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Complementary killing effect of tirapazamine in combination with radiation therapy on cells with high aldehyde dehydrogenase activity in SAS cell line

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► ***Original article***

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ABSTRACT

***Background*:** Tumor cells with high aldehyde dehydrogenase activity ($ALDH^{high}$ cells) are induced by an intratumoral hypoxic condition and lead to radioresistance, tumor recurrence, and metastasis. Therefore, to enhance the anti-tumor effect of radiotherapy, it is reasonable to efficiently control $ALDH^{high}$ cells by targeting them. In this study, we evaluated the effect of tirapazamine, a hypoxic toxin, combined with irradiation on $ALDH^{high}$ cells. ***Materials and Methods*:** Human tongue squamous cell carcinoma SAS cells were used in this study. Spheroids were irradiated with 6 Gy following treatment with 40 μM tirapazamine. After 24, 48, and 72 hours, the populations of $ALDH^{high}$ cells were analyzed. The frozen sections of spheroids were prepared, and hypoxia-inducible factor-1α-positive areas and ALDH1-positive areas were detected. ***Results*:** Compared with the cells grown in monolayer culture, the SAS cells grown in spheroids exhibited radioresistance. Furthermore, the proportion of $ALDH^{high}$ cells was significantly higher in spheroids than in monolayer culture. The $ALDH^{high}$ cells were sustained in a hypoxic fraction localized at the center of the spheroids after irradiation. Tirapazamine effectively reduced these $ALDH^{high}$ cells. The combination of tirapazamine with irradiation showed an additive cytotoxic effect in spheroids, but not in parental cells, which was consistent with a preferential killing effect of tirapazamine on $ALDH^{high}$ cells. ***Conclusion*:** Combined tirapazamine and radiotherapy administration appeared to be a reasonable approach to control $ALDH^{high}$ cells in hypoxic regions that may be involved in recurrence and metastasis.

INTRODUCTION

According to the World Cancer Report 2014, an estimated 529,000 new cases of oral-cavity and pharynx cancers were observed in 2012, with 292,000 deaths [1]. Despite recent technological advances that enable high tumor radiation dose concentrations, no recent dramatic improvements in locoregional control and the overall survival rate after radiotherapy have been observed. In recent years, research has shown that cell populations with high aldehyde dehydrogenase activity ($ALDH^{high}$ cells) are responsible for radioresistance in head and neck squamous cell carcinoma cells [2-4]. Cancer stem cell-like cells (CSC-like cells) are also radioresistant [5]. Therefore, radiotherapeutic outcomes for the head and neck cancer can be improved by establishing new approaches to targeting $ALDH^{high}$ cells. Many studies have shown that a hypoxic environment contributes to the growth and maintenance of $ALDH^{high}$ cells [6-8]. Consequently, controlling the hypoxic fraction in the tumor, which is thought to be a niche of CSC-like cells with high ALDH activity, can reduce the radiation-resistant $ALDH^{high}$ fraction and improve radiotherapeutic outcomes.

Tirapazamine is an aromatic N-oxide. In a hypoxic environment, tirapazamine is reduced by single-electron reductases to generate a radical species, leading to DNA damage. Many reports have shown that tirapazamine and its analogs have remarkable hypoxia-selective cytotoxicity [9-12]. Some clinical and preclinical studies have indicated that the combination of radiotherapy and hypoxic cytotoxins, including tirapazamine, with treatment individualization based on the extent of tumor hypoxia can improve treatment outcomes [13-16]. However, in a phase III clinical trial for head and neck cancer, the combination of tirapazamine with radiotherapy without confirming the presence of hypoxic fractions in each case failed to show an

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overall benefit. Consequently, research on tirapazamine combined with radiotherapy has been suspended, and the effect of tirapazamine on the recently discovered radioresistant fraction of $ALDH^{high}$ cells has not been clarified.

This study aimed to assess the efficacy of tirapazamine combined with irradiation on the radioresistant hypoxic fraction of $ALDH^{high}$ cells.

MATERIALS AND METHODS

Materials

Tirapazamine and fetal bovine serum (FBS) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Phosphate-buffered saline (PBS) was obtained from Fujifilm (Osaka, Japan). Tirapazamine was diluted in PBS. Penicillin and streptomycin were obtained from Gibco-Invitrogen Corp. (Carlsbad, CA, USA). A dead cell removal kit (#130-090-101) was obtained from Miltenyi Biotec (Auburn, CA, USA). An Aldefluor kit (#01700) was obtained from Stemcell Technologies (Vancouver, BC, Canada). Alexa Fluor® 555 conjugated-rabbit anti-human ALDH1 polyclonal antibody (#bs-10162R-A555) was obtained from Bioss, Inc. (Woburn, MA, USA). Mouse anti-human HIF-1α antibody (#610959) was obtained from BD Pharmingen (Franklin Lakes, NJ, USA). FITC-conjugated donkey anti-mouse IgG (#sc-2099) was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Vectashield® mounting medium for fluorescence with 4′, 6-diamidino-2-phenylindole (DAPI) (#H-1200) was obtained from Vector Laboratories, Inc. (Burlingame, CA, USA).

Cell culture and growth conditions

The human tongue squamous cell carcinoma cell line, SAS, was provided by the Riken BioResource Center (Ibaraki, Japan). The human oral squamous cell carcinoma cell lines HO1-u-1, HSC-2, HSC-3, and Ca9-22 were provided by the Cell Resource Center for Biomedical Research, Institute of Development, Aging, and Cancer, Tohoku University (Miyagi, Japan). Cells were grown in RPMI1640 (Gibco) supplemented with 10% FBS and 1% penicillin and streptomycin antibiotics at 37°C in a 5% CO_2 incubator with 21% O_2. Hypoxia was defined as 1% O_2. This environment was achieved by culturing cells in modular incubator chambers (Billups-Rothenberg, Del Mar, CA, USA), which were flushed with gas mixtures (95% N_2/5% CO_2) and sealed to maintain hypoxia after checking oxygen concentrations by using an O_2 monitor (JKO-02 Ver. III; JIKCO, Tokyo, Japan).

Spheroid generation and culture

Cells were seeded on low attachment prime surface dishes or plates (Sumitomo Bakelite, Tokyo, Japan), which were incubated for 7 days under normoxia (21% O_2) at 37°C. Spheroids were trypsinized with 0.25% trypsin in PBS for 7 minutes and incubated in Hank's balanced salt solution with 0.1 mg/mL DNase I (#11284932001; Sigma-Aldrich) for 7 minutes at 37°C and dissociated into single cells by pipetting using a 1000 μL pipette tip.

Irradiation

Cells were exposed to X-rays (6 MV) by using a Clinac® iX system linear accelerator (Varian Medical Systems, Inc.). Irradiation doses of 1 to 6 Gy were given at a dose rate of 100 MU/minute at room temperature.

Trypan blue dye exclusion assay

Parental monolayer cells (parental cells) of SAS, HO1-u-1, HSC-2, HSC-3, and Ca9-22 were plated into 24-well plates (BD Labware, Franklin Lakes, NJ, USA) at 1.5×10^5 cells/well and cultured with tirapazamine at a concentration of 0 and 40 μM under normoxia or hypoxia at 37°C. After 24 hours of incubation, the cells were stained with 0.5%-trypan blue stain solution, and cell viability was assessed by using a hemocytometer to count the number of stained and unstained cells. In the same way, only SAS cells were treated with tirapazamine at concentrations of 0, 0.1, 1.0, 5.0, 10, 20, 40, and 100 μM under normoxia or hypoxia for 24 hours, and cell viability was assessed.

Clonogenic assay

Parental cells of SAS were plated into 60-mm dishes (Iwaki, Shizuoka, Japan) at 200–2000 cells per dish and cultured under normoxia at 37°C. After 30 hours, the cells were treated with tirapazamine at concentrations of 0, 0.5, 1.0, 5.0, 10, 20, 50, and 100 μM under normoxia or hypoxia at 37°C. After 6 hours of incubation, the medium was exchanged with fresh RPMI1640. After incubation for 8 days under normoxia, the cells were fixed with methanol and stained with 4% Giemsa dye.

Parental cells or spheroids were treated with/without tirapazamine at a concentration of 40 μM under normoxia. After 6 hours of incubation, the cells were irradiated with 0, 1, 3, or 6 Gy. After irradiation, the cells were trypsinized and dissociated into single cells. Then, the cells were seeded into 60-mm dishes at 200–4000 cells per dish based on the dose of irradiation and concentration of tirapazamine. After incubation for 8 days under normoxia, the cells were fixed with methanol and stained with 4% Giemsa dye.

Real-time polymerase chain reaction (PCR)

Total cellular RNA was isolated from the cells by using the RNeasy® Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. First-strand cDNA was synthesized with an iScript RT Supermix for RT-qPCR® (Bio-Rad, Hercules, CA, USA) from extracted total RNA according to the manufacturer's protocol. Gene expression was assessed by performing real-time polymerase chain reaction with SsoAdvanced Universal SYBR Green

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Supermix® (Bio-Rad), with typical amplification parameters (95°C for 30 seconds, followed by 40 cycles at 95°C for 10 seconds, and 60°C for 20 seconds) on a CFX connect™ real-time PCR detection system (Bio-Rad). The mRNA expression values were analyzed using the ΔΔCt method after normalization with GAPDH. The oligonucleotide primer sets used for real-time PCR were purchased from Takara Bio Inc. (Shiga, Japan) and are described as follows:

HIF1A:
forward 5′-CTCATCAGTTGCCACTTCCACATA-3′
reverse 5′-AGCAATTCATCTGTGCTTTCATGTC-3′

CA9:
forward 5′- ACCAGACAGTGATGCTGAGTGCTAA-3′
reverse 5′-TCAGCTGTAGCCGAGAGTCACC-3′

GAPDH:
forward 5′-GCACCGTCAAGGCTGAGAAC-3′
reverse 5′-TGGTGAAGACGCAGTGGA-3′

Flowcytometry

A single-cell suspension of 1.5 × 10^5 cells was centrifuged at 1200 rpm for 3 minutes. The cell pellet was resuspended in 150 μL of Aldefluor® buffer containing 0.75 μL of activated Aldefluor® reagent. One half of the sample was transferred to a second tube containing 0.75 μL of the ALDH-specific inhibitor diethylaminobenzaldehyde (DEAB). Samples were incubated for 30 minutes at 37°C and analyzed by using a Cytoflex (Beckman Coulter, Inc.). The cells treated with DEAB were used as the negative control.

Immunofluorescence microscopy

A covered glass was placed in each 35-mm dish (Iwaki), and SAS cells were seeded at 2.0 × 10^5 cells per dish and incubated for 48 hours at 37°C under normoxia. Then, the cells were fixed for 20 minutes with methanol at 4°C and rinsed gently three times with PBS. Spheroids were fixed for 3 hours with methanol at 4°C, and then washed gently three times with PBS. After fixation, spheroids were incubated in 30% sucrose in PBS for 3 hours at 4°C and then processed to give 5-μm frozen sections. The sections and cells on the cover glasses were incubated with 4% Block Ace solution (DS Pharma Biomedical, Osaka, Japan) for 30 minutes at room temperature. After washing with PBS, Alexa Fluor® 555 conjugated-rabbit anti-human ALDH1 polyclonal antibody (#bs-10162R-A555) (1:500) in Can Get Signal® solution 2 (TOYOBO, Tokyo, Japan) were added, and the mixture was incubated overnight at 4° C. After washing with PBS, mouse anti-human hypoxia-inducible factor (HIF)-1α antibody (#610959) (1:200) in Can Get Signal® solution 1 was added, and the mixture was incubated overnight at 4° C. Then, fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse immunoglobulin G (#sc-2099) (1:100) in Can Get Signal® solution 2 was added, and the mixture was incubated 3 hours at room temperature and counterstained with Vectashield® mounting medium for fluorescence with DAPI (#H-1200). The slides were examined under a FluoView FV10i confocal laser scanning microscope (Olympus Corporation, Tokyo, Japan) with 60× water immersion objective lens and filters for DAPI (excitation: 405 nm; emission: 461 nm), FITC (excitation: 473 nm; emission: 519 nm) and TRITC (excitation: 559 nm; emission: 578 nm). Images were analyzed using FV10-ASW4.2 software (Olympus Corporation).

Statistical analysis

All experiments were performed at least three times. The significance of differences was determined by using the two-sided Student's t-test and Welch's t-test depending on data distribution. The significance level was set to $P < 0.05$. Excel 2016 software (Microsoft, Redmond, WA, USA) with the add-in software Statcel 4 was used for statistical analysis.

RESULTS

Evaluation of the potential efficacy of tirapazamine against head and neck cancer cell lines in monolayer culture

The cytotoxicity of tirapazamine in different cell lines of head and neck cancers was examined using the trypan blue exclusion assay. In SAS, Ca9-22, HO-1-u-1, HSC-2, and HSC-3 cells in monolayer culture, tirapazamine showed stronger growth inhibition under hypoxia with 1% O_2 than under normoxia with 21% O_2. The cytotoxic effects were lowest in the SAS cell line (figure 1A). To examine the mechanism of anti-tumor effects of tirapazamine and effectiveness of tirapazamine in combination with irradiation, we planned to use a three-dimensional tumor spheroid model to reproduce the tumor microenvironment. However, in the process of collecting cells by dissociation of spheroids to evaluate each cell in the spheroids, only SAS cells could be collected with >90% cell viability from spheroids, so they were chosen for subsequent experiments. The cells of all cell lines other than SAS were not used for subsequent experiments because >40% of the cells were damaged and lost during the process under the same conditions of spheroid dispersal. The cytotoxicity of tirapazamine under hypoxia was concentration-dependent in SAS (figures. 1B, C).

Relationship between hypoxia and the ALDH activity phenotype in SAS cells

To investigate the oxygen properties in SAS spheroids, the spheroids were cultured from a monolayer parental cell line. In the grown spheroids, cells accumulating HIF-1α were localized at the

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center (figure 2A). In the parental and spheroid cells, the mRNA expression level of HIF-1α did not change, but the mRNA expression level of carbonic anhydrase IX (CAIX), a hypoxia marker whose expression was enhanced in response to the intracellular accumulation of HIF-1α, was increased (figures 2B, C). Reportedly, hypoxia induces cells that highly express ALDH [6-8]. Therefore, the populations of ALDH^{high} cells in the parental cells and spheroids were evaluated by performing the Aldefluor assay. In the absence of the ALDH-specific inhibitor DEAB, more ALDH^{high} cells were observed in spheroid than in parental cells (figure 2D), and as shown in figure 2E, the population of ALDH^{high} cells was higher in spheroids than in parental cells (26.83% ± 2.71% vs. 37.85% ± 1.39%, P = 0.02). High ALDH activity has been linked to tumor radioresistance in head and neck squamous cell carcinoma (HNSCC) [2-4]. The clonogenic assay results are shown in figure 2F. Spheroids exhibited greater resistance to irradiation than parental cells. In summary, ALDH^{high} cells increased in spheroids, and spheroids were more resistant to irradiation.

Effect of tirapazamine on ALDH^{high} cells

We evaluated the effect of tirapazamine and irradiation on the hypoxic fraction and ALDH^{high} cell fraction. The population of ALDH^{high} cells treated with irradiation alone was sustained 72 hours after treatment. Conversely, after administering tirapazamine alone or in combination with irradiation, the population of ALDH^{high} cells decreased (figure 3A, B). In addition, 72 hours after irradiation, the frozen sections of spheroids were prepared, and ALDH^{high} cells treated with irradiation were sustained in the HIF-1α-positive areas (figure 3C). However, after irradiation with tirapazamine, the HIF-1α-positive areas inside spheroids were decreased, and ALDH^{high} cells were decreased. In the assessment of the anti-tumor effect of tirapazamine on spheroids, tirapazamine combined with irradiation showed an additive cytotoxic effect relative to that of irradiation alone (irradiation alone, 0.061 ± 0.016; tirapazamine alone, 0.222 ± 0.090; tirapazamine + irradiation, 0.008 ± 0.003) (figure 3E). Although, no additive effects were observed for tirapazamine combined with irradiation in parental cells (figure 3D). These results suggested that tirapazamine preferentially killed the cells in the hypoxic fraction of the spheroids, including ALDH^{high} cells, and thereby complementarily killing cells that are difficult to injure with irradiation.

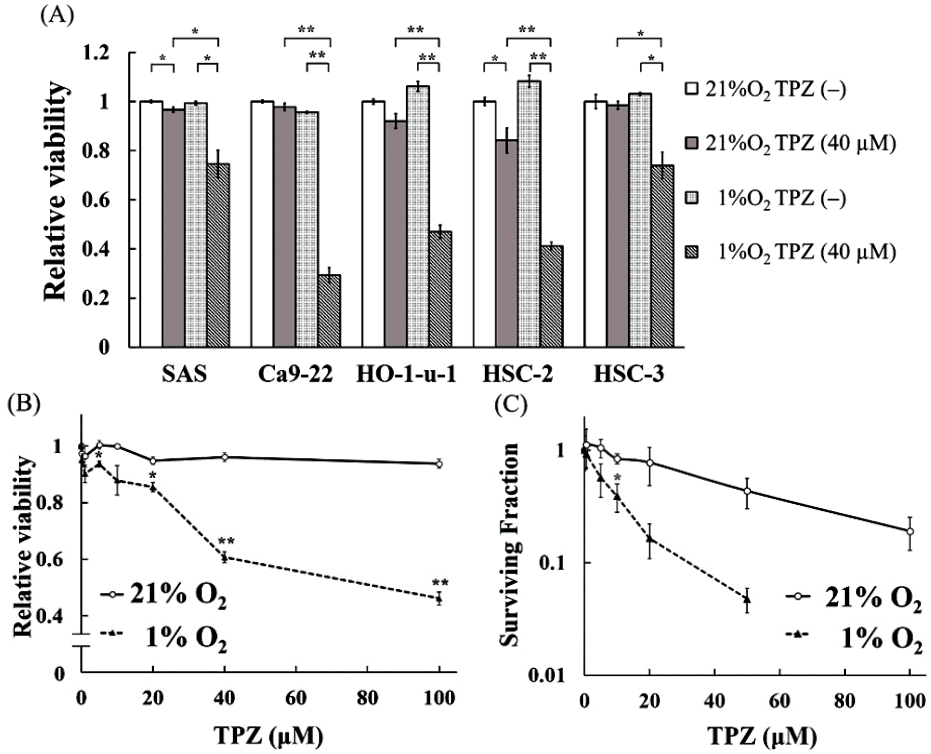

Figure 1. Tirapazamine exhibited higher cytotoxicity in parental cells under hypoxia than under normoxia. **(A, B)** Results of trypan blue dye exclusion assay for parental cells. **(A)** SAS, HO1-u-1, HSC-2, HSC-3, and Ca9-22 cells were treated with tirapazamine at a concentration of 0 and 40 μM for 24 hours under normoxia or hypoxia. The cell viability was normalized by each untreated control sample under normoxia. *P < 0.05, **P < 0.01. **(B)** SAS cells were treated with tirapazamine at a concentration of 0, 0.1, 1.0, 5.0, 10, 20, 40, and 100 μM for 24 hours under normoxia or hypoxia. The cell viability was normalized by each untreated control sample. * = significantly different relative to cells treated at the same concentration under normoxia (* P < 0.05, **P < 0.01). **(C)** The results of the clonogenic assay for SAS parental cells. Cells were treated with tirapazamine at a concentration of 0, 0.5, 1.0, 5.0, 10, 20, 50, and 100 μM for 6 hours under normoxia or hypoxia. Surviving fractions were normalized by each untreated control sample. * = significantly different relative to cells treated at the same concentration under normoxia (*P < 0.05). Abbreviation: TPZ, tirapazamine.

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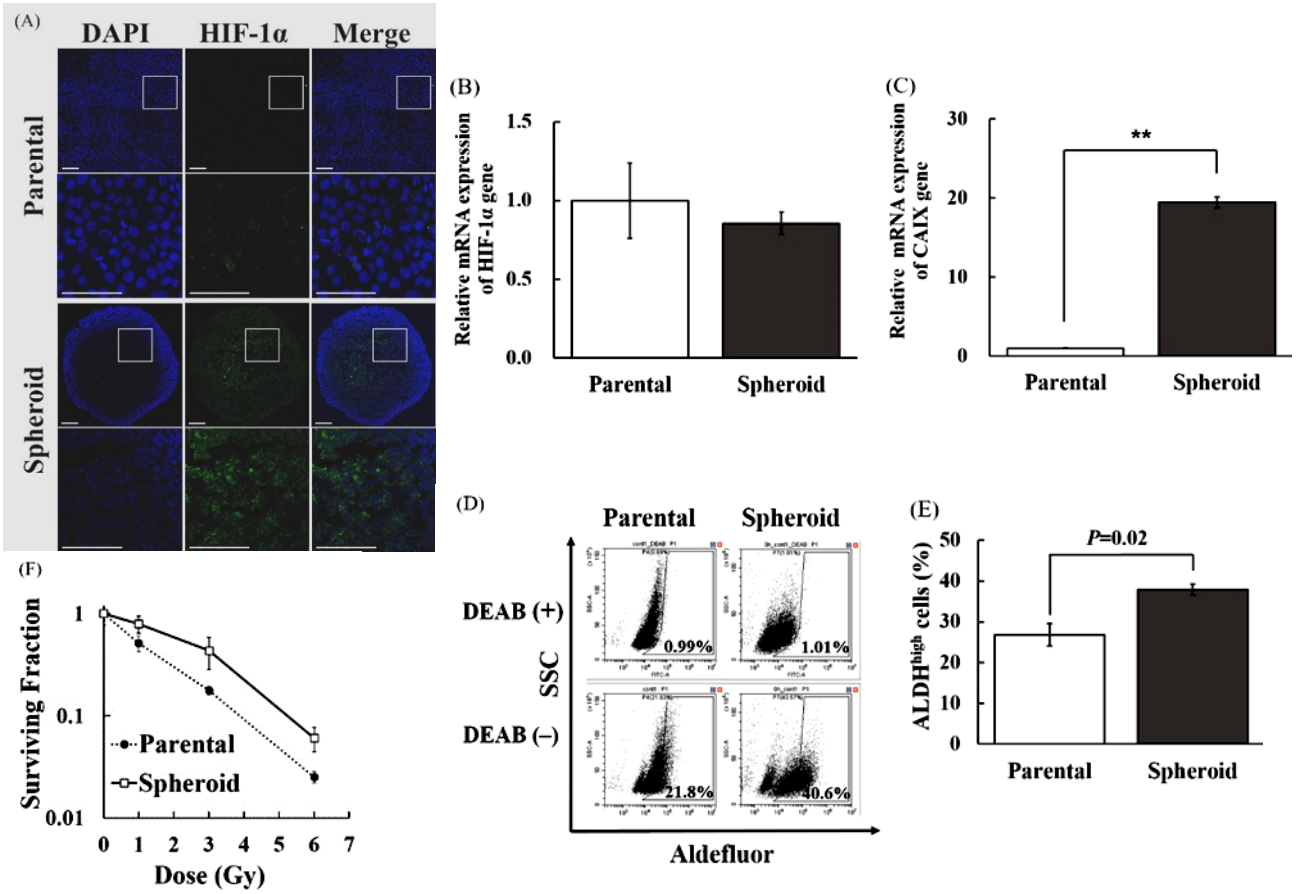

Figure 2. The hypoxic or ALDH^{high} cell fraction in parental cells and spheroids. **(A)** Detection of HIF-1α on SAS parental cells or spheroids after 7 days of culture under normoxia. Sections were observed under a confocal microscope with 60× water immersion objective lens. Scale bar, 100 μm. **(B, C)** Relative mRNA expressions of **(B)** HIF-1α and **(C)** CAIX genes in parental cells or spheroids after 7 days of culture under normoxia were detected by real-time PCR. Results were analyzed using the ΔΔCt method after normalization with GAPDH. $^{**}P < 0.01$. **(D, E)** The results of the Aldefluor assay for parental cells or spheroids. **(D)** The typical histogram and **(E)** the population of ALDH^{high} cells. Cells were dissociated into a single-cell suspension and analyzed by flow cytometry. The cells treated with DEAB were used as a negative control. **(F)** Results of the clonogenic assay. Parental cells or spheroids were irradiated with 1, 3, or 6 Gy under normoxia. After irradiation, the cells were dissociated into single cells and reseeded into new dishes. Surviving fractions were normalized by each unirradiated control sample. Abbreviations: ALDH, aldehyde dehydrogenase; ALDH^{high} cells, cells with high aldehyde dehydrogenase activity; CAIX, carbonic anhydrase IX; DAPI, 4',6-diamidino-2-phenylindole; DEAB, diethylaminobenzaldehyde; HIF, hypoxia-inducible factor.

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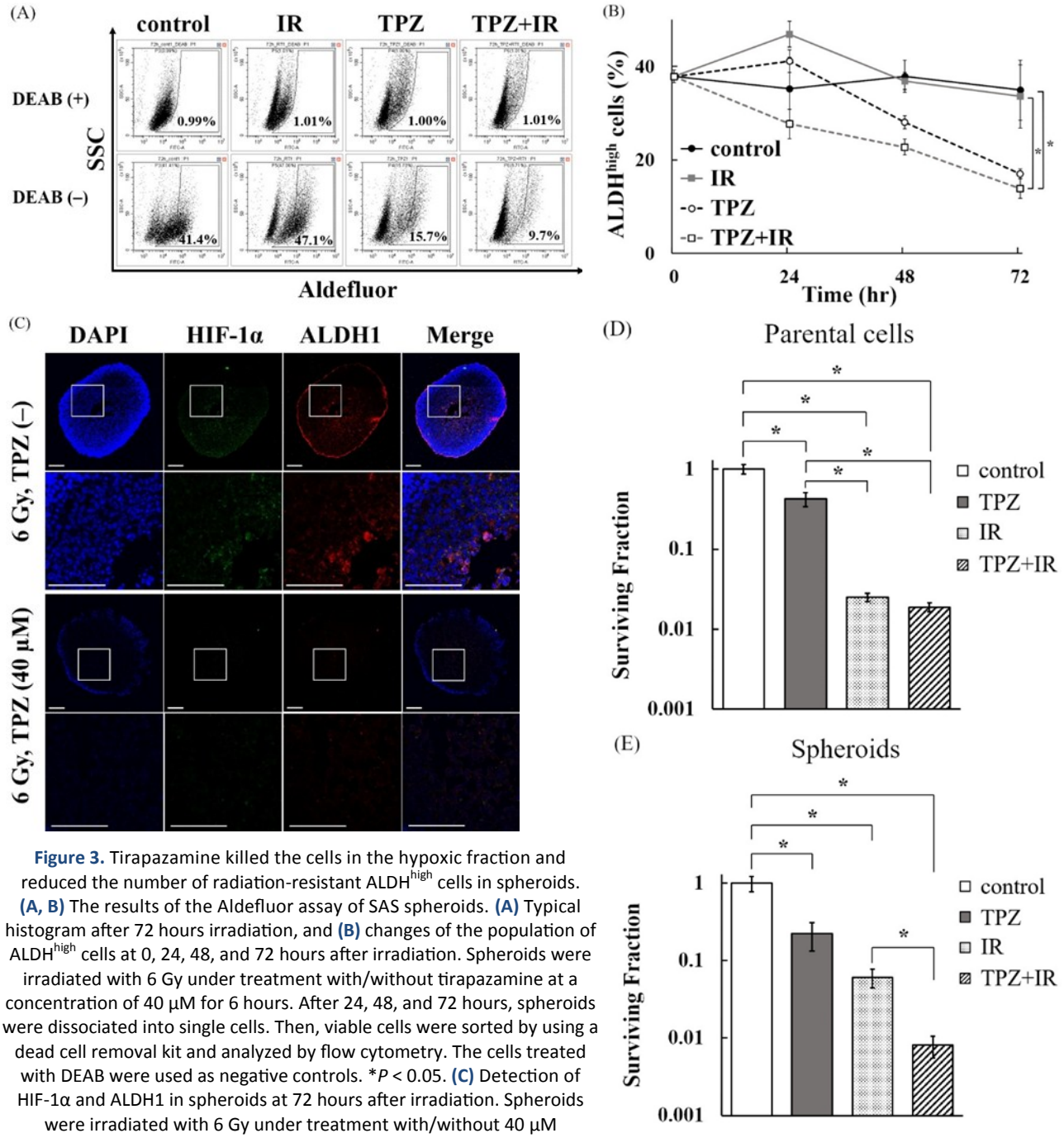

Figure 3. Tirapazamine killed the cells in the hypoxic fraction and reduced the number of radiation-resistant ALDH^{high} cells in spheroids. **(A, B)** The results of the Aldefluor assay of SAS spheroids. **(A)** Typical histogram after 72 hours irradiation, and **(B)** changes of the population of ALDH^{high} cells at 0, 24, 48, and 72 hours after irradiation. Spheroids were irradiated with 6 Gy under treatment with/without tirapazamine at a concentration of 40 μM for 6 hours. After 24, 48, and 72 hours, spheroids were dissociated into single cells. Then, viable cells were sorted by using a dead cell removal kit and analyzed by flow cytometry. The cells treated with DEAB were used as negative controls. *P < 0.05. **(C)** Detection of HIF-1α and ALDH1 in spheroids at 72 hours after irradiation. Spheroids were irradiated with 6 Gy under treatment with/without 40 μM tirapazamine for 6 hours in normoxia. After 72 hours, the frozen sections of spheroids were prepared, and HIF-1α- and ALDH1-positive areas were detected. Sections were observed under a confocal microscope with 60× water immersion objective lens. Scale bar, 100 μm. **(D, E)** The results of the clonogenic assay for **(D)** parental cells and **(E)** spheroids. Cells were cultured with tirapazamine at a concentration of 40 μM under normoxia for 6 hours. Then, cells were irradiated with 6 Gy, dissociated into single cells, and reseeded into new dishes. Surviving fractions were normalized by each untreated control sample. *P < 0.05. Abbreviations: ALDH, aldehyde dehydrogenase; ALDH^{high} cells, cells with high aldehyde dehydrogenase activity; DAPI, 4′,6-diamidino-2-phenylindole; DEAB, diethylaminobenzaldehyde; HIF, hypoxia-inducible factor; IR, irradiation; TPZ, tirapazamine.

DISCUSSION

CSC-like cells that have high ALDH activity can cause radioresistance and recurrence, whereas ALDH^{high} cells that do not necessarily have all CSC-like cell markers have also been proven to be radioresistant [2-4]. Furthermore, irradiation itself can induce ALDH^{high} cells [17, 18]. The present study also showed that ALDH hyperactive cells were induced by radiation and survived in hypoxic regions. Therefore, to improve the outcome of radiation therapy, it is important to develop therapies targeting ALDH^{high} cells. Hypoxic niches directly protect CSC-like cells that have high ALDH activity from irradiation through lack of oxygen. And the activation of the HIF signaling pathway, at least in part, contributes to the

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growth and survival of CSC-like cells [7, 19]. Therefore, ALDH^{high} cells can be reduced with treatments that target the hypoxic environment of tumors.

In a recent study using the spheroid model of ovarian cancer cells, the internal areas of spheroids, which contained Oct4-expressing cells, disappeared following 24-hour treatment with TX-402, which is an analog of tirapazamine [20]. In our study, 72 hours after irradiation, tirapazamine reduced the HIF-1α- and ALDH1-positive areas at the central areas of spheroids in the same section (figure. 3C). In addition, this is possibly the first study to evaluate the time course of ALDH^{high} cells in spheroids after treatment with hypoxic cytotoxins and irradiation (figures. 3A, B). Furthermore, regarding the anti-tumor effect of tirapazamine on spheroids, the combination of tirapazamine and irradiation significantly reduced the surviving fractions relative to those for irradiation alone (figure 3E). ALDH^{high} cells show high tumorigenicity, invasion ability, and radiation and chemotherapy resistance. Therefore, the high activities of ALDH are often used as a CSC-like phenotype [2-4]. Croker [21] showed that treatment with the specific ALDH inhibitor, DEAB, sensitized ALDH^{high} CD44+ human breast cancer cells to radiation. Kurth [2] found that there was at least a partial association between high ALDH activity and ALDH1A3 expression in HNSCC, and knockdown of ALDH1A3 decreases tumor radioresistance. Therefore, reduction of ALDH^{high} cells and ALDH1-positive areas with tirapazamine would contribute to the anti-tumor effect in combination with radiation therapy.

As the Trans-Tasman Radiation Oncology Group 98.02 showed, for patients with HNSCC with hypoxic tumors detected by ^{18}F-fluoromisonidazole (FMISO), the combination of tirapazamine and radiation improved locoregional control rates [14]. The present study results support the effectiveness of using hypoxic cytotoxins combined with radiotherapy if they can reduce radioresistant CSC-like cells. In recent years, tracers, such as ^{18}F-fluoroazomycin arabinoside (FAZA), ^{18}F-flortanidazole (HX4), and ^{18}F-FRP170—which are more water soluble and more transferrable to tissues than ^{18}F-FMISO—have been developed [22-24]. Reportedly, ^{18}F-FAZA and ^{18}F-HX4 can predict the effect of radiotherapy combined with tirapazamine or with TH-302 at preclinical levels [13, 15]. We believe that the combination of radiotherapy and hypoxic cytotoxins, including tirapazamine, when used with individualized treatment based on the extent of tumor hypoxia, can improve treatment outcomes.

CONCLUSION

In this study, we showed that the combination of tirapazamine and irradiation had a complementary anti-tumor effect on tumor spheroids in human tongue squamous cell carcinoma SAS cells. Our study results also suggested that tirapazamine not only reduced the hypoxic fraction but also ALDH^{high} cells and thereby lowered tumor radioresistance. The combination of tirapazamine and radiotherapy potentially can be an effective treatment modality for patients with an intratumoral hypoxic fraction.

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***Conflicts of Interest*:** None declared.
***Ethical considerations*:** Not applied.
***Authors' Contributions*:** MS and KH conceived the study and helped run the study, collect data, and draft the manuscript. KI carried out experiments and performed data acquisition, data analysis, and drafting of the manuscript. FK, MT, IF, HK, YH YT and MA performed the support of data analysis and drafting of the manuscript during the study. All authors read and approved the final manuscript.
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