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Thioridazine Sensitizes Esophageal Carcinoma Cell Lines to Radiotherapy-Induced Apoptosis *In Vitro* and *In Vivo*

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Background: Radiotherapy is one of the primary treatments for esophageal squamous cell carcinoma (ESCC). Identification of novel radio-sensitizing agents will improve the therapeutic outcome of radiotherapy. This study aimed to determine the radio-sensitizing effect of the antipsychotic agent thioridazine in ESCC and explored the underlying mechanisms.

Material/Methods: ECA-109 and TE-1 ESCC cells were treated with thioridazine and radiotherapy alone and in combination. Cell survival was measured by MTT assay. Cell cycle and apoptosis were monitored by flow cytometry. Western blot analysis was used to analyze the expression of phospho-PI3K, phosphor-AKT, phospho-mTOR, Caspase-3, Caspase-9, Bax, Bcl-2, Bal-xl, Bak, and p53. The xenograft mouse model was used to study the *in vivo* anticancer effect of thioridazine and irradiation.

Results: Combined treatment with thioridazine and irradiation significantly reduced viability of ESCC cells compared with thioridazine or irradiation treatment alone. Thioridazine and irradiation treatment induced G0/G1 phases cell cycle arrest through down-regulation of CDK4 and cyclinD1. In addition, thioridazine and irradiation treatment induced apoptosis through up-regulation of cleaved capase-3 and 9, as well as an increase in the expression of Bax and Bak and a decrease in the expression of Bcl-2 and Bcl-xl. Furthermore, thioridazine and irradiation treatment inhibited the PI3K-AKT-mTOR pathway and up-regulated the expression of p53. In xenograft mice, thioridazine and irradiation reduced ESCC tumor growth.

Conclusions: Thioridazine sensitizes ESCC cells to radiotherapy. Thioridazine may play a role in ESCC radiation therapy as a promising radiosensitizer.

MeSH Keywords: **Apoptosis • Esophageal Neoplasms • Radiotherapy, Adjuvant • Thioridazine**

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Background

Esophageal cancer is the eighth most common cancer and the sixth most common cause of cancer deaths worldwide [1]. Esophageal squamous cell carcinoma (ESCC) is the major histopathological subtype of esophageal cancer [2]. Radiotherapy is an important form of nonsurgical management of ESCC [3,4]. Unfortunately, systemic radiotherapy alone is ineffective in patients with unresectable, recurrent, or metastatic ESCC [5]. Radiation-induced apoptosis is the major cell death mechanism involved in cancer radiotherapy. The evasion of apoptosis is a prominent hallmark of cancer, and induces resistance to radiotherapy [6]. Therefore, agents that inhibit apoptosis may be a novel strategy for antagonizing cancer resistance to radiotherapy.

Thioridazine (10-[2-(1-methyl-2-piperidyl) ethyl]-2-(methylthio) phenothiazine) is a first-generation antipsychotic drug that has been used to treat psychotic disorders such as psychosis and schizophrenia [7]. Thioridazine has antimicrobial activity, and is thus used to treat antibiotic-resistant organisms such as *Mycobacterium tuberculosis* [8]. In addition, thioridazine has anticancer effects via its anti-proliferation and anti-survival activities [9]. Thioridazine also induces cell apoptosis in cervical cancer, endometrial cancer [10], ovarian cancer [11], activated B-cell subtype of diffuse B-cell lymphoma [12], neuroblastoma and glioma [13], gastric cancer [14], leukemia [15], and melanoma cells [16]. It has been reported that thioridazine induces apoptosis by targeting the PI3K-Akt-mTOR pathway [17]. Activation of the PI3K-Akt-mTOR pathway has been reported to contribute to resistance of esophageal cancer to several commonly used classes of chemotherapeutic agents [18]. Therefore, thioridazine is currently considered as a potential anticancer drug in chemotherapy or radiotherapy.

Since high concentrations of thioridazine cause adverse effects such as dysrhythmia and sudden death, low concentrations of thioridazine may be favorable for thioridazine-based combination cancer therapy by reducing the occurrence of adverse effects and improving the anticancer effects. However, the role and mechanisms of thioridazine in radiation-induced apoptosis in ESCC remains unknown. In the current study, we explored the anticancer and radio-sensitizing effects of thioridazine in ESCC *in vitro* and *in vivo* and investigated the underlying molecular mechanisms.

Material and Methods

Cell culture

The ECA-109 and TE-1 ESCC cell lines were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) and 100 mg/mL of penicillin and streptomycin (Invitrogen, USA) in 5% CO_2 at 37°C.

MTT assay

MTT assay was performed to determine cell survival. Cells were seeded in 96-well plates at a density of 3000 cells per well. After culturing for 24 h, cells were treated with 0, 1, 5, 10, 15, 20, 25, and 30 μM thioridazine for 12 h. To investigate the effect of thioridazine and irradiation on cell proliferation, cells received X-ray irradiation for 12 h at a single dose of 2, 6, and 8 Gy after thioridazine treatment. Control dishes were sham-irradiated under the same conditions. MTT (3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma, USA) was added to each well at a final concentration of 0.5 mg per milliliter, and incubated for 4 h at 37°C. The supernatant was then removed and formazan precipitates were dissolved using 150 μl dimethyl sulfoxide. Absorbance was read at 570 nm wavelength. All experiments were repeated 3 times.

Flow cytometry

Cell cycle analysis and quantification of cell apoptosis was performed by flow cytometry, as previously reported [19]. Briefly, cells were seeded in 96well plates at a density of 3000 cells per well and treated with 15 μM thioridazine, followed by 4-Gy irradiation. Cells were fixed in 2% paraformaldehyde, and then stained with an Annexin V-FITC Apoptosis Kit (Keygene Biotechnology). Data were acquired on a FACSCalibur flow cytometer (BD, USA) using Cell-Quest software (BD, Bioscience). For each experiment, 10 000 events per sample were recorded. All experiments were repeated 3 times.

Western blot

Cells were lysed with RIPA lysis buffer. Protein lysates (10 μL) were subjected to electrophoresis on 6–15% SDS-polyacrylamide gel (Beyotime Biotechnology) and transferred to Polyvinylidene fluoride Membranes (Millipore, Billerica, USA). The membranes were blocked in the solution containing 5% BSA and 1×PBS-0.1% Tween20. The membranes were incubated with primary antibodies overnight at 4°C followed by incubation with secondary antibodies at room temperature for 2 h. Primary antibodies used in this study were antibodies against Caspase-3, Caspase-9, Bax, Bcl-2, Bcl-xl, Bak, phospho-PI3K, phospho-AKT, phospho-mTOR, P53 (Cell Signaling Technology), or β-actin (Sigma-Aldrich, USA). Goat polyclonal anti-mouse IgG-horseradish peroxidase (HRP) or goat polyclonal anti-rabbit IgG-HRP were used as secondary antibodies. Bands were visualized by chemiluminescence detection kit (Pierce, USA). All experiments were repeated 3 times.

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Mouse xenograft model

Animal experiments were approved by the Ethics Committee of Anhui Medical University. BALB/c-nu/nu mice (female, 5–6 weeks old) were subcutaneously inoculated with 0.1 milliliter of ECA109 cells (1×10^7 cells per mL) at the right flank of the abdomen. When the tumors reached a volume of 100 mm^3 after inoculation on day 10, the animals were randomly assigned into 4 groups (n=12 per group): the control group, the thioridazine group (THZ group), the irradiation (IR) group, and the THZ + IR group treated with THZ and IR. The mice in the control group were treated with PBS orally, whereas the mice in the THZ and THZ+IR groups were treated with oral administration of 25 mg per kg thioridazine every 3 days for 27 days. For mice in the IR and THZ+IR group, tumors were irradiated with an RS-2000 biological irradiator using X-rays at a single dose of 4 Gys at 2 Gee/min on day 11. Six mice were randomly selected from each group and used for measuring tumor volume. Tumors were measured using a caliper, and tumor volume (in mm^3) was calculated using the following formula: tumor volume (mm^3)=($A\times B^2$)/2, where A is the larger diameter and Bis the smaller diameter of the tumor. The other 6 mice were used to observe the survival period. Tumors were then excised, frozen, and stored.

Data analysis and statistics

Dose-response curves, combination indices, and dose reduction indices were generated using CompuSyn 1.0 software (Paramus, USA). Concentrations and corresponding effect levels for all data points were input to generate a complete report of analytic results. The effect of a treatment was expressed as a decimal between 0 (no effect) and 1 (100% effect). The combination index (CI) was used to determine whether the interaction of 2 or more drugs is synergistic, additive, or antagonistic as follows: CI=1, additive; CI<1, synergistic; and CI>1, antagonistic. The CI was calculated using the following equation: CI=((D1/Dx1)+(D2/Dx2)), D1 and D2 were the dose of drug 1 and drug 2 in combination treatment, respectively, and Dx1 and Dx2 were the dose of drug 1 and drug 2 used alone, respectively.

Statistical analysis was performed using SSPS 13.0 software (SPSS Inc.) and Prism 5.0 software (GraphPad Prism). Data are presented as mean ±SD. Statistical comparisons between groups were performed using Student's t-tests, and a P value of <0.05 was considered to indicate statistical significance.

Results

Thioridazine enhances radio-sensitivity of ESCC cells

MTT assay was used to evaluate the effects of thioridazine and irradiation on cell survival in ECA-109 and TE-1 cells. Thioridazine treatment inhibited cell viability of ECA-109 and TE-1 cells in a dose-dependent manner (Figure 1A) (P<0.01). Irradiation alone inhibited cell viability of ECA-109 and TE-1 cells in a dose-dependent manner (Figure 1B). Combination treatment with both thioridazine and irradiation further reduced the percentage of cell viability of both cells (Figure 1B, 1C). The CI of thioridazine (15μM) and irradiation (4 Gy) were 0.71 in ECA-109 cells and 0.91 in TE-1 cells (CI≤1), respectively (Figure 1D, 1E), indicating that thioridazine and irradiation synergistically inhibited cell viability in ECA-109 and TE-1 cells.

Thioridazine and irradiation induces cell cycle arrest at the G0/G1 phases in ECA-109 and TE-1 cells

We further investigated the effect of thioridazine and irradiation on cell cycle progression in ECA-109 and TE-1 cells using flow cytometry. The percentage of ECA-109 cells and TE-1 cells at the G0/G1 phases was reduced after the treatment with thioridazine and irradiation alone compared with the control group (Figure 2A–2D). Combination treatment with thioridazine and irradiation significantly increased the percentage of cells at the G0/G1 phases compared with thioridazine and irradiation treatment alone (Figure 2A–2D). Western blot analysis showed that thioridazine and irradiation treatment alone significantly reduced the expression of CDK4 and cyclinD1 in ECA-109 and TE-1 cells. Combination treatment with thioridazine and irradiation significantly decreased the expression of CDK4 and cyclinD1 compared with thioridazine and irradiation treatment alone (Figure 2E, 2F).

Thioridazine and irradiation induces apoptosis in ECA-109 and TE-1 cells

We investigated the effect of thioridazine and irradiation on cell apoptosis in ECA-109 and TE-1 cells using flow cytometry. Thioridazine and irradiation treatment alone significantly increased the percentage of apoptotic cells in ECA-109 and TE-1 cells compared with the control group (Figure 3A–3D). Combination treatment with thioridazine and irradiation significantly increased the percentage of apoptotic cells in ECA-109 and TE-1 cells compared with thioridazine and irradiation treatment alone (Figure 3A–3D). In addition, combination treatment with thioridazine and irradiation increased the expression of cleaved capase-3 and -9 in ECA-109 and TE-1 cells (Figure 3E). Furthermore, combination treatment with thioridazine and irradiation increased the expression of Bax and Bak, the pro-apoptotic proteins, and decreased the expression of Bcl-2 and Bcl-xl, the anti-apoptotic proteins, in ECA-109 and TE-1 cells (Figure 3F).

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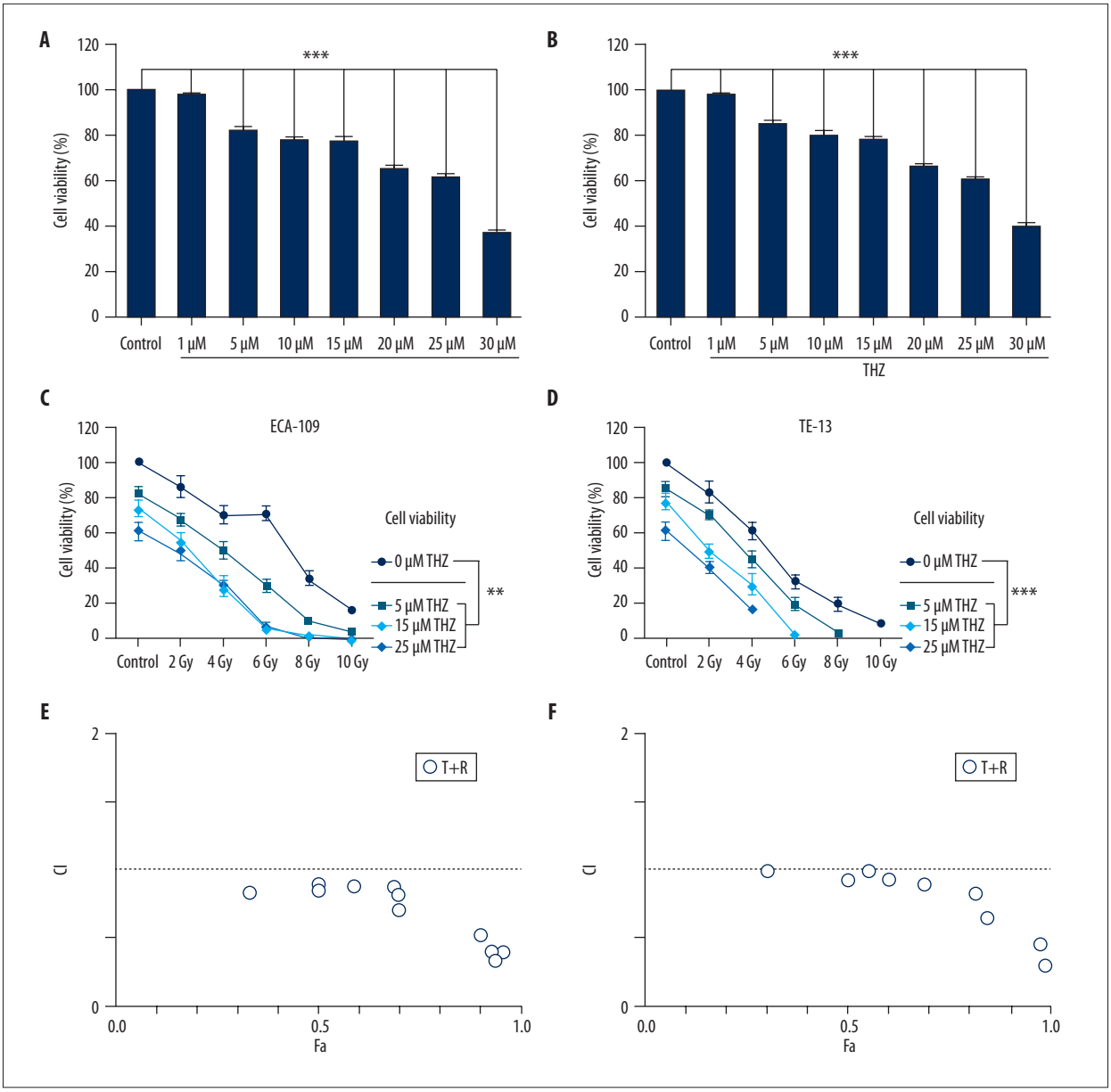

Figure 1. Effects of thioridazine and irradiation on survival of ECA-109 and TE-1 cells. (**A**) Thioridazine inhibited cell survival of ECA-109 and TE-1 cells in a dose-dependent manner. ECA-109 and TE-1 cells were treated with various doses of thioridazine (0, 1,5, 10, 15, 20, 25, and 30 μM) for 12 h. MTT assay was used to determine cell viability. (**B, C**) Combination treatment with thioridazine and irradiation inhibited cell survival in ECA-109 cells (**B**) and TE-1 cells (**C**). ECA-109 and TE-1 cells were treated with various doses of thioridazine (0, 5, 15, and 25 mM) for 12 h, followed by treatment with 0, 2, 4, 6, or 8 Gy irradiation for 12 h. MTT assay was used to determine cell viability. Data are represented as the means±SD. n=3. ** P<0.01, *** P<0.001 *vs.* THZ alone. (**D, E**) CIs as a function of effect levels of thioridazine and irradiation combination treatment in ECA-109 (**D**) and TE-1 (**E**) cells.

Thioridazine and irradiation treatment resulted in inhibition of the PI3K-AKT-mTOR pathway and up-regulation of P53 expression in ECA-109 and TE-1 cells

Western blot analysis was used to evaluate the effect of thioridazine and irradiation on activation of the PI3K-AKT-mTOR pathway and the expression of P53 in ECA-109 and TE-1 cells (Figure 4A–4D). Compared with the control, the expression of phosphorylated PI3K, AKT and mTOR was significantly lower in ECA-109 and TE-1 cells treated with thioridazine (Figure 4). Irradiation did not significantly alter the expression of phosphorylated PI3K, AKT, and mTOR (Figure 4). Combination

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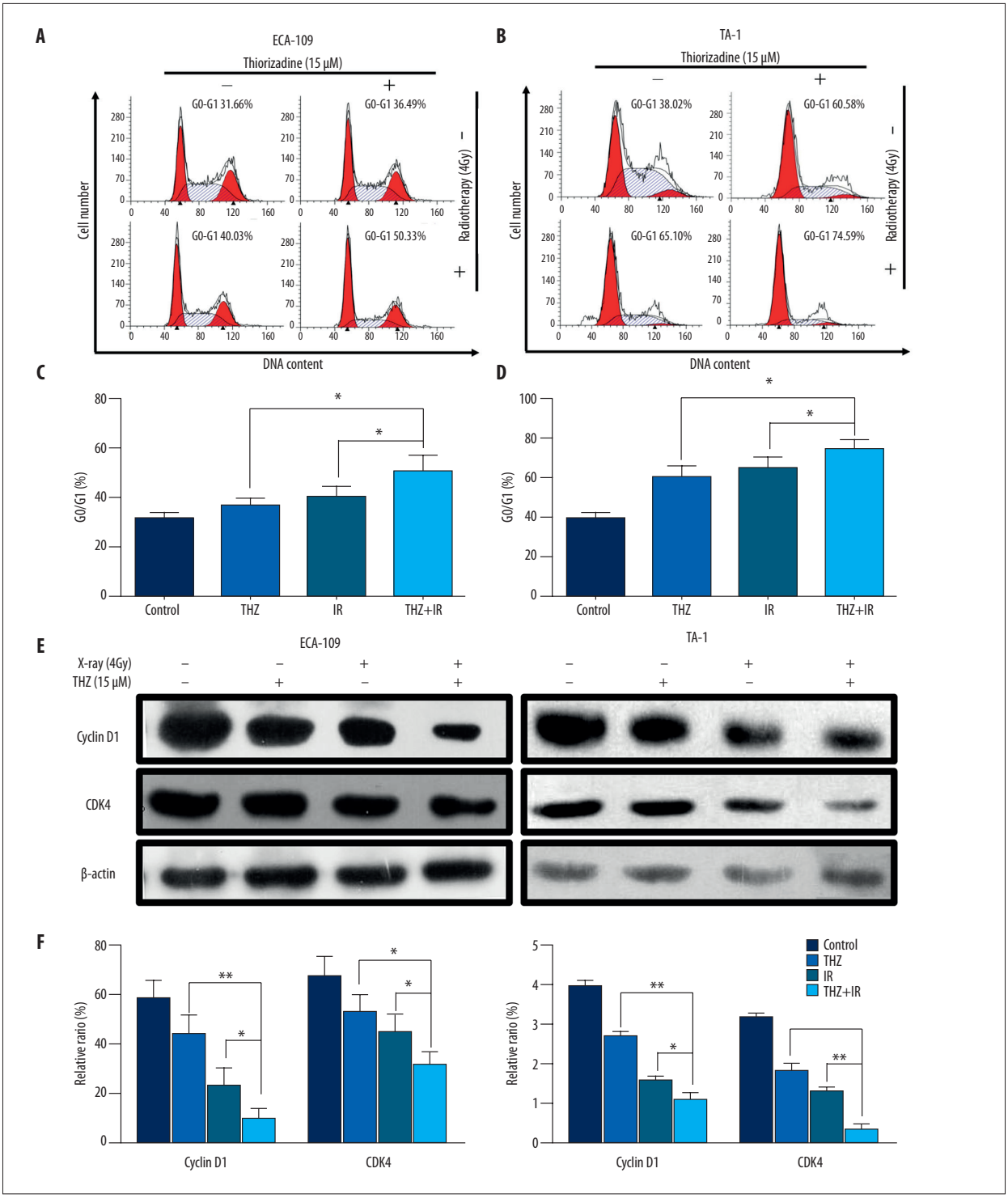

Figure 2. Thioridazine and irradiation treatment induced cell cycle arrest at the G0/G1 phases in ECA-109 and TE-1 cells. (**A, C**) Representative flow cytometry showed the cell cycle distribution in ECA-109 cells (**A**) and TE-1 cells (**C**) in the control group, thioridazine group, irradiation group, and combination group. (**B, D**) The percentages of ECA-109 cells (**B**) and TE-1 cells at the G0/G1 phases. * P<0.05 *vs.* thioridazine group or irradiation group. (**E**) Representative Western blot showing the expression ofCyclinD1 and CDK4 in ECA-109 and TE-1 cells in the control group, thioridazine group, irradiation group, and combination group. (**F**) Quantification of the expression of CyclinD1 and CDK4. β-actin was used as a control. n=3. * P<0.05 and ** P<0.01 *vs.* thioridazine group or radiation group, respectively.

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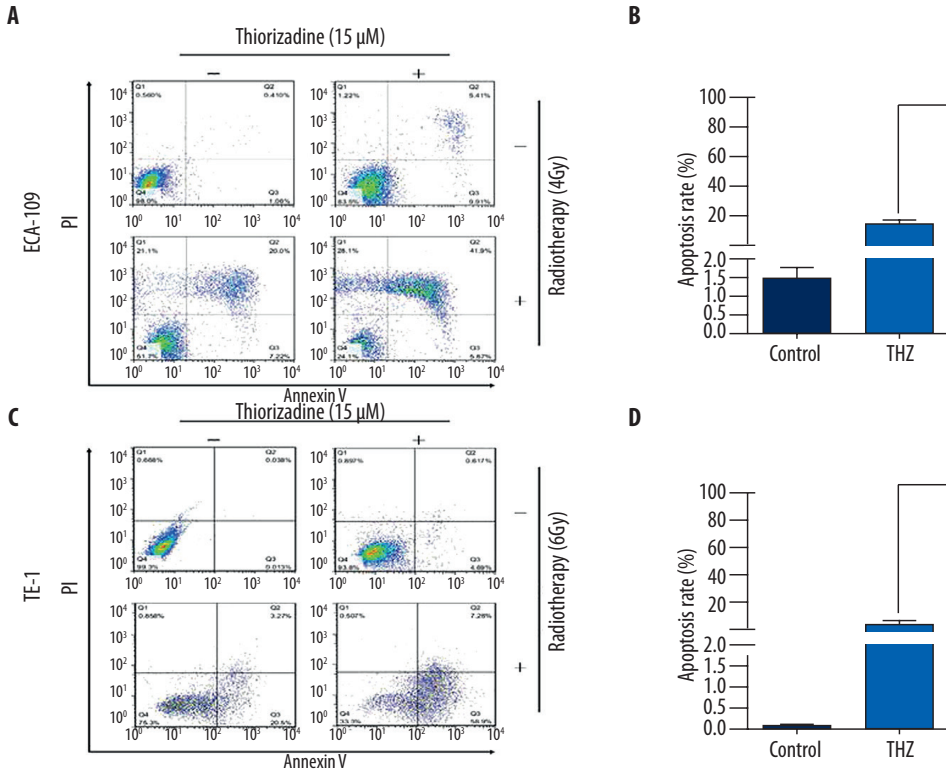

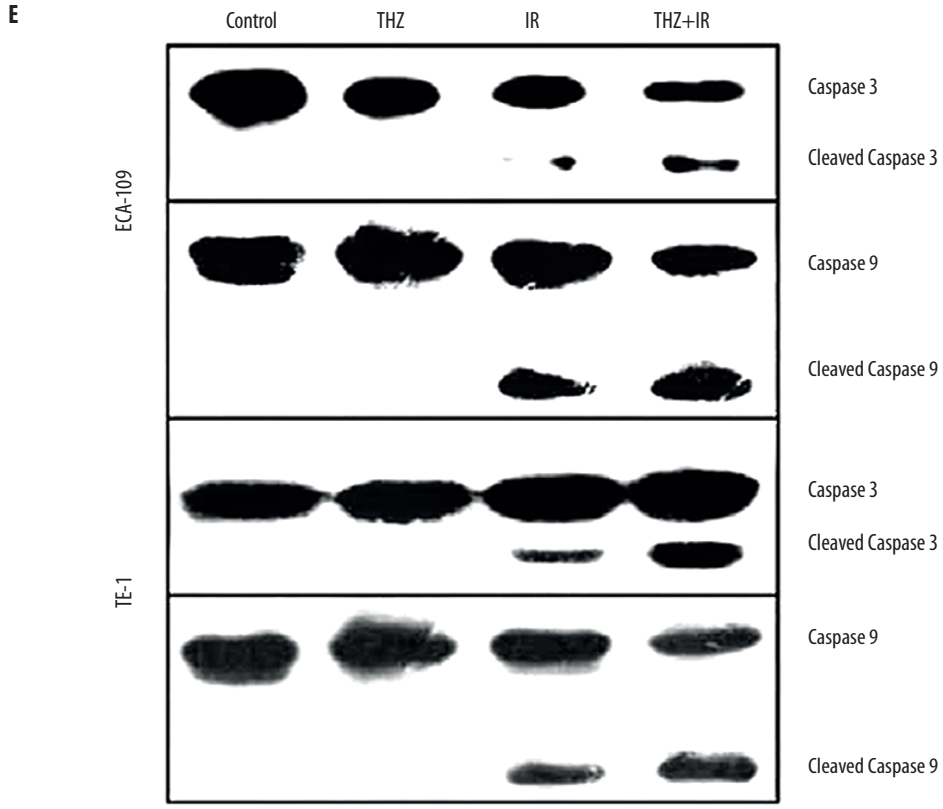

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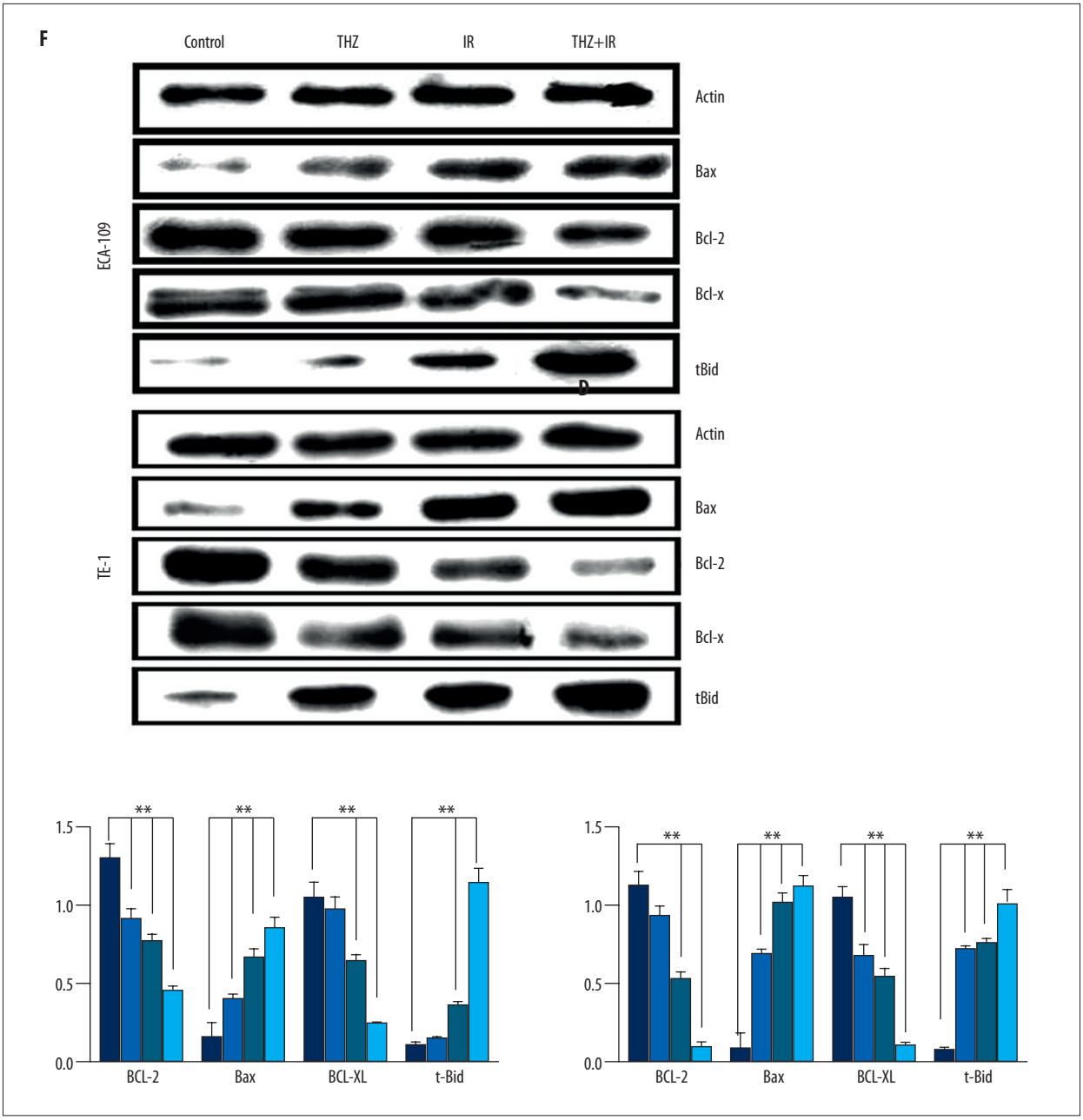

Figure 3. Thioridazine and irradiation induced apoptosis in ECA-109 and TE-1 cells. (**A, C**) Flow cytometry showed the percentage of apoptotic cells in ECA-109 (**A**) and TE-1 (**C**) cells in the control group, thioridazine group, irradiation group, and combination group. Cells were treated with 0 or 15 μM of thioridazine for 12 h, followed by 0 or 8 Gy irradiation. Quantitative analysis of apoptotic ECA-109 (**B**) and TE-1 (**D**) cells. *** P<0.05 *vs.* thioridazine group or irradiation group. (**E**) Western blot analysis showed the expression caspase-3, Cleaved caspase-3, caspase-9, and Cleaved caspase-9 in ECA-109 and TE-1 cells in the control group, thioridazine group, irradiation group, and combination group. (**F**) Western blot analysis showed the expression of Bax, Bcl-2, Bcl-xl, and Bak in the control group, thioridazine group, irradiation group, and combination group.

treatment with thioridazine and irradiation significantly inhibited the expression of phosphorylated PI3K, AKT, and mTOR compared with the treatment with thioridazine or irradiation alone (Figure 4). In addition, compared with the control, the expression of P53 was significantly increased in cells treated with thioridazine or irradiation (Figure 4). Combination treatment with thioridazine and irradiation significantly increased the expression of p53 compared with the treatment with thioridazine or irradiation alone (Figure 4).

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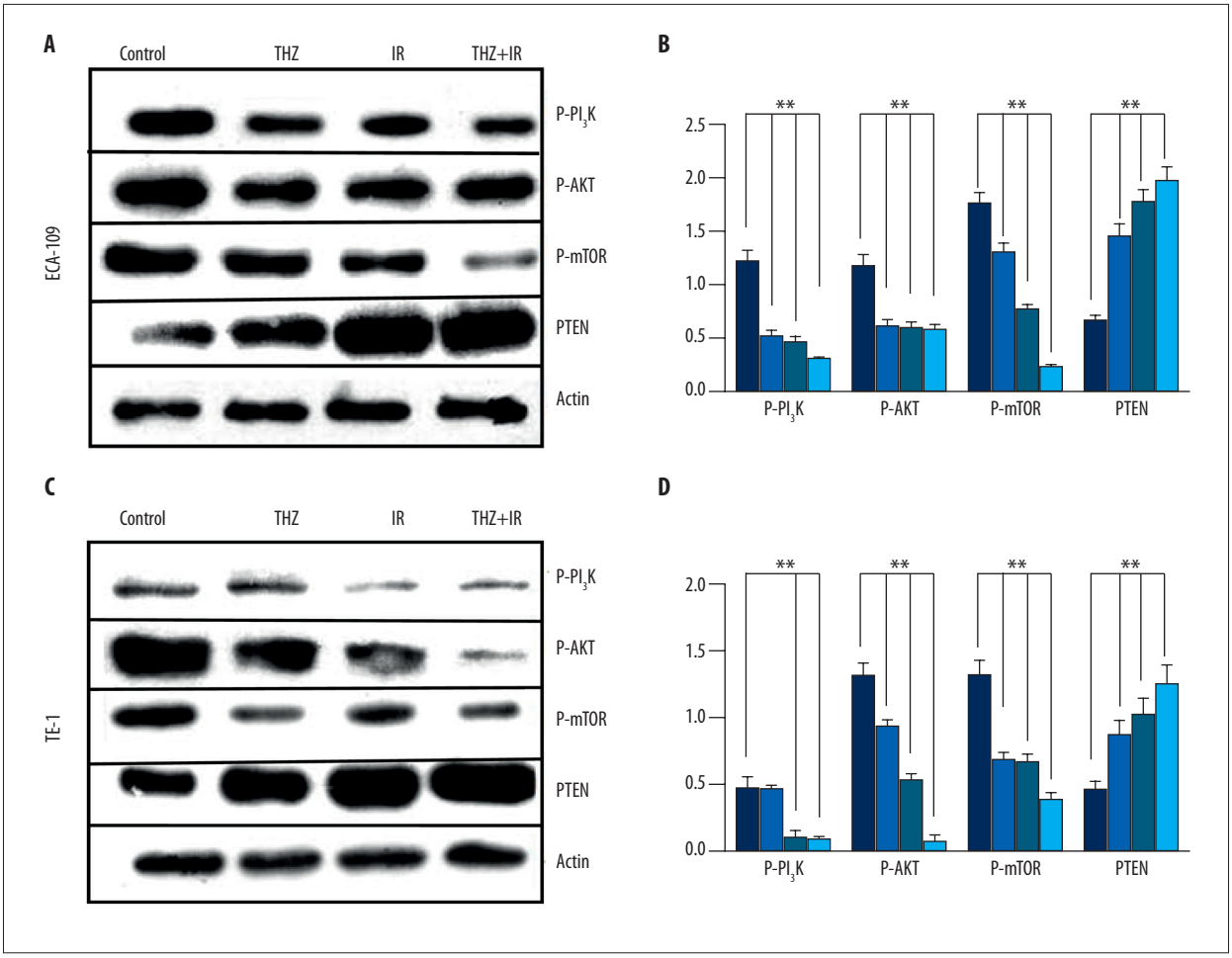

Figure 4. Thioridazine and irradiation inhibited the PI3K-AKT-mTOR pathway and up-regulation of P53 expression in ECA-109 and TE-1 cells. (**A, C**) Representative Western blot analysis showed the expression of phosphorylatedPI3K, AKT and mTOR and p53 in ECA-109 (**A**) and TE-1 (**C**) cells in the control group, thioridazine group, irradiation group, and combination group. β-actin was used as a loading control. (**B, D**) Quantitative analysis of the expression of phosphorylated PI3K, AKT, and mTOR and p53 in ECA-109 (**A**) and TE-1 (**C**) cells. n=3. * P<0.05, ** P<0.01, *vs.* thioridazine group or irradiation group.

Thioridazine and irradiation reduced ESCC tumor growth in xenograft mice

We further studied the effect of thioridazine and irradiation on tumor growth in nude mice transplanted with ECA-109 cells. The tumor volume was significantly less in the THZ and RT group than in the control group. The tumor volume was significantly less in the THZ + RT group than in the THZ group and the RT group (Figure 5A). More mice survived in the THZ and RT groups than in the control group. More mice survived in the THZ + RT group than in the THZ or RT groups (Figure 5B).

Discussion

Currently, although chemo-radiotherapy has significantly improved the treatment effects for unresectable esophageal cancer, radio-resistance limits the utilization of radiotherapy [20,21]. This unfavorable therapeutic outcomes is due to radio-resistance related to various biological factors, while increasing radiation may induce the increasing occurrence of adverse events, including bleeding, perforation, radiation esophagitis and pneumonitis, and other adverse effects [22]. Complicated molecular mechanisms could include high activity of Akt, overexpression of Bcl-2 and Bcl-xL, and defects in the release of mitochondrial proteins, and unclear mechanisms are involved in radio-resistance in various cancer cell types [23–25]. Therefore, a novel radiosensitizer interrupting the tumor signal pathway followed by synchronous radiotherapy is urgently required to improve the curative effect of radiotherapy, with minimal adverse effects.

Thioridazine is an antipsychotic drug widely used to treat schizophrenia and psychosis. Recently, it has been reported

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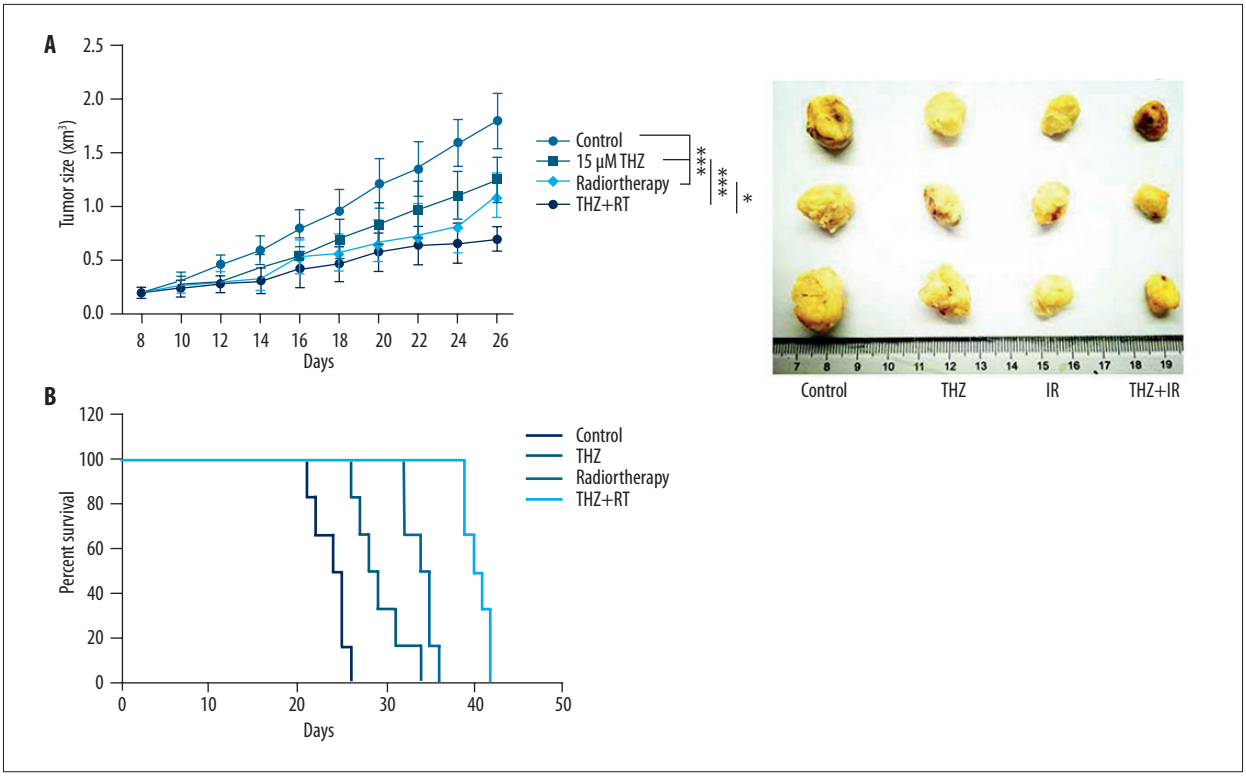

Figure 5. Thioridazine enhances radio-sensitivity inhibits tumor growth *in vivo*. (**A**) ECA-109 tumor cells were injected subcutaneously. Mice were divided into 4 groups: control, thioridazine, irradiation, and combination groups. Data represent the mean tumor volume of 6 mice. (**B**) Six mice were used to observed the survival period in all 4 groups. The percent of surviving mice in the combination group was significant different from that in the individual radiation group and individual thioridazine group, * P<0.05, ** P<0.01, *** P<0.01.

that thioridazine suppresses cancer cell growth by inhibiting proliferation, inducing apoptosis, and regulating the activity of the anti-apoptosis signaling pathway [10–14]. Thioridazine can significantly enhance the anti-proliferative activity of doxorubicin in treating breast cancer cells and cancer stem cell-like cells, which shows that thioridazine has significant chemo-sensitizing ability [26,27]. Moreover, thioridazine can increase the TRAIL-mediated apoptosis via the ROS mediated inhibition of Akt signaling and down-regulation of Mcl-1 and c-FLIP(L) [28]. Based on these studies, we propose that thioridazine should be developed as an effective radiosensitizer and used for cancer radiotherapy.

The radiotherapy reaction of cancer cells may depend on the phase in the cell cycle [29,30]. Tumor cells are least sensitive in S phase. In the present study, we found that thioridazine combined irradiation inhibited proliferation of ECA-109 and TE-1 cells by inducing a G0/G1 phase cell cycle arrest. Cyclin D1 and cyclin-dependent kinase 4 (CDK4) have been reported to be involved in the regulation of cell proliferation by mediating the G1 to S phase transition [31]. We found that combination treatment decreased the expression of CDK4 and cyclinD1 in both esophagus cancer cells compared with thioridazine and irradiation treatment alone, indicating that thioridazine can enhance the irradiation-mediated cell suppression via cell cycle arrest.

Radiotherapy plays a paramount role in the treatment of solid tumors, largely by inducing cell apoptosis, mainly through the intrinsic pathway [32]. However, radiotherapy resistance in various cancers present a huge challenge in clinical treatment. Research has demonstrated that activation of PI3K-Akt-mTOR and overexpression of Bcl-2 and Bcl-XL, important members of Bcl-2 family involving in intrinsic apoptosis pathway and frequently overexpressed in human tumors, are vital molecular mechanisms in radiotherapy resistance [33,34]. The intrinsic pathway of apoptosis starts with BH3-only protein activation, which inhibits the pro-survival Bcl-2-like proteins, followed by activation of pro-apoptotic proteins BAX and BAK. BAX and BAK begin to form the oligomers, destroy the mitochondrial outer membrane, and promote cytochrome c release, which activate caspase-9 to cleaved caspase-9, depending on activated APAF1. Therefore, caspase-3 is activated and followed by apoptosis [35,36]. Recent research suggests that in head and neck squamous cell cancer (HNSCC) and small-cell lung cancer(SCLC), the combination of radiation and inhibitor of anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-xL induced

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more apoptosis than the summation of their separate effects, which means down-regulation of Bcl-2 and Bcl-xL possess the capacity to radio-sensitize cancer cells [24,37,38]. In the current study, compared with thioridazine and radiation treatment alone, the combined treatment with thioridazine and irradiation dramatically induced the cleavage of caspase-9 and caspase-3, which was consistent with the annexin V-FITC/PI double-labeled flow cytometry analysis, suggesting that thioridazine and radiation induce apoptosis via the mitochondrial-mediated intrinsic pathway. We found that thioridazine and radiation co-treatment reduced the level of Bcl-2/Bcl-xL with concomitantly elevated expression of pro-apoptotic Bax/Bak in both TE-1 and ECA-109 cells, suggesting that cell apoptosis induced by thioridazine and radiation may be partly facilitated by regulating the proportion of Bax, Bcl-2, and Bcl-xL proteins.

The PI3K-Akt-mTOR pathway plays a pivotal role in cell growth, proliferation, and metabolism, which is dysregulated in a variety of cancers [39,40]. Recent advances have also demonstrated that PI3K-Akt activity contributes to cancer radiotherapy resistance, mainly through 3 mechanisms: intrinsic radio-resistance, hypoxia, and tumor-cell proliferation [41,42]. PI3K-Akt-mTOR inhibitors, including single and dual inhibitors, sensitize various cancer types to radiotherapy, which is a promising approach for cancer treatments. LY294002 and wortmannin, well-studied specific PI3K inhibitors, respectively sensitize cancer cells to radiation through inactivation of PKB and lead to inhibition of cellular DNA-PK. Palomid 529 (Akt inhibitor), RAD001 (mTOR inhibitor), and BEZ235 (dual PI3K/mTOR inhibitor) lead to more apoptosis and DNA double-strand breaks, increased apoptosis, and decreased number of tumor blood vessels, cell death in a PTEN-independent manner, and autophagic cell death [42–44]. Thioridazine has been shown to have pro-apoptosis effects on cervical and endometrial cells, possibly by inhibition of PI3K-Akt-mTOR-p70S6K signaling, accompanied by the inhibition of tumor growth and metastasis *in vivo* by cervical, endometrial, and melanoma tumor xenografts in mice [45,46]. Moreover, evidence shows that thioridazine exerts anti-angiogenic activity by inhibiting VEGFR2-PI3K-mTOR signal transduction, and induced G0/G1 arrest and cancer cell apoptosis [47,48]. In the present study, we show that thioridazine can inhibit the PI3K/Akt/mTOR pathway, and, combined with radiation, can significantly increase the expression of p53 compared with the treatment of thioridazine. Our results suggest that thioridazine may increase sensitivity of esophagus cancer cells to radiation via inhibiting the PI3K/mTOR pathway.

Conclusions

To confirm the anti-tumor effect of combination therapy with thioridazine and irradiation *in vitro*, we found this higher activity after combination therapy was also supported in a tumor xenograft model. Tumor volume was significantly reduced in the combination group after the treatment, and survival time was much longer than in the control group. Our results suggest that thioridazine is a promising candidate for radiotherapy sensitization in ESCC treatment.

References:

1. Sheng Q, Wang H.-Q., Das R et al: Abstract 2612: Ijm716, an anti her3 antibody that inhibits her3 dimerization, displays anti-tumor activity in esophageal squamous cell cancer. Cancer Research, 2014; 74(19 Suppl.): 2612
2. Zhang L: Identification and characterization of tumor suppressor gene and cancer stemness gene in esophageal squamous cell carcinoma. The University of Hong Kong (Pokfulam, Hong Kong), 2015
3. Qin Q, Zuo Y, Yang X et al: Smac mimetic compound LCL161 sensitizes esophageal carcinoma cells to radiotherapy by inhibiting the expression of inhibitor of apoptosis protein. Tumour Biol, 2014; 35(3): 2565–74
4. Kato H, Nakajima M, Sasaki K [Esophageal cancer]. Kyobu Geka, 2011; 64(8 Suppl.): 776–81 [in Japanese]
5. Enzinger PC, Mayer RJ: Esophageal cancer. New Engl J Med, 2003; 349(23): 2241–52
6. Hinz S, Pagerols-Raluy L, Oberg HH et al: Foxp3 expression in pancreatic carcinoma cells as a novel mechanism of immune evasion in cancer. Cancer Res, 2007; 67(17): 8344–50
7. Purhonen M, Koponen H, Tiihonen J, Tanskanen A: Outcome of patients after market withdrawal of thioridazine: A retrospective analysis in a nationwide cohort. Pharmacoepidemiol Drug Saf, 2012; 21(11): 1227–31
8. Amaral L, Kristiansen JE, Viveiros M, Atouguia J: Activity of phenothiazines against antibiotic-resistant *Mycobacterium tuberculosis*: A review supporting further studies that may elucidate the potential use of thioridazine as anti-tuberculosis therapy. J Antimicrob Chemother, 2001; 47(5): 505–11
9. Mu J, Xu H, Yang Y et al: Thioridazine, an antipsychotic drug, elicits potent antitumor effects in gastric cancer. Oncol Rep, 2014; 31(5): 2107–14
10. Kang S, Dong SM, Kim BR et al: Thioridazine induces apoptosis by targeting the PI3K/Akt/mTOR pathway in cervical and endometrial cancer cells. Apoptosis, 2012; 17(9): 989–97
11. Rho SB, Kim BR, Kang S: A gene signature-based approach identifies thioridazine as an inhibitor of phosphatidylinositol-3'-kinase (PI3K)/AKT pathway in ovarian cancer cells. Gynecol Oncol, 2011; 120(1): 121–27
12. Nagel D, Spranger S, Vincendeau M et al: Pharmacologic inhibition of MALT1 protease by phenothiazines as a therapeutic approach for the treatment of aggressive ABC-DLBCL. Cancer Cell, 2012; 22(6): 825–37
13. Gil-Ad I, Shtaif B, Levkovitz Y et al: Characterization of phenothiazine-induced apoptosis in neuroblastoma and glioma cell lines. J Mol Neurosci, 2004; 22(3): 189–98
14. Mu J, Xu H, Yang Y et al: Thioridazine, an antipsychotic drug, elicits potent antitumor effects in gastric cancer. Oncol Rep, 2014; 31(5): 2107–14
15. Moraes V, Rodrigues T: Overexpression of the oncoprotein BCL-XL inhibits thioridazine-induced apoptosis in acute promyelocytic leukemia cells (LB162). FASEB J, 2014; 28(1 Suppl.): p. LB162
16. Mao M, Yu T, Hu J, Hu L et al: Dopamine D2 receptor blocker thioridazine induces cell death in human uterine cervical carcinoma cell line SiHa. J Obstet Gynaecol Res, 2015; 41(8): 1240–45
17. Kang S, Dong SM, Kim BR et al: Thioridazine induces apoptosis by targeting the PI3K/Akt/mTOR pathway in cervical and endometrial cancer cells. Apoptosis, 2012; 17(9): 989–97
18. Hildebrandt M, Yang H, Hung M.-C. et al: Genetic variation in AKT1 and AKT2 is associated with variation in recurrence-free survival following cisplatin treatment of esophageal cancer. Cancer Research, 2008; 68: 4787
19. Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C et al: A novel assay for apoptosis flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin V. J Immunol Methods, 1995; 184(1): 39–51

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20. Kato H, Nakajima M: Treatments for esophageal cancer: A review. Gen Thorac Cardiovasc Surg, 2013; 61(6): 330–35
21. Nakajima M, Kato H: Treatment options for esophageal squamous cell carcinoma. Expert Opin Pharmacother, 2013; 14(10): 1345–54
22. Main BG, Strong S, McNair AG et al: Reporting outcomes of definitive radiation-based treatment for esophageal cancer: A review of the literature. Dis Esophagus, 2015; 28(2): 156–63
23. Zhang T, Cui GB, Zhang J et al: Inhibition of PI3 kinases enhances the sensitivity of non-small cell lung cancer cells to ionizing radiation. Oncol Rep, 2010; 24(6): 1683–89
24. Loriot Y, Mordant P, Dugue D et al: Radiosensitization by a novel Bcl-2 and Bcl-XL inhibitor S44563 in small-cell lung cancer. Cell Death Dis, 2014; 5: e1423
25. Ezekwudo D, Shashidharamurthy R, Devineni D et al: Inhibition of expression of anti-apoptotic protein Bcl-2 and induction of cell death in radioresistant human prostate adenocarcinoma cell line (PC-3) by methyl jasmonate. Cancer Lett, 2008; 270(2): 277–85
26. Ke XY, Lin Ng VW, Gao SJ et al: Co-delivery of thioridazine and doxorubicin using polymeric micelles for targeting both cancer cells and cancer stem cells. Biomaterials, 2014; 35(3): 1096–108
27. Castaing M, Loiseau A, Cornish-Bowden A: Synergy between verapamil and other multidrug-resistance modulators in model membranes. J Biosciences, 2007; 32(32): 737–46
28. Min KJ, Seo BR, Bae YC et al: Antipsychotic agent thioridazine sensitizes renal carcinoma Caki cells to TRAIL-induced apoptosis through reactive oxygen species-mediated inhibition of Akt signaling and downregulation of Mcl-1 and c-FLIP(L). Cell Death Dis, 2014; 5(2): 701–5
29. Kim MY, Park SJ, Shim JW et al: Naphthazarin enhances ionizing radiation-induced cell cycle arrest and apoptosis in human breast cancer cells. Int J Oncol, 2015; 46(4): 1659–66
30. Lin S, Yan Y, Liu Y et al: Sensitisation of human lung adenocarcinoma A549 cells to radiotherapy by Nimotuzumab is associated with enhanced apoptosis and cell cycle arrest in the G2/M phase. Cell Biol Int, 2015; 39(2): 146–51
31. Kim R: Recent advances in understanding the cell death pathways activated by anticancer therapy. Cancer, 2005; 103(8): 1551–60
32. Fulda S: Targeting IAP proteins in combination with radiotherapy. Radiat Oncol, 2015; 10: 105
33. Park SS, Kim YJ, Ju EJ et al: Ibulocydine sensitizes human cancers to radiotherapy by induction of mitochondria-mediated apoptosis. Radiother Oncol, 2014; 112(2): 295–301
34. Lee JU, Hosotani R, Wada M et al: Role of Bcl-2 family proteins (Bax, Bcl-2 and Bcl-X) on cellular susceptibility to radiation in pancreatic cancer cells. Eur J Cancer, 1999; 35(9): 1374–80
35. Czabotar PE, Lessene G, Strasser A, Adams JM: Control of apoptosis by the BCL-2 protein family: Implications for physiology and therapy. Nat Rev Mol Cell Biol, 2014; 15(1): 49–63
36. Waterhouse NJ, Ricci JE, Green DR: And all of a sudden it's over: Mitochondrial outer-membrane permeabilization in apoptosis. Biochimie, 2002; 84(2–3): 113–21
37. Zerp SF, Stoter TR, Hoebers FJ et al: Targeting anti-apoptotic Bcl-2 by AT-101 to increase radiation efficacy: Data from *in vitro* and clinical pharmacokinetic studies in head and neck cancer. Radiat Oncol, 2015; 10: 158
38. Zerp SF, Stoter R, Kuipers G et al: AT-101, a small molecule inhibitor of anti-apoptotic Bcl-2 family members, activates the SAPK/JNK pathway and enhances radiation-induced apoptosis. Radiat Oncol, 2009; 4: 47
39. García-Carracedo D, Ángeles Villaronga M, Álvarez-Teijeiro S et al: Impact of PI3K/AKT/mTOR pathway activation on the prognosis of patients with head and neck squamous cell carcinomas. Oncotarget, 2016 [Epub ahead of print]
40. Kourea HP, Nikolaou M, Tzelepi V et al: Expression of phosphorylated Akt, mTOR and MAPK in type I endometrial carcinoma: Clinical significance. Anticancer Res, 2015; 35(4): 2321–31
41. Wang Z, Huang Y, Zhang J: Molecularly targeting the PI3K-Akt-mTOR pathway can sensitize cancer cells to radiotherapy and chemotherapy. Cell Mol Biol Lett, 2014; 19(2): 233–42
42. Chang L, Graham PH, Ni J et al: Targeting PI3K/Akt/mTOR signaling pathway in the treatment of prostate cancer radioresistance. Crit Rev Oncol Hematol, 2015; 96(3): 507–17
43. Horn D, Hess J, Freier K et al: Targeting EGFR-PI3K-AKT-mTOR signaling enhances radiosensitivity in head and neck squamous cell carcinoma. Expert Opin Ther Targets, 2015; 19(6): 795–805
44. Chen YH, Wei MF, Wang CW et al: Dual phosphoinositide 3-kinase/mammalian target of rapamycin inhibitor is an effective radiosensitizer for colorectal cancer. Cancer Lett, 2014; 357(2): 582–90
45. Kang S, Dong SM, Kim BR et al: Thioridazine induces apoptosis by targeting the PI3K/Akt/mTOR pathway in cervical and endometrial cancer cells. Apoptosis, 2012; 17(9): 989–97
46. Gil-Ad I, Shtaif B, Levkovitz Y et al: Phenothiazines induce apoptosis in a B16 mouse melanoma cell line and attenuate *in vivo* melanoma tumor growth. Oncol Rep, 2006; 15(1): 107–12
47. Park MS, Dong SM, Kim BR et al: Thioridazine inhibits angiogenesis and tumor growth by targeting the VEGFR-2/PI3K/mTOR pathway in ovarian cancer xenografts. Oncotarget, 2014; 5(13): 4929–34
48. Seung Bae R, Boh-Ram K, Sokbom K: A gene signature-based approach identifies thioridazine as an inhibitor of phosphatidylinositol-3'-kinase (PI3K)/AKT pathway in ovarian cancer cells. Gynecol Oncol, 2011; 120(1): 121–27

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