Transcutaneous carbon dioxide enhances the antitumor effect of radiotherapy on oral squamous cell carcinoma

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Abstract. Radiotherapy (RT) is one of the main treatment modalities for oral squamous cell carcinoma (OSCC), however, radioresistance is a major impediment to its clinical success and poses as a concern that needs to be addressed. Tumor hypoxia is known to be significantly associated with radioresistance in various malignancies, hence, resolving the hypoxic state of a tumor may improve the antitumor effect of RT on OSCC. We have previously revealed that transcutaneous CO_2 induced mitochondrial apoptosis and suppressed tumor growth in OSCC by resolving hypoxia. Considering the previous study, we hypothesized that transcutaneous CO_2 may enhance the antitumor effect of RT on OSCC by improving intratumoral hypoxia, thereby overcoming radioresistance. In the present study, the combination of transcutaneous CO_2 and RT significantly inhibited tumor growth compared with other treatments. This combination therapy also led to decreased expression of HIF-1α in parallel with increased expression of the cleaved forms of caspase-3-8-9 and PARP, which play essential roles in mitochondrial apoptosis. Additionally, the combination therapy increased the expression of ROS modulator 1 and subsequent mitochondrial ROS production, compared to RT alone. These results indicated that transcutaneous CO_2 could potentially improve the antitumor effect of RT by decreasing the intratumoral hypoxia and increasing the mitochondrial apoptosis. Our findings indicated that CO_2 therapy may be a novel adjuvant therapy in combination with RT for OSCC.

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Introduction

At present, the main treatments for oral squamous cell carcinoma (OSCC) are surgery, chemotherapy and radiotherapy (RT). RT is a particularly effective strategy for OSCC, since it is employed as both a primary modality and an adjuvant treatment following surgery. Despite modern radiation techniques, many studies have highlighted several complications associated with RT, including bone marrow suppression (1-3). Disregarding the associated complications, the therapeutic success of RT is also challenged by the development of tumor-cell radioresistance, which can be attributed to cell cycle, differentiation and hypoxia (4-6). Among these, tumor hypoxia is known to exhibit a significantly strong association with radioresistance (7,8). Therefore, resolving tumor hypoxia could be a key strategy in overcoming radioresistance and improving the antitumor effect of RT.

The importance of improving tumor oxygenation for potential clinical use has already been recognized and some strategies, including the use of radio-sensitizers and hypoxic cytotoxins, have been developed to overcome hypoxia-mediated tumor radioresistance (9). However, these strategies often lead to the development of more severe side-effects (10,11).

Tumor hypoxia is a characteristic feature of malignant tumors, including OSCC (12). In hypoxic conditions, hypoxia inducible factor-1α (HIF-1α), an oxygen-dependent α subunit of HIF, activates the transcription of specific metastatic genes, thus playing an important role in the growth and survival of malignant tumors (13,14). In addition, a recent study has revealed that HIF-1α plays an important role in hypoxia-related tumor radioresistance (15).

We previously revealed that transcutaneous CO_2 application caused absorption of CO_2 and an increase in the O_2 pressure in treated tissues, potentially causing an 'artificial Bohr effect' (16). We have also reported that CO_2 therapy induced mitochondrial apoptosis and suppressed tumor growth in OSCC by resolving hypoxia (17). Furthermore, we have revealed that transcutaneous CO_2 decreased the expression of HIF-1α in

OSCC. Considering our previous study, we hypothesized that transcutaneous CO_2 may enhance the antitumor effect of RT on OSCC by improving intratumoral hypoxia, thereby overcoming radioresistance.

In the present study, we investigated the antitumor effects of a combination treatment of transcutaneous CO_2 with RT by using human OSCC xenograft models *in vivo*.

Materials and methods

Cell culture. A human OSCC cell line, HSC-3, was used in our study (Health Science Research Resources Bank, Osaka, Japan). The HSC-3 cell line was established from a metastatic deposit of poorly differentiated OSCC of the tongue in a mid-internal jugular lymph node from a 64-year-old man (18). The HSC-3 cells were routinely cultured in Eagle's minimum essential medium (EMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) and 1,000 U/ml penicillin/streptomycin solution (Sigma-Aldrich) in an incubator with 5% CO_2 at 37˚C. Trypsin (0.25%) and ethylenediaminetetraacetic acid (EDTA, 0.02%) (Sigma-Aldrich) solution were used to isolate the cells for subculture, as previously described (17,19).

X-ray irradiation. X-ray irradiation was performed at a dose rate of 0.75-0.78 Gy/min using a 150 kV X-ray generator unit operating at 5 mA with an external 0.1 mm aluminum filter (MBR-1505122; Hitachi Medical Co., Tokyo, Japan).

Colony formation assay. Colony formation assays for the X-ray irradiated HSC-3 cells were performed to evaluate the response of these cells to X-ray irradiation. HSC-3 cells in T-25 flasks (Corning Japan, Tokyo, Japan) were treated by X-ray irradiation at three different doses (0, 2 or 8 Gy). Immediately after X-ray irradiation, the cells were trypsinized, seeded at a density of 1,000 cells/well in 6-well plates, and incubated in a humidified atmosphere of 5% CO_2 at 37˚C. After 2 weeks of incubation, the cells were stained by Giemsa (Muto Pure Chemicals Co., Tokyo, Japan) and the number of colonies was counted.

Animal models. Twenty-four male athymic BALB/c nude mice, 7-week-old, were obtained from CLEA Japan (Tokyo, Japan). The animals were maintained under pathogen-free conditions, in accordance with the institutional guidelines. All animal experiments were performed in accordance with the Guidelines for Animal Experimentation of Kobe University Animal Experimentation Regulations (permission no. P120602) and were approved by the Institutional Animal Care and Use Committee. HSC-3 cells at doses of 4.0x106 cells in 500 μl phosphate buffered saline (PBS) were implanted into the back of 24 mice to create the human OSCC xenograft models.

Transcutaneous CO_2 therapy. Transcutaneous CO_2 therapy was performed as previously described (17,19). Briefly, the area of skin around the implanted tumor was covered with CO_2 hydrogel. This area was then sealed with a polyethylene bag, and 100% CO_2 gas was administered into the bag. Treatment was performed for 20 min each, twice a week for 2 weeks.

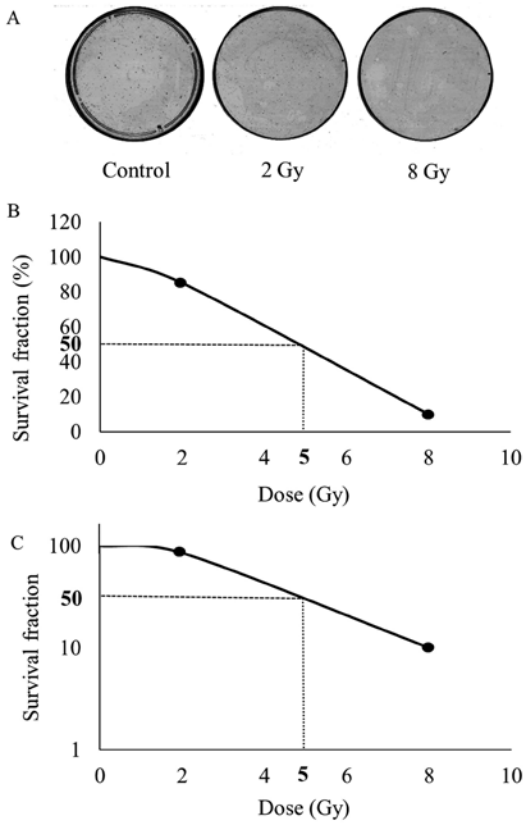

Figure 1. Effect of X-ray irradiation on the survival of human HSC-3 cells *in vitro*. (A) Colony formation assay at three different doses (0, 2 and 8 Gy). (B) IC_{50} in colony formation. (C) IC_{50} in colony formation by log scale.

Animals in the control group were treated in a similar manner by replacing CO_2 with room air.

In vivo studies. The mice implanted with HSC-3 cells were randomly divided into four groups: control group (n=6), RT group (n=6), CO_2 group (n=6) or the combination group (n=6). RT was performed at a dose of 5.0 Gy each and in the RT group, the mice were treated by RT alone twice a week for 2 weeks (20 Gy in total). Mice in the CO_2 group were treated by transcutaneous CO_2 therapy alone. In the combination group, the mice were treated by CO_2 therapy, immediately followed by RT. Tumor volume and body weight were monitored and calculated as previously described (17,19).

Treated tumors were removed 24 h after the final treatment and the total RNA and cell lysates were immediately extracted from the half of each tumor. The other half of each tumor was formalin-fixed and paraffin-embedded for staining. Serial 10-µm thick transverse sections were prepared from each block. Bone marrow suppression was investigated by the collection of 10 μl blood samples by inserting a heparinized capillary tube just below the eye ball. The obtained samples were diluted with Turk's solution (190 μl) and the leukocytes (white blood cells) in the samples were counted using a heamocytometer by a clinical laboratory technician.

Fluorescence activated cell scanning (FACS) assay. Flow cytometry was performed using a FACS Calibur™ flow cytometer (BD Pharmingen; BD Biosciences, Franklin Lakes, NJ, USA) as previously described (17). The apoptotic activity in

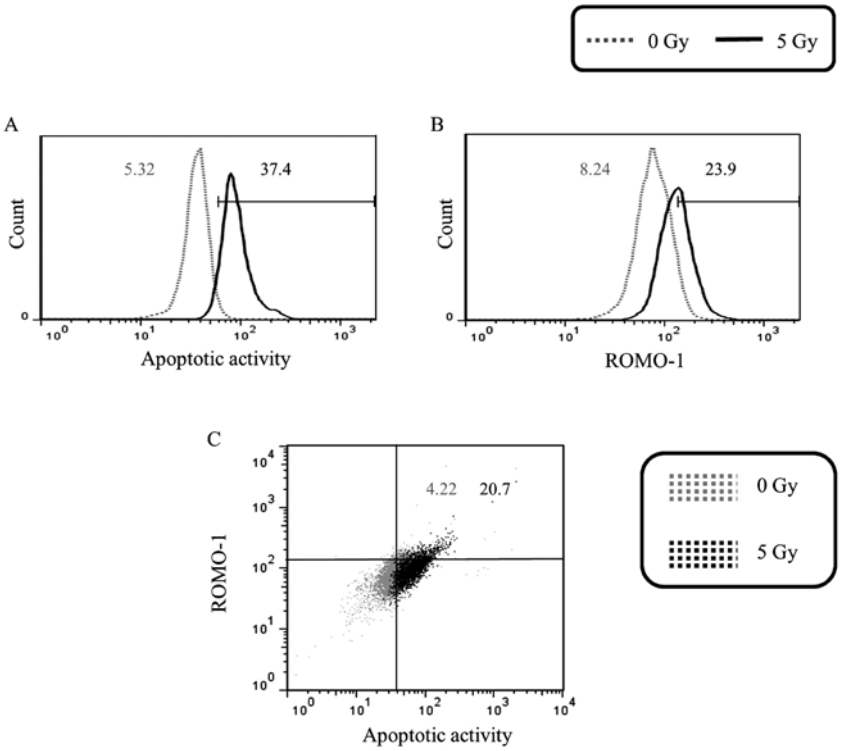

Figure 2. Effect of RT on ROS-related apoptotic activity in human HSC-3 cells *in vitro*. (A) Apoptotic activity and (B) ROS production were evaluated in human HSC-3 cells which were irradiated. (C) Correlation between apoptotic activity and ROS production in HSC-3 cells which were irradiated.

treated tumors was evaluated by DNA fragmentation using the Apo-Direct kit according to the manufacturer's protocol (BD Pharmingen; BD Biosciences). In addition, ROS production was evaluated using anti-human ROS modulator 1 (ROMO-1) antibody (1:1,000; cat. no. ab121379; Abcam, Cambridge, UK) as an indicator. The data were analyzed using BD CellQuest software (BD Biosciences), and apoptotic or ROMO-1 expression cells were quantified as a percentage of the total number of cells.

Immunohistochemical analysis. The formalin-fixed and paraffin-embedded tumor sections were pretreated with citrate buffer for 40 min at 95˚C, quenched with 0.05% H_2O_2 and incubated overnight at 4˚C with the following primary antibodies in Can Get Signal immunostain solution A (Toyobo, Osaka, Japan): rabbit anti-human HIF-1α antibody (1:1,000; cat. no. ab82832; Abcam) and anti-human ROS modulator 1 (ROMO-1) antibody (1:1,000; cat. no. ab121379; Abcam). Following the treatment, the sections were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG polyclonal antibody (1:1,000; cat. no. 6721; Nichirei Bioscience, Tokyo, Japan) for 30 min at room temperature. Signals were developed as a brown reaction product using peroxidase substrate 3',3'-diaminobenzidine (Nichirei Bioscience). The sections were counterstained with hematoxylin and examined with a BZ-8000 confocal microscope (Keyence, Osaka, Japan). Immunohistochemical-positive staining was semi-quantified by densitometric analysis using the ImageJ software, version 1.47 (National Institutes of Health, Bethesda, MD, USA) (http://rsbweb.nih.gov/ij/download.html). Values were normalized against the control group and were presented as ratios.

Immunofluorescence staining. To assess the apoptotic activity in treated tumors, we performed the immunofluorescence staining by using the Apo-Direct kit following the manufacturer's protocol (BD Biosciences). The nucleus was stained with DAPI. The images were captured using a BZ-8000 confocal microscope (Keyence).

Immunoblot analysis. The cell lysates were prepared from treated tumors by using a whole cell lysis buffer supplemented with Halt protease and phosphatase inhibitor cocktail (Mammalian Protein Extraction Reagent; Thermo Fisher Scientific, Rockford, IL, USA). The protein samples were processed using standard western immunoblot procedures. The membranes were incubated overnight at 4˚C with the following primary antibodies in Can Get Signal Immunoreaction Enhancer Solution 1 (Toyobo): anti-human caspase-8 antibody (1:1,000; cat. no. 9496; Cell Signaling Technology, Danvers, MA, USA), anti-human caspase-9 antibody (1:1,000; cat. no. 7237; Cell Signaling Technology), anti-human caspase-3 antibody (1:1,000; cat. no. 9664; Cell Signaling Technology), anti-human PARP antibody (1:1,000; cat. no. 5625; Cell Signaling Technology) and anti-human α-tubulin antibody (1:2,000; cat. no. 6074; Sigma-Aldrich). After washing, the membranes were incubated with the appropriate secondary antibody conjugated to horseradish peroxidase (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) in Can Get Signal Immunoreaction Enhancer Solution 2 (Toyobo) and exposed with ECL Prime Plus Western Blotting Detection System Reagent (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). The signals were detected using a Chemilumino analyzer LAS-3000 mini (Fujifilm, Tokyo, Japan). Positive bands in the immunoblots

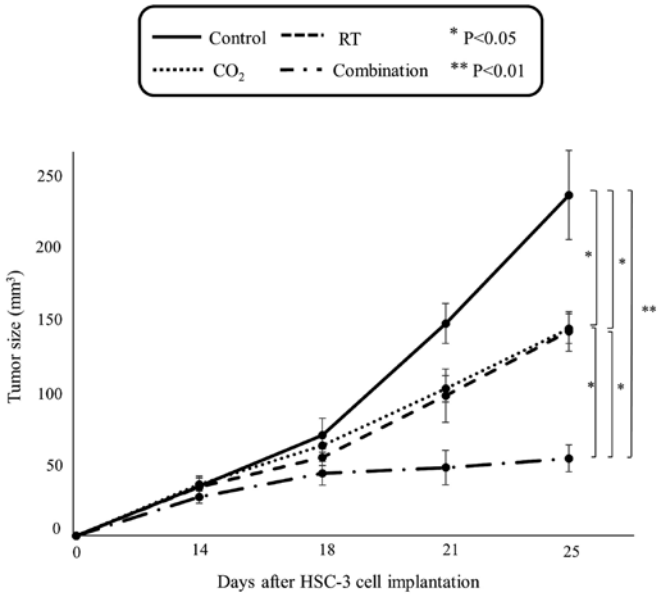

Figure 3. Tumor growth after multiple treatments *in vivo*. Tumor growth was monitored twice weekly until the end of the treatments. *P<0.05; **P<0.01.

were semi-quantified by densitometric analysis using ImageJ software version 1.47. The values were normalized against α-tubulin and were presented as ratios.

Statistical analysis. StatView J-4.5 software (Hulinks, Inc., Tokyo, Japan) was used for statistical analysis performed on all data for four groups by the Scheffe's test. Data was presented as the mean value ± standard error. P-value <0.05 was considered to indicate a statistically significant difference.

Results

RT induces ROS-related apoptosis in human HSC-3 cells in vitro. Colony formation assays for HSC-3 cells irradiated by various doses of X-rays were performed to evaluate the response of these cells to RT. The number of HSC-3 cell colonies decreased dose-dependently after RT (Fig. 1A). The half maximal inhibitory concentration (IC_{50}) in colony formation was achieved at a dose of 5.0 Gy (Fig. 1B).

Subsequently, the reliability of ROS-related apoptosis in X-ray irradiated HSC-3 cells in vitro was studied. There was a correlation between the apoptotic activity and the expression of ROMO-1 in the cells irradiated with 5.0 Gy X-rays (Fig. 2).

Transcutaneous CO_2 enhances the effect of RT on OSCC tumor growth with no observable side-effects in vivo. The effect of the transcutaneous CO_2 therapy on RT was investigated *in vivo*. Tumor volume in the combination group significantly decreased compared with that in the control group (P<0.01), in the RT group (P<0.05) and in the CO_2 group (P<0.05). At the end of the experiment, tumor volume in the combination group was reduced to 23, 38 and 37% of that in the control, RT, and CO_2 groups, respectively (Fig. 3). In addition, significant decrease in tumor volume was observed in the CO_2 and the RT groups compared with the control group (P<0.05). No significant difference in body weight was observed among the

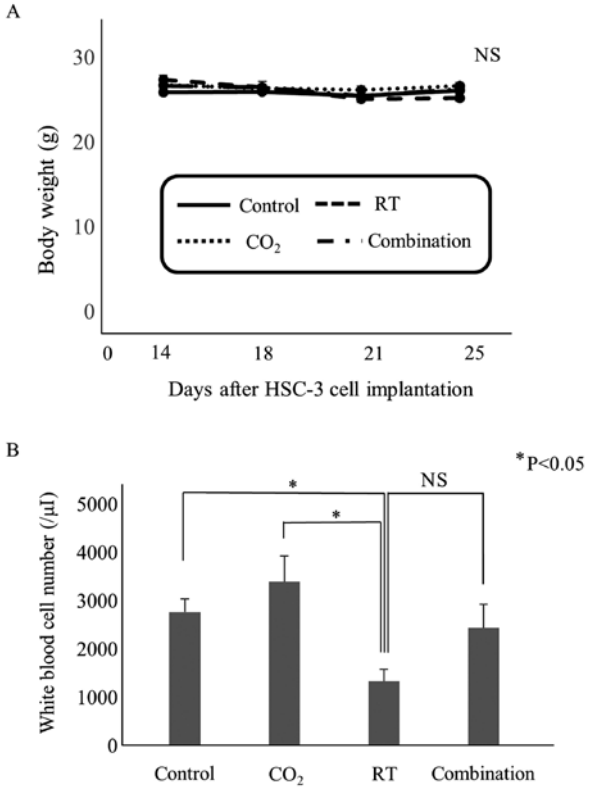

Figure 4. Presence or absence of side-effects of the CO_2 therapy. Body weight was monitored twice weekly until the end of the treatments. White blood cell number after multiple treatments *in vivo*. Leukocytes (white blood cells) count was performed using a heamocytometer. *P<0.05.

four groups (Fig. 4A), however the white blood cell number in the RT group was significantly decreased compared with that in the control and the CO_2 group (Fig. 4B).

Transcutaneous CO_2 with RT suppresses tumor growth by inducing apoptosis and ROS production in HSC-3 cells in vivo. Subsequently, we examined the *in vivo* effects of transcutaneous CO_2 with RT on the expression of HIF-1α, the apoptotic activity and the production of ROS in HSC-3 cells. Immunohistochemical positive staining for HIF-1α was hardly detectable in the CO_2 and the combination group compared with the other two groups (Fig. 5A). In contrast, positive staining for ROMO-1 (indicative of ROS production) was significantly higher in the combination group compared with that in the other three groups (Fig. 5B). Furthermore, immunofluorescence staining revealed that apoptotic activity was increased in both the CO_2 and the combination groups (Fig. 6A). Quantitative analysis of the immunofluorescence-positive staining revealed that apoptotic cells were significantly increased. Relative positive staining compared to the control was increased 19.0-, 46.0-, and 66.0-fold in the CO_2, RT and combination groups, respectively (Fig. 6B).

Immunoblot analysis revealed an increased protein expression of the cleaved forms of caspase-3 and PARP in the combination group compared with the other groups (Fig. 7A). The protein expression of cleaved caspase-8 was increased in the RT and the combination group, compared with that in the other two groups, whereas increased protein expression of

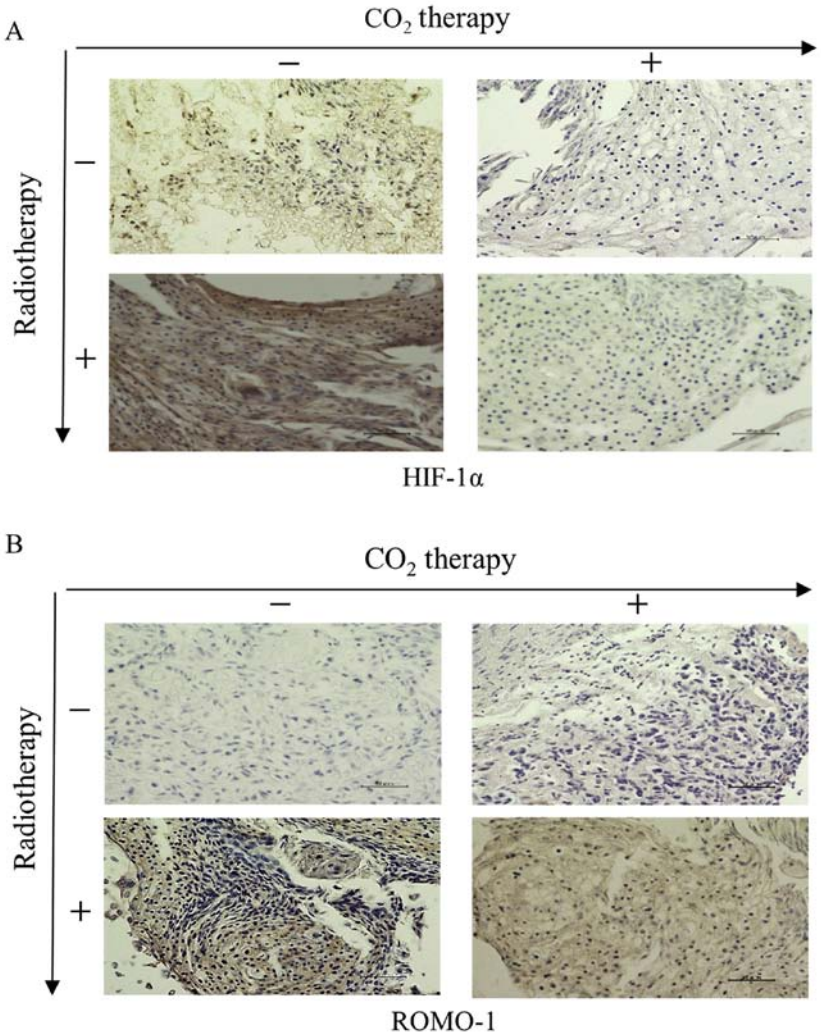

Figure 5. Immunohistochemical staining for (A) HIF-1α and (B) ROMO-1 in implanted tumors from multiple treatment groups *in vivo*.

cleaved caspase-9 was observed in the CO_2 and the combination group (Fig. 7A). Positive bands in the immunoblots were semi-quantified by densitometric analysis based on the concentrations of α-tubulin: caspase-3 (0.22, 0.62, 0.71, 0.89), caspase-8 (0.39, 0.41, 0.83, 0.93), caspase-9 (0.41, 0.79, 0.41, 1.00) and PARP (0.22, 0.53, 0.52, 0.93) in the control, CO_2, RT and combination group, respectively (Fig. 7B).

Discussion

RT is one of the main treatments for OSCC. However, radioresistance is a recognized obstacle responsible for poor clinical success and patient prognosis. Radioresistance of tumors is caused by cell cycle, differentiation and hypoxia of which, tumor hypoxia is known to demonstrate a strong correlation with radioresistance (4-8). Hypoxia, characterized by an inadequate supply of oxygen to the tissues, disturbs the radiolysis of H_2O and reduces the production of reactive and cytotoxic species, and that radiation-induced DNA damage is fixed under normoxic conditions (20,21).

Recent studies have revealed that HIF-1α plays a major role in hypoxia-related tumor radioresistance (15). Under hypoxic conditions, HIF-1α in tumor cells is activated; this activation promotes tumor growth. In contrast, normoxic conditions induce the proteolysis of HIF-1α and the loss of HIF-1α activity, which results in a dramatic decrease in tumor growth, angiogenesis and cellular energy metabolism (22).

RT results in tumor reoxygenation, upregulated production of ROS and activation and stabilization of HIF-1α in solid tumors, including OSCC (23). The cytokines induced by HIF-1α activate anti-apoptotic signals to tumor vessels, thereby imparting radioresistance to tumor cells (24). Therefore, it stands to reason that blocking either HIF-1α or these cytokines could considerably increase the radiosensitivity of tumor vasculature, thus causing a decreased overall tumor radioresistance (25-28). Hence, resolving hypoxia via blocking the activation of HIF-1α may prevent the development of radioresistance and improve the prognosis of OSCC patients.

In support of this, several studies have revealed the correlation between the survival rate and the value of hypoxia in irradiated solid tumors, including OSCC (29-33). Several strategies to address hypoxia have been explored, including the use of radio-sensitizers and hypoxic cytotoxins (9). However, when used in combination, these strategies resulted in a significant increase in the rate of both severe radiation tissue injury and the chance of seizures during therapy, presumably due to

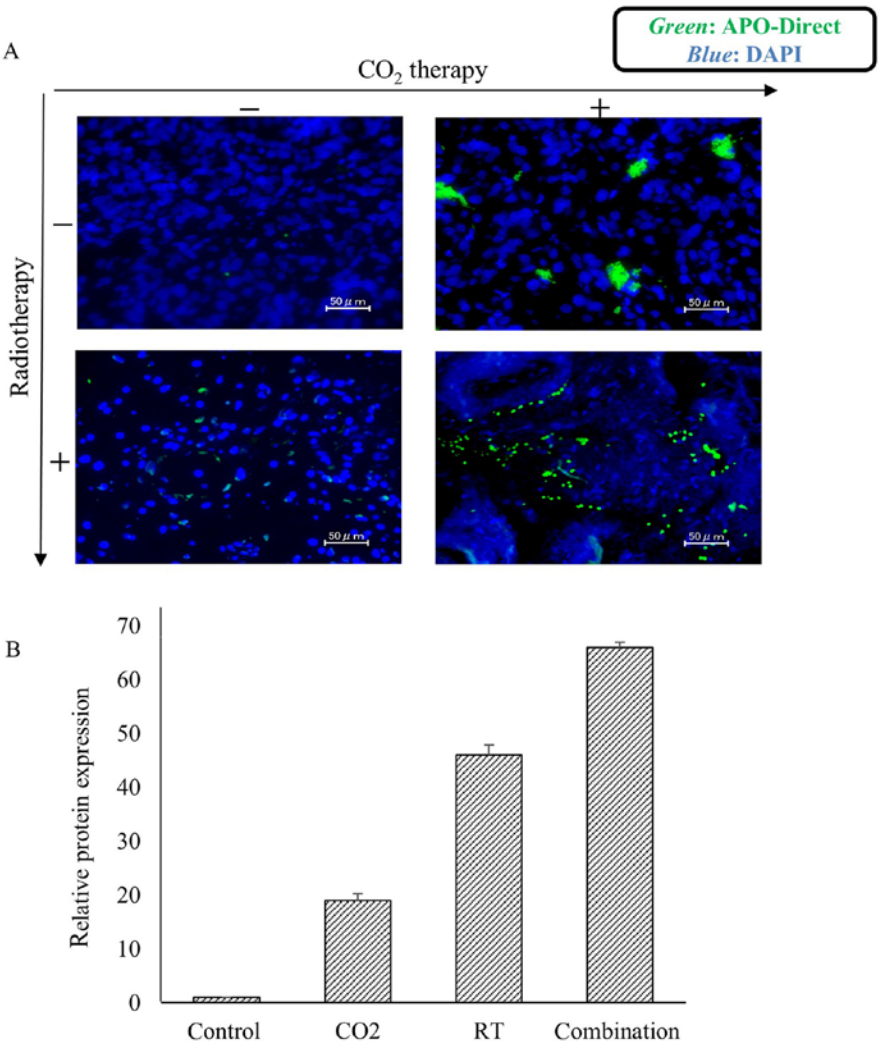

Figure 6. Immunofluorescence staining for mitochondria and apoptotic cells in treated human HSC-3 cells *in vivo*. (A) Immunofluorescence staining of apoptotic nuclear (green) cells in implanted tumors from multiple treatment groups *in vivo*. (B) Immunohistochemical-positive staining was semi-quantified by densitometric analysis using the ImageJ software. Values were normalized against the control group and presented as ratios.

oxygen (O_2) toxicity (10,11). Strategies for improving hypoxia employ 100% O_2 or carbogen (95% O_2 + 5% CO_2), involve direct inhalation and do not inflict any toxicity (34). Breathing carbogen in combination with nicotinamide administration was used to improve the tumor response to accelerated RT in head-and-neck tumors (35).

We have previously reported that a transcutaneous CO_2 system could combat hypoxia in treated tissues, potentially resulting in an 'artificial Bohr effect' (16). We have also demonstrated that transcutaneous CO_2 induced mitochondrial apoptosis and suppressed tumor growth in OSCC by resolving hypoxia (17) and decreasing the expression of HIF-1α in OSCC. In light of these findings, we hypothesized that transcutaneous CO_2 could improve hypoxic conditions and enhance the antitumor effect of RT on OSCC. CO_2 was used instead of O_2 because O_2 has an inferior ability to dissolve in tissues and has no apoptotic action. Antitumor effects did not arise when O_2 was directly used. Compared to carbogen, CO_2 therapy is localized because the tumor is covered directly with CO_2 hydrogel and the administered 100% CO_2 gas.

In the present study, the combination therapy of transcutaneous CO_2 with RT decreased the expression of HIF-1α and significantly suppressed *in vivo* OSCC tumor growth compared with RT alone. The results indicated that addressing hypoxia by transcutaneous CO_2 could mitigate intratumoral radioresistance.

Apoptosis plays an important role in determining the cellular fate (36,37) and is mediated by two distinct cell death pathways: the intrinsic and the extrinsic pathway. The intrinsic cell death pathway is initiated by mitochondrial outer membrane damage, followed by the release of cytochrome *c* from the mitochondria into the cytoplasm, activation of an initiator caspase, caspase-9, which activates the downstream protein, caspase-3. The extrinsic cell death pathway is initiated when a ligand binds to its receptor causing the activation of an initiator caspase, caspase-8, which then activates the downstream protein caspase-3. In both pathways, caspase-3 is responsible for the cleavage of poly (ADP-ribose) polymerase (PARP) during cell death (38-40). In the present study, the expression of cleaved caspase-8 was observed in both the RT and the combination groups, whereas cleaved caspase-9 was observed in both the CO_2 and the combination groups. These results indicated that the effect of RT on OSCC results from the activation of the death receptor-mediated pathway, whereas

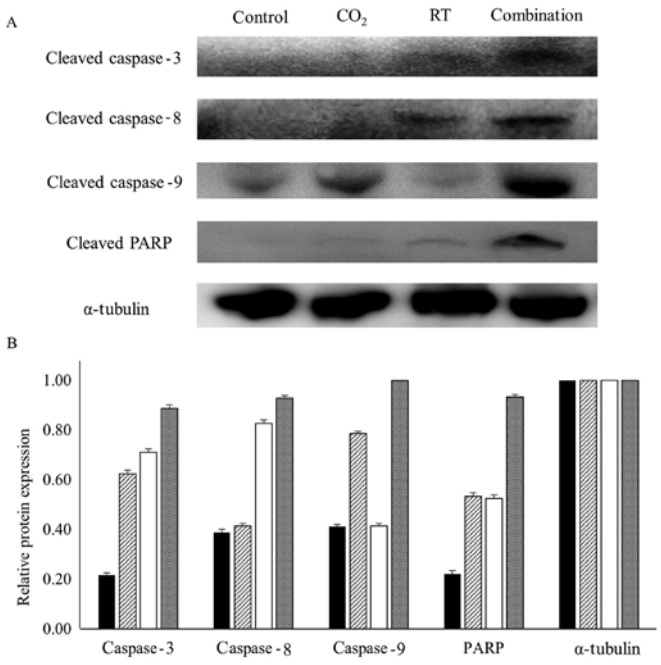

Figure 7. Effects of the combination therapy on mitochondrial apoptosis in human HSC-3 cells *in vivo*. (A) Immunoblot analysis of the expression of cleaved forms of caspase-3, -8, -9, and PARP in implanted tumors from multiple treatment groups *in vivo*. (B) Positive bands were semi-quantified by densitometric analysis using ImageJ software. Values were normalized against α-tubulin and presented as ratios.

the effect of transcutaneous CO_2 occurs via the mitochondrial apoptotic pathway. Furthermore, the combination therapy of transcutaneous CO_2 and RT demonstrated a strong antitumor effect mediated by both the death receptor and the mitochondrial apoptotic pathways.

In the present study, CO_2 did not induce significant oxidative stress but did induce obvious apoptosis. We previously reported that transcutaneous CO_2 inhibited SCC tumor growth *in vivo* by increasing the number of mitochondria and activating mitochondrial apoptosis by reducing intratumoral hypoxia (17). Mitochondria play important roles in cellular energy metabolism and apoptosis. Mitochondria proliferate independently from cancer cells, and the rates of cancer cell division and proliferation are likely faster than those of mitochondria under hypoxic conditions (41). Therefore, while cancer cells exhibit abnormal proliferation, the number of mitochondria decreases in cancer cells under hypoxic conditions, and mitochondrial dysfunction (the Warburg effect) prevents apoptosis in tumor tissue (42).

The antitumor effect of RT is also impacted by the generation of ROS (43), through a relationship that still requires further clarification. Radiation generates ROS when it passes through biological systems. ROS are very reactive, attack the critical cellular macromolecules such as DNA, and lead to cell damage and death (44). ROS have an important function in cell survival and cell death triggered by tumor necrosis factor-α (TNF-α) signaling, and the main source of these ROS is the mitochondria (45,46). Specifically, ROMO-1 is a mitochondrial membrane protein responsible for TNF-α-induced ROS production (47). ROMO-1 expression is upregulated in most cancer cells and in senescent cells, and it is induced by external stimuli (48). TNF-α treatment triggers the interaction between TNF complex II and the C-terminus of ROMO-1 exposed to the outside of the mitochondrial outer membrane (49). Simultaneously, ROMO-1 reduces the mitochondrial membrane potential, resulting in ROS generation and apoptosis. Thus, the ROS level is significantly increased by ROMO-1 overexpression. In the present study, we observed a correlation between apoptotic activity and ROMO-1 expression in HSC-3 cells after X-ray irradiation *in vitro*, indicating that RT could induce ROS-related apoptosis in human HSC-3 cells. Additionally, the *in vivo* overexpression of ROMO-1 was higher in the combination group than that in the RT group. In summary, transcutaneous CO_2 may not interfere with ROS-related apoptosis.

In the present study, RT was performed *in vivo* at the *in vitro* IC_{50} dose of 5.0 Gy. Various studies have revealed that mice can be irradiated with different doses (50-52). In addition, Fujii *et al* reported that a single dose of radiation at 5.0 Gy rapidly increased pO_2 in SCC compared to other doses (53). Thus, we hypothesized that a combination therapy with CO_2 and 5.0 Gy (IC_{50}) would be effective in the present study. However, it may be more appropriate to use a dose other than 5.0 Gy.

In conclusion, this is the first study to demonstrate that transcutaneous CO_2 enhanced the antitumor effect of RT on OSCC by improving intratumoral hypoxia, whilst eliciting no observable side-effects. Although further studies are required, transcutaneous CO_2 may be a novel adjuvant therapy in combination with RT for OSCC.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

EI, TH, TU, DT, IS and TKa conceived and designed the study. EI and TU performed the experiments. EI, TH and DT wrote the paper. TU, TKa and RK reviewed and edited the manuscript. TA, YS, RS, RK and TKo supervised all aspects of this study. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All animal experiments were performed in accordance with the Guidelines for Animal Experimentation of Kobe University Animal Experimentation Regulations (permission

no. P120602) and were approved by the Institutional Animal Care and Use Committee.

Consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

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