


Aripiprazole sensitizes head and neck cancer cells to ionizing radiation by enhancing the production of reactive oxygen species

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Abstract

Drug repositioning is an alternative process for drug development in cancer. Specifically, it is a strategy for the discovery of new antitumor drugs by screening previously approved clinical drugs. On the basis of this strategy, aripiprazole, an antipsychotic drug, was found to have anticancer activity. In this study, we investigated the radiosensitizing effects of aripiprazole on head and neck cancer cells at sublethal doses of ionizing radiation (IR) *in vitro* and *in vivo*. Treatment with aripiprazole suppressed the growth of head and neck cancer cells in a concentration-dependent manner, as evidenced by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Intriguingly, aripiprazole significantly enhanced the sensitivity of these cells to the IC₅₀ dose of IR. The combination of aripiprazole with IR synergistically increased annexin and propidium iodide double-positive and terminal deoxynucleotidyl transferase dUTP nick end labeling-positive cell populations, and induced cleaved poly(ADP-ribose) polymerase and caspase-3 expression, indicating the induction of apoptosis in these cells. Aripiprazole and IR-induced apoptosis were accompanied by an increase in reactive oxygen species and was almost completely suppressed by the addition of the antioxidant, N-acetylcysteine. Finally, aripiprazole greatly sensitized xenograft tumors to IR at doses that did not affect tumor growth. Taken together, these results suggest that aripiprazole could be considered a potent radiosensitizer for head and neck cancer.

KEYWORDS

aripiprazole, head and neck cancer, ionizing radiation, radiosensitizer

Abbreviations: 4-HNE, 4-hydroxynonenal; Ari, aripiprazole; CFA, colony-forming assay; DMSO, dimethyl sulfoxide; HNC, head and neck cancer; IHC, immunohistochemistry; IR, ionizing radiation; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; PI, propidium iodide; ROS, reactive oxygen species; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

Hyeon-Jeong Jeong and Chan-Woong Jung contributed equally.

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1 | INTRODUCTION

Head and neck cancer (HNC) is the sixth most common cancer worldwide, with over 500,000 new cases each year. HNC mainly occurs in the oral cavity, oropharynx, hypopharynx, and larynx, and its representative histology is squamous cell carcinoma.^{1,2} The development of HNC is increased mainly by continuous exposure to tobacco and alcohol.³ Surgery and radiation therapy are the main treatments for HNC, with chemotherapy added as an adjunct. Treatment of HNC is difficult because sufficient surgical resection is limited owing to the occurrence of functional and/or cosmetic disorders, severe side effects after radiation and chemotherapy, and local and distant metastases.⁴ Therefore, treatments that can minimize the side effects of radiation and chemotherapy while preserving the function of the surgical site as much as possible, are being actively researched.

Aripiprazole (Ari) is a second-generation atypical antipsychotic drug that is very effective in treating mental disorders, including schizophrenia and bipolar disorder.⁵ Ari exhibits unique pharmacological activities, acting as a serotonin 5-HT_{1A} and partial dopamine D₂ agonist, and 5-HT_{2A} antagonist. The action of Ari depends on the amount of dopamine secreted. When dopamine is secreted excessively, it acts as an antagonist to lower the concentration of dopamine. In contrast, when dopamine secretion is insufficient, it acts as a dopamine agonist, thereby maintaining the secretion of dopamine at a certain level.⁶ Recently, in addition to its effects on mental disorders, Ari has been shown to have anticancer and radiosensitizing effects in various cancer cells.⁷⁻¹³ In this study, we examined the radiosensitizing effect of Ari against HNC cells *in vitro* and *in vivo*, and the possible mechanism involved in this effect.

2 | MATERIALS AND METHODS

2.1 | Reagents and antibodies

Ari and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich. Dimethyl sulfoxide (DMSO) was obtained from Ducheфа Biochemistry. Antibodies against cleaved poly(ADP-ribose) polymerase (PARP) (#5625), caspase-3 (#9668), and cleaved caspase-3 (#9664) for immunohistochemistry (IHC) were purchased from Cell Signaling Technology. The β -actin antibody (sc-47778) was obtained from Santa Cruz Biotechnology. The antibody against 4-hydroxynonenal (4-HNE; ab46545) was purchased from Abcam. Secondary fluorescent and horseradish peroxidase-conjugated antibodies were obtained from Bethyl Laboratories.

2.2 | Cell culture

HNC cell lines (FaDu, CAL27, and Detroit562) were cultured in Minimum Essential Media Eagle, Dulbecco's modified Eagle's medium (Welgene) containing 10% fetal bovine serum (ATCC) and

antibiotic-antimycotic solution (Welgene). The cells were maintained in a humidified incubator at 37 °C with 5% CO₂.

2.3 | Proliferation assay

HNC cells were seeded in 96-well plates (1×10^3 cells/ml) and incubated for 24, 48, or 72 h. After incubation, the cells were treated with 10 μ l of MTT solution (20 mg/ml) for 4 h at 37 °C under 5% CO₂. The supernatant was discarded and the formazan crystals produced by viable cells were solubilized with 100 μ l of DMSO. The absorbance of the solubilized formazan solution was measured at 490 nm using a microplate reader (Bio-Rad).

2.4 | Irradiation and colony-forming assay (CFA)

To measure ionizing radiation (IR) resistance, cells were seeded in six-well plates (2×10^4 cells/ml) and exposed to γ -rays from a ¹³⁷Cs γ -ray source (BIOBEAM 8000, 2.6 Gy/min, Gamma-Service Medical) at the indicated dose rate. After 5 days of incubation, the cells were washed with phosphate-buffered saline (PBS) and stained with 0.05% crystal violet dissolved in 20% methanol. After washing three times with distilled water, the colonies were counted.

2.5 | Analysis of the cellular reactive oxygen species (ROS) level

To investigate the level of intracellular ROS, cells were seeded in a 60-mm dish (3×10^5 cells/well). After 48 h of incubation, the cells were stained with 20 μ M chloromethyl dichlorofluorescein diacetate (Sigma-Aldrich) for 30 min at 37 °C under 5% CO₂. Cells were then harvested and washed with PBS, and ROS levels were analyzed using a flow cytometer (FACS CytoFlex, Beckman Coulter).

2.6 | Annexin V/propidium iodide (PI) assay

To measure the effect of Ari treatment on cell death, Annexin V/PI staining was performed. HNC cells were seeded in a 60-mm dish (3×10^5 cells/well) and incubated for 48 h, followed by harvesting for staining. Cell death was measured using the Annexin V-FITC Apoptosis Detection kit (BD Biosciences) according to the manufacturer's protocol using a FACS CytoFlex.

2.7 | Cell cycle analysis

Cells were resuspended in 100% cold ethanol and chilled on ice overnight. The cells were then washed with PBS and stained with PBS containing 50 μ g/ml PI, 10 μ g/ml ribonuclease A, and 0.05% Triton X-100 for 40 min in the dark. After centrifugation, the cells

were resuspended in PBS. The flow cytometry analysis was performed using a FACS CytoFlex.

2.8 | Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay

HNC cells were seeded onto chamber slides in complete medium. The cells were fixed with 4% paraformaldehyde for 10 min at room temperature, then washed twice with PBS. The cells were then permeabilized by adding 0.2% Triton X-100 in PBS at room temperature for 5 min and washed twice with PBS. Cells were stained using the DeadEnd™ Fluorometric TUNEL System (Promega) as described in the manufacturer's protocol, and viewed under a confocal laser scanning microscope (Carl Zeiss).

2.9 | Western blot analysis

HNC cells were lysed in radioimmunoprecipitation assay buffer (50mM Tris-HCl [pH 7.4], 100mM NaCl, 5mM EDTA, 0.5% Nonidet P-40, phosphatase inhibitor cocktail set II [Calbiochem], and a protease inhibitor cocktail tablet [Roche]). The protein content was determined using the Bradford protein assay reagent (Bio-Rad). Proteins were separated using 10%–15% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk in TBST (20mM Tris-HCl [pH 7.6], 137mM NaCl, and 0.01% Tween-20) for 1 h at room temperature, then incubated with the indicated primary antibodies overnight at 4°C with gentle shaking. After extensive washing with TBST, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. After washing three times with TBST for 10 min, the membrane was visualized by enhanced chemiluminescence (Amersham) according to the manufacturer's protocol.

2.10 | Animal experiments

All animal experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee of the Korea Institute of Radiological & Medical Sciences, Republic of Korea. The appropriateness of the drug doses used in mice was calculated by converting the human doses into the animal doses according to FDA guidelines and literatures (Supplemental Method). Athymic BALB/c female mice (Orient Bio) were injected subcutaneously with FaDu cells (2.5×10^6 cells in medium) in the right thigh. FaDu tumor-bearing mice were randomized into eight groups ($n = 8/\text{group}$). Treatment was performed with Ari alone, IR alone, Ari combined with IR, or the control. The Ari alone and Ari/IR combination groups were divided into groups according to the concentration of the drug being administered (0.5, 1, 2, and 3 mg/kg). Treatment with Ari was started 1 day before IR and was maintained for 3 days. Ari was administered orally. IR was performed with an X-ray unit operated at 260 kVp with a dose rate of

2 Gy/min (10 mA with added filtration of 2 mm copper, distance from X-ray source to the target of 41 cm). Mice were exposed to 5 Gy/day for 3 days; a total of 15 Gy. The tumor volume (mm^3) was calculated using the following formula: long diameter \times (short diameter) $^2 \times 0.5$. Tumor growth rates were compared using the tumor volume doubling time. The body weights of mice were monitored during all treatments. No significant loss of body weight (less than 10%) was observed.

2.11 | IHC

For observing histological features, samples fixed in 4% paraformaldehyde were embedded in paraffin. Xenograft tumor tissues were sectioned using a microtome into 5- μm thick slices. IHC was performed to assess the expression of cleaved PARP (1:100), cleaved caspase-3 (1:100), and 4-HNE (1:100) in the tumor xenograft sections. Secondary antibodies were conjugated with the fluorescent dyes DyLight 488, DyLight 555, and DyLight 680 (1:100; Bethyl Laboratories). The stained sections were visualized under a confocal laser scanning microscope (Carl Zeiss). The intensity of staining in each tumor section was evaluated using ImageJ software (<http://imagej.net/>).

2.12 | Statistical analysis

Data are presented as the mean \pm SEM of at least three independent experiments. Differences between groups were analyzed with Student's *t* test and were considered significant at $*.01 < p < .05$; $**.005 < p < .01$; $***p < .005$. Statistical analyses and graphing were performed using Microsoft Excel 2013 and Prism 6.0 (GraphPad) software.

3 | RESULTS

3.1 | Ari sensitized HNC cells to IR

We first examined the effect of Ari on the sensitivity of the HNC cell lines (FaDu, CAL27, and Detroit562) to IR. Ari treatment suppressed the growth of HNC cells in a concentration-dependent manner at 72 h (Figure 1A, Figure S1A). At sublethal doses of IR ($< \text{IC}_{50}$), Ari treatment significantly sensitized HNC cells as shown by the CFA, a method for evaluating the *in vitro* IR sensitivity of cancer cells (Figure 1B, Figure S1B). The reduction of HNC cell numbers by the combination of Ari and IR was clearly revealed by microscopic examination (Figure 1C). These results indicate that Ari enhanced the sensitivity of HNC cells to sublethal doses of IR.

3.2 | Ari and IR combination synergistically enhanced apoptosis in HNC cells

We next investigated the effect of the combination of Ari and IR on apoptosis in FaDu and CAL27 cells. The flow cytometric analysis of FaDu

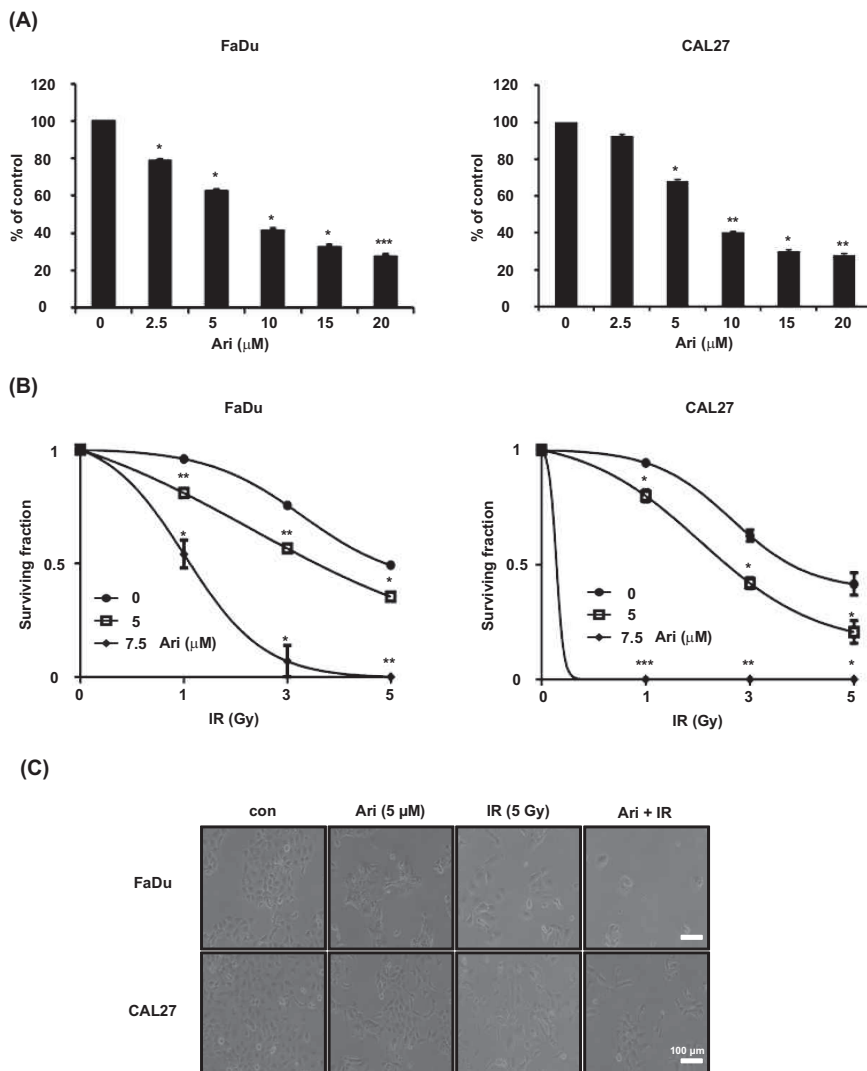


FIGURE 1 Effect of aripiprazole (Ari) on growth and ionizing radiation (IR) resistance in head and neck cancer FaDu and CAL27 cells. (A) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay of FaDu and CAL27 cells treated with increasing concentrations of Ari. (B) Colony-forming assay of head and neck cancer (HNC) cells treated with IR with or without Ari at the indicated concentrations. (C) Representative pictures of FaDu and CAL27 cells treated with or without Ari, and with or without IR. Magnification 100 \times . * $p < .05$, ** $p < .01$, *** $p < .005$.

cells showed that the combination of Ari (5mM) and IR (5 Gy) synergistically increased the Annexin V+/PI+ population, which indicates an augmentation of apoptotic cell death compared to Ari or IR treatment alone (Figure 2A, left). The Ari and IR combination also significantly enhanced Annexin V/PI double-positive populations in CAL27 cells, although its effect was to a lesser extent than that of FaDu cells (Figure 2A, right). However, in TUNEL staining of HNC cells, another method to measure apoptosis, the Ari and IR combination synergistically elevated TUNEL-positive cells in both FaDu and CAL27 cells (Figure 2B). In addition, combination treatment with Ari and IR increased the expression of cleaved PARP and caspase-3 compared to Ari or IR treatment alone in these cells (Figure 2C). Taken together, these results suggest that Ari significantly enhances IR-induced apoptotic cell death in HNC cells.

3.3 | Ari and IR combination elevated the generation of ROS in HNC cells

To examine whether the increased generation of ROS by the Ari and IR combination was the cause of elevated apoptotic cell death in HNC cells, we treated cells with N-acetylcysteine (NAC), an antioxidant,

before Ari and IR treatment. As shown in Figure 3A, the combination of Ari and IR significantly enhanced ROS generation in FaDu and CAL27 cells, which was almost completely suppressed by NAC pretreatment. Pretreatment with NAC abrogated apoptotic cell death as measured by Annexin V/PI staining (Figure 3B). NAC treatment also reduced the number of TUNEL-positive cells induced by the combination of Ari and IR (Figure 3C). Additionally, NAC treatment significantly restored the colony-forming ability of HNC cells, which was suppressed by the combination of Ari and IR (Figure 3D). Finally, pretreatment with NAC reduced the expression of cleaved PARP and caspase-3 induced by the combination of Ari and IR (Figure 3E). These results strongly indicate that the Ari and IR combination enhanced ROS generation, which was partly responsible for the synergistic cell death in HNC cells.

3.4 | Ari sensitized a FaDu cell xenograft tumor to IR in vivo

Finally, we evaluated the effects of the combination of Ari and IR on tumor growth in vivo. FaDu cells were implanted into nude mice via subcutaneous injection. As shown in Figure 4A, Ari treatment

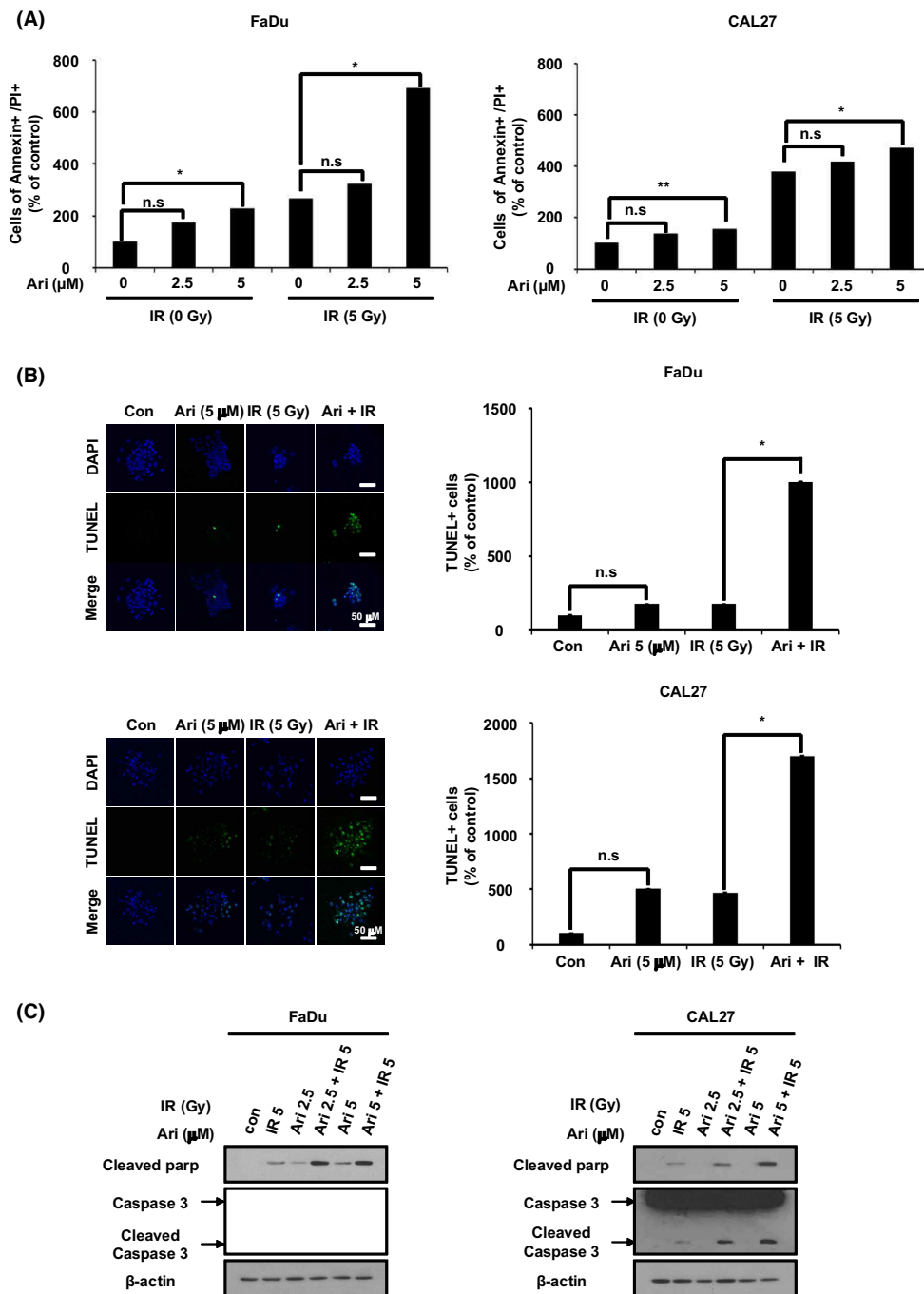


FIGURE 2 Effect of combining aripiprazole (Ari) with ionizing radiation (IR) on apoptosis of head and neck cancer FaDu and CAL27 cells. (A) Flow cytometry following annexin V and propidium iodide staining of FaDu and CAL27 cells. (B) Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining of FaDu and CAL27 cells. Representative pictures of the staining (left, magnification 40x) and quantification of the staining (right). (C) Western blots of FaDu and CAL27 cells probed with the indicated antibodies. * $p < .05$, ** $p < .01$.

did not affect tumor growth at the 0.5 and 3 mg/kg concentrations. However, the Ari and IR combination treatment synergistically suppressed tumor growth compared with IR treatment alone. A Kaplan-Meier survival analysis also demonstrated the IR-sensitizing effect of Ari in this xenograft model (Figure 4B). The IHC analysis of xenograft tissues clearly demonstrated that the Ari and IR combination significantly increased the expression of 4-HNE, an indicator of ROS generation in vivo, and cleaved PARP and caspase-3 (Figure 4C).

Taken together, these results indicate that Ari is a promising sensitizer of IR in an in vivo xenograft model of HNC.

4 | DISCUSSION

Ari, an atypical antipsychotic agent, is well-known to be very effective in the treatment of schizophrenia and schizoaffective

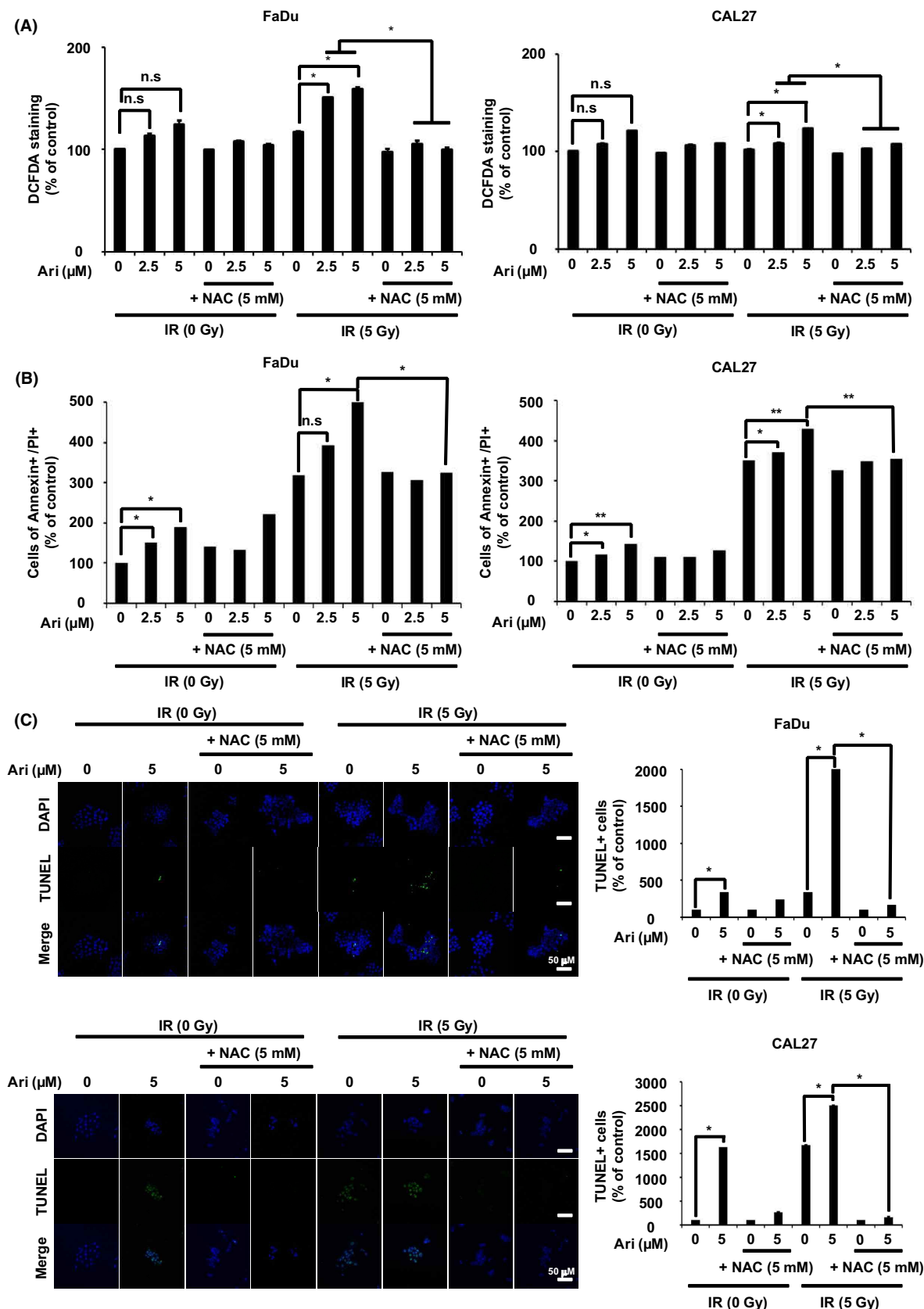


FIGURE 3 Effect of N-acetylcysteine (NAC) on reactive oxygen species generation, apoptosis, and clonogenic survival of head and neck cancer FaDu and CAL27 cells after combined treatment of aripiprazole (Ari) and ionizing radiation (IR). (A) Chloromethyl 2,7-dichlorofluorescein diacetate (DCFDA) staining. (B) Flow cytometry of annexin V and propidium iodide staining. (C) Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining. (left, magnification 40 \times) and quantification of the staining (right). (D) Colony-forming assay. (E) Western blots of FaDu and CAL27 cells pretreated with NAC (5 mM) with or without Ari and IR. * $p < .05$, ** $p < .01$.

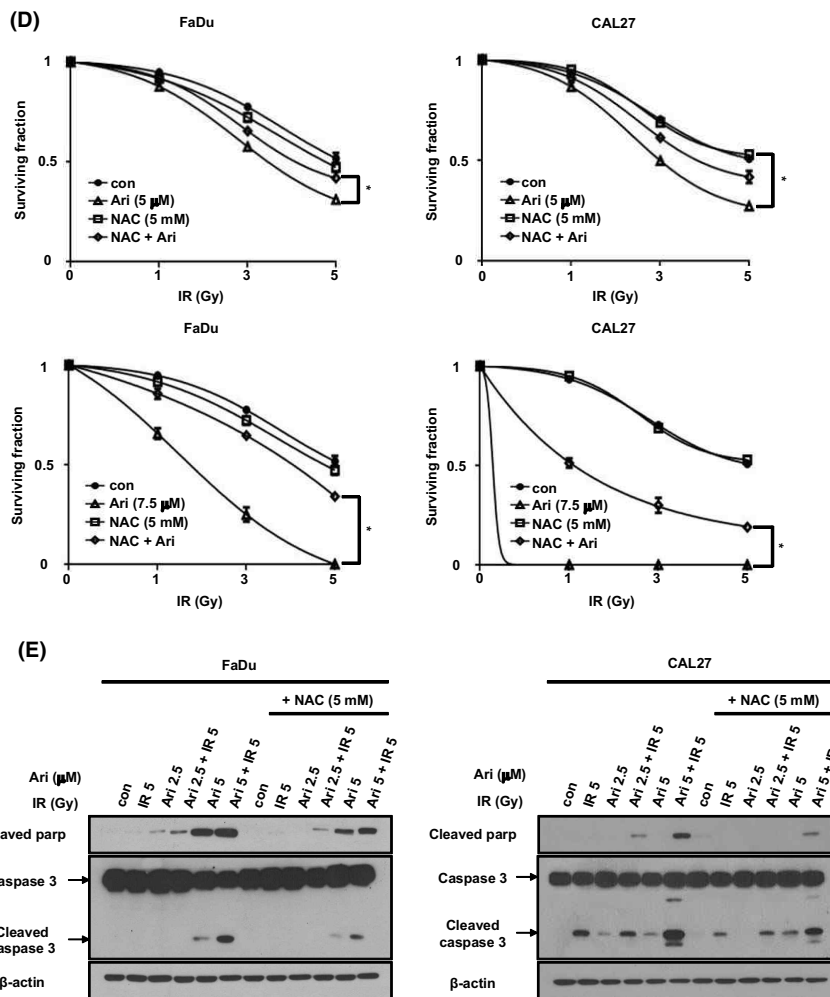


FIGURE 3 (Continued)

disorders. In several recent studies applying drug repositioning strategies, Ari has been reported to exhibit anticancer effects.⁷⁻¹² In addition, Ari has been reported to have an IR-sensitizing effect on breast cancer cells.¹³ In this study, we found that Ari acted as a potent radiosensitizer in HNC cells. In vitro, Ari showed a significant IR-sensitizing effect at sublethal doses in the CFA, and this result was due, at least in part, to the increase in cell apoptosis by promoting the production of ROS. These in vitro results were reproduced in an in vivo xenograft tumor model in mice. After oral administration of Ari before IR, the combined therapy showed a significant tumor-suppressive effect compared to IR alone. This is consistent with a previous report that a relatively high dose (30 mg/kg) of Ari had a partial antitumor effect in vivo in a colon cancer cell xenograft model.⁸ In the current study, Ari exhibited a strong IR-sensitizing effect even at low concentrations where the antitumor effect does not appear. Therefore, Ari is highly likely to show IR-sensitizing effects even at low concentrations used in the treatment of patients with depression in clinical practice. Thus, it is judged to be a very promising drug that can dramatically increase the radiation therapy efficacy of HNC.

In a study on the mechanism, Lee et al. reported that the IR sensitization effect of Ari was halved due to the activation of AMP-activated protein kinase because Ari acts as a partial agonist of dopamine receptor D2.¹³ However, the mechanism underlying the IR-sensitizing effect of Ari has not been elucidated. In the present study, the IR-sensitizing effect of Ari was thought to be due to promotion of the known production of ROS by IR. Thus, the ROS produced by adding Ari increases the therapeutic effect of IR. Forno et al. reported that the cytotoxicity of Ari in human hepatic cells was due to activation of the unfolded protein response.¹⁴ Although the unfolded protein response is a mechanism that is generally induced when endoplasmic reticulum stress occurs, and this stress accompanies the generation of ROS^{15,16}, the IR-sensitizing effect of Ari can be attributed to the enhanced generation of ROS by Ari and IR, although further studies are needed to define the exact mechanism.

In this study, we found that Ari, a FDA-approved antipsychotic drug, showed excellent radiosensitizing effects in both in vitro and in vivo models of head and neck cancer. However, the results of this study have a limitation in that they do not provide evidence for whether Ari can exhibit a similar radiosensitizing

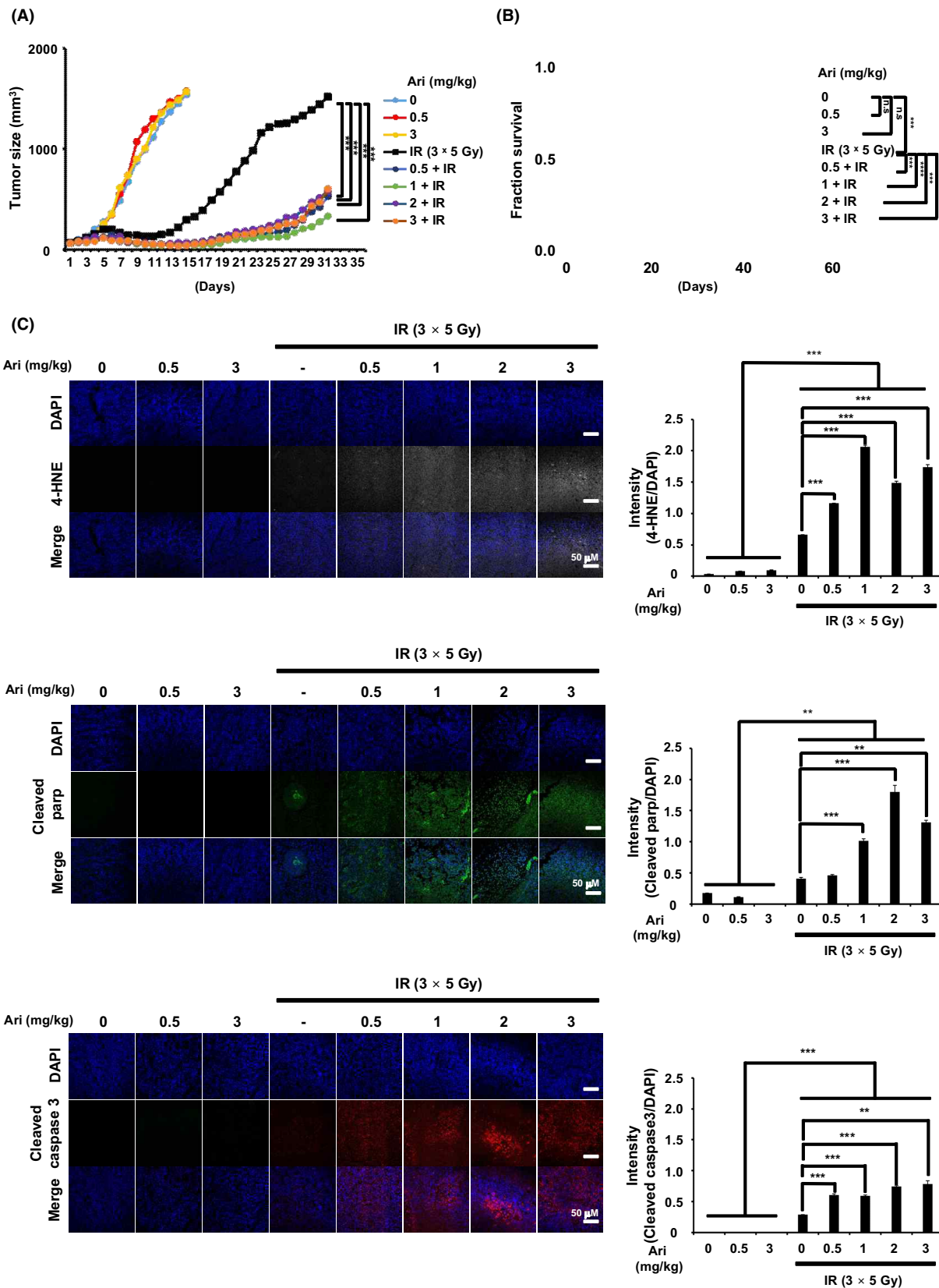


FIGURE 4 Effect of combined aripiprazole (Ari) and ionizing radiation (IR) treatment on xenograft tumor growth of head and neck cancer FaDu cells. (A) Measurements of the in vivo tumor growth rate. (B) Kaplan–Meier survival curves of control, Ari only, IR only, and the Ari and IR combination treatment. Survival curves were generated when the tumor size reached 1500 mm³, which was considered lethal. (C) Immunohistochemistry of xenograft tissues probed with the indicated antibodies. DAPI, 4',6-diamidino-2-phenylindole. Representative pictures of the staining (left, magnification 40x) and quantification of the staining (right). ****p* < .005.

effect in other cancers for which radiotherapy is frequently administered and whether it can actually exhibit a radiosensitizing effect in clinical practice. Therefore, it would be recommended to investigate whether Ari shows radiosensitizing effect in other cancer types other than head and neck cancer through additional research, and to apply whether Ari shows radiosensitizing effect in actual patients by conducting clinical research.

In conclusion, we found that Ari exhibited a strong IR-sensitizing effect on HNC cells and HNC xenograft tumors *in vitro* and *in vivo*, respectively, by promoting ROS production. Ari is a United States Food and Drug Administration approved antidepressant currently in clinical use; therefore, it is expected to be useful immediately as an excellent adjuvant that can dramatically increase the efficacy of IR therapy for cancer.

AUTHOR CONTRIBUTIONS

B. Park, Y. Moon, and M.-J. Park participated in research design. H.-J. Jeong and H.-J. Kim conducted experiments. J.-Y. Kim and C.-W. Jung performed data analysis. J.-Y. Kim and M.-J. Park wrote or contributed to the writing of the manuscript.

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DISCLOSURE

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

All animal experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee of the Korea Institute of Radiological & Medical Sciences, Republic of Korea.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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