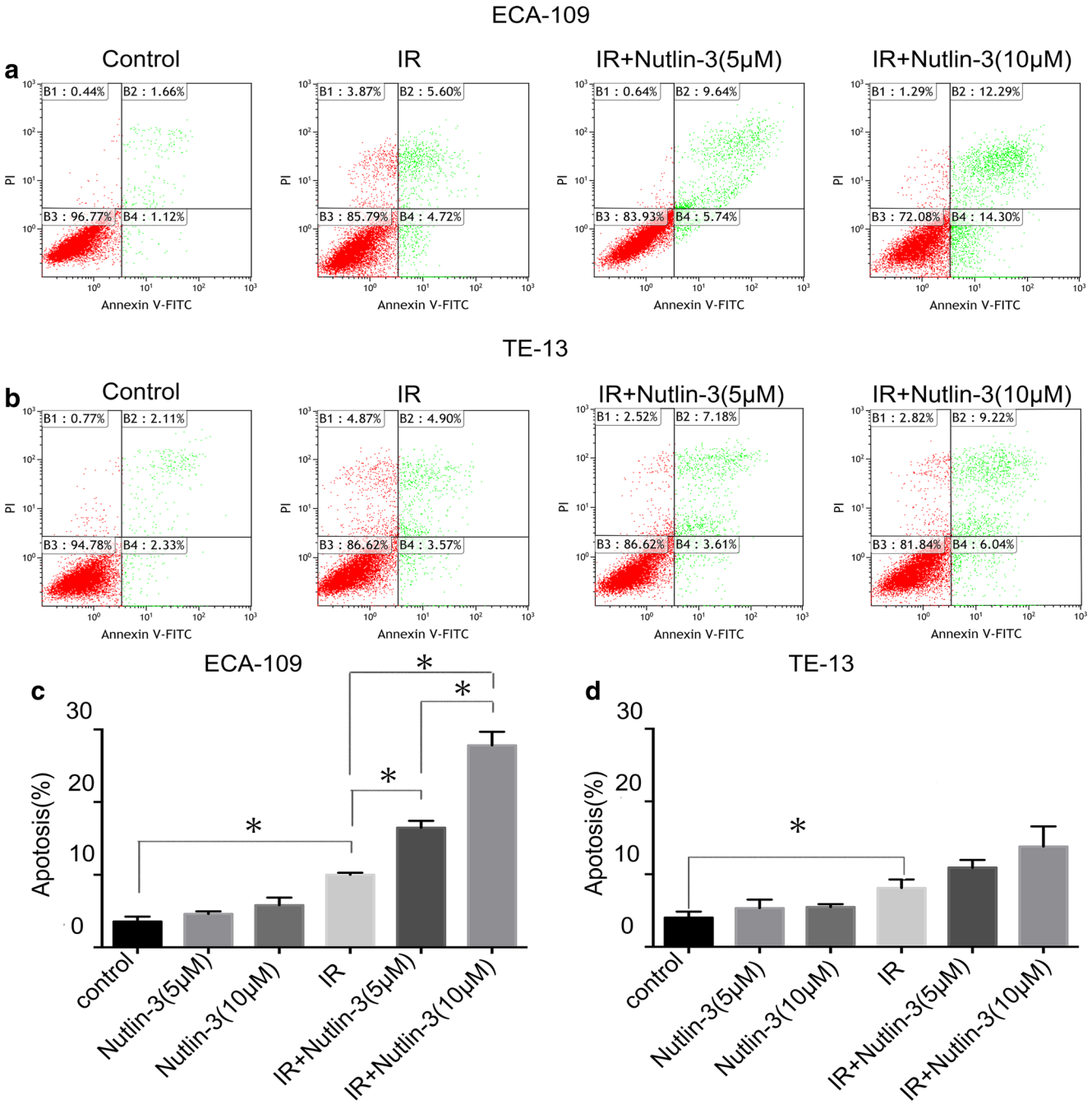

Fig. 3 Flow cytometric analysis showing that nutlin-3 induces apoptosis in ECA-109 cells. Nutlin-3 (5 μM, 10 μM) significantly enhanced irradiation-induced apoptosis of ECA-109 cells (*$p < 0.05$). There was no significant difference in apoptosis in TE-13 cells

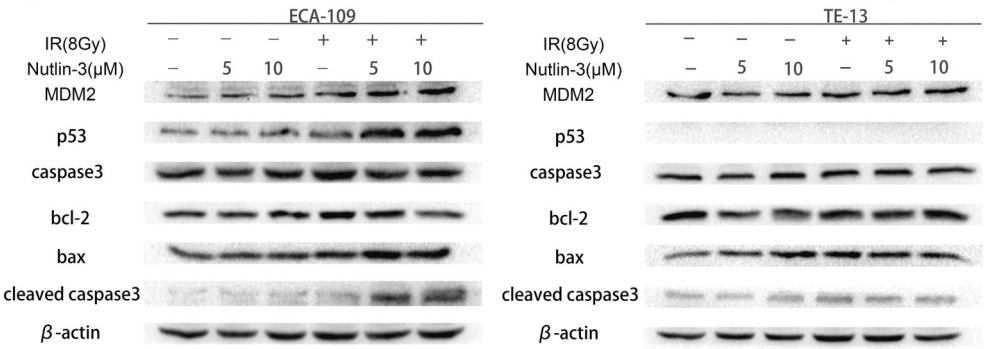

Fig. 4 The expression of apoptosis-associated proteins. Nutlin-3 leads to the downregulation of bcl-2 expression and promotes caspase-3 and bax expression in ECA-109 cells

The Influence of Nutlin-3 on the Expression of Apoptosis-Associated Proteins in ESCC Cells

To assess the mechanism underlying the effects of nutlin-3, we examined the expression of p53 and apoptosis-associated proteins. Western blotting indicated that p53, MDM2, cleaved caspase3 and bax were up-regulated in the presence of nutlin-3 in ECA-109 cells. By contrast, the expression of bcl-2 was suppressed by nutlin-3 (Fig. 4). This effect was not observed in the TE-13 cell line (Fig. 4).

Nutlin-3 Enhances the Radiosensitivity of ECA-109 ESCC Cells in Vivo

To detect the effect of nutlin-3 on ESCC xenografts in vivo, nude mice bearing ECA-109 cells were exposed to X-ray (6 Gy), nutlin-3 (50 mg/kg, per day), or both treatments. As shown in Fig. 5, compared with the control group, IR and the combined treatment more effectively suppressed tumor growth and decreased tumor weight (tumor weight, $p < 0.05$ for the nutlin-3 and IR combination group versus the IR alone group, Fig. 5c).

Moreover, to compare tumor growth, we calculated the doubling time required for growth in the four groups in the ECA-109 xenograft mouse model. Table 2 indicates that the doubling times of the IR alone group (10.5 ± 6.5 days) and nutlin-3 group (4.4 ± 3.5 days) were significantly reduced compared with the combination treatment group (20.2 ± 6.9 days). However, the doubling time of the control group was 1.6 ± 0.5 days (Table 2). The normalized tumor growth delay in the combination treatment group suggests that nutlin-3 enhances irradiation with an enhancement factor of 1.8 (Table 2).

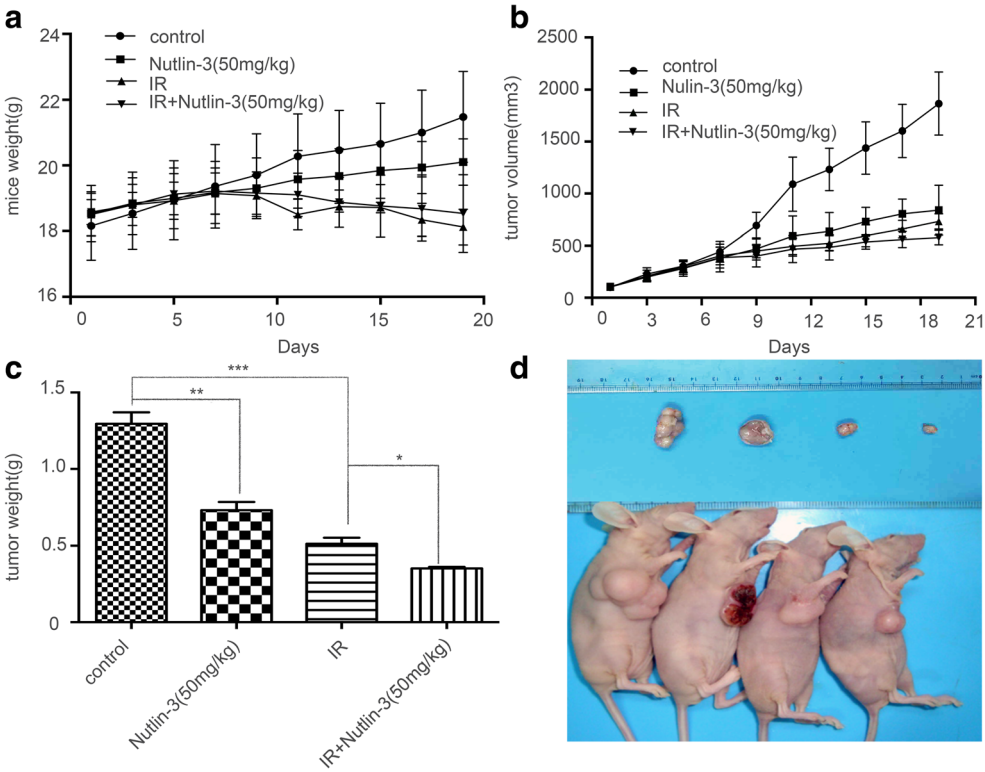

Fig. 5 Nutlin-3 enhanced radiosensitivity in the ECA-109 xenograft mouse model. a, Measurement of mouse weight every other day. b, Measurement of tumor size every other day. The data represent the average tumor volume; error bar, SD. c, Measurement of tumor weight in nude mice after sacrifice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. d, Representative mouse and tumor images

Table 2 Radiosensitization effect of nutlin-3 in an ECA-109 xenograft model

Treatment	Doubling time(days)	Absolute growth delay(days)[a]	Normalized growth delay(days)[b]	Enhancement factor
Control	1.6 ± 0.5			
Nutlin-3	4.4 ± 3.5	2.8(4.4–1.6)		
IR	10.5 ± 6.5	8.9(10.5–1.6)		
IR + Nutlin-3	20.2 ± 6.9	18.6(20.2–1.6)	15.8(18.6–2.8)	1.8(15.8/8.9)

[a] Absolute growth delay (days) calculated by subtracting the doubling time of the tumor in the treatment group from that in the control group

[b] Normalized growth delay (days) calculated by subtracting the absolute growth delay of the tumor in the group treated with nutlin-3 alone from that of the group treated with both irradiation and nutlin-3

Discussion

In this study, we investigated whether nutlin-3 enhances the sensitivity of ESCC cells to irradiation in vitro and in vivo. To clarify the effect of nutlin-3 on ESCC cells, we performed cell proliferation assays and clonogenic survival experiments. We also detected the apoptosis rate of ESCC cells in response to nutlin-3. Pretreatment with nutlin-3 suppressed cell proliferation and colony formation, resulting in a significant increase in radiosensitivity in ECA-109 cells with wild-type p53. We also observed that the combination of nutlin-3 and irradiation had a pronounced synergistic pro-apoptosis effect on ECA-109 compared with the IR or nutlin-3 alone group. Nutlin-3 improved irradiation-induced cell apoptosis to enhance the radiosensitization of ECA-109 cells. Furthermore, we evaluated the expression of apoptosis-associated proteins. Consistent with the above results, nutlin-3 was sufficient to induce the activation of bax, cleave caspase-3 and suppress the expression of bcl-2. By contrast, these effects were not observed in the TE-13 cell line, suggesting that it may be dependent on the expression of p53.

Using a xenograft nude mouse model, we demonstrated that nutlin-3 enhances the radiosensitivity of ECA-109 cells in vivo. The combination of nutlin-3 and IR notably suppressed tumor weight compared with the other groups. Collectively, we conclude that nutlin-3 enhances the radiosensitivity of ESCC cells with wild-type p53 in vitro and in vivo.

MDM2 plays a central role in the regulation of p53 function [13, 14]. Inhibition of the MDM2 may reduce the chemoradioresistance of ESCC cells [12]. Several studies [20–23] in related fields have clearly demonstrated that inhibition of MDM2 enhances the chemosensitivity or radiosensitivity of cancer cells with wild-type p53. And MDM2 mediated apoptosis various apoptosis mediators [15–18]. Similarly, in this study, we provided evidence that nutlin-3, an antagonist of MDM2, improves the radiosensitivity of ESCC cells with wild-type p53. In addition, we observed that nutlin-3 pre-treatment of ESCC cells with wild-type p53 enhanced cell apoptosis and increased the expression of bax and caspase-3 in response to irradiation.

This study is the first to demonstrate that nutlin-3 can be used to enhance the sensitivity of ESCC cells with wild-type p53 to irradiation. We also determined that nutlin-3 suppresses tumor weight and tumor volume in a xenograft nude mouse model employing ESCC cells harboring wild-type p53. The in vivo results are consistent with the findings in cell lines. Because we only examined 5 nude mice, our results must be further tested.

In summary, nutlin-3 enhances the radiosensitivity of ESCC cells with wild-type p53. Our result suggests that nutlin-3 may be a potent therapeutic strategy for ESCC, but this finding must be tested further.

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Compliance with Ethical Standards

Competing Interests The authors declare that they have no competing interests.

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