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Suppression of cancer stem-like cell radioresistance by inhibiting AMPK signaling

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ABSTRACT

Cancer stem cell (CSC) radioresistance is a major cause of radiotherapy (RT) failure and tumor recurrence. The molecular target for eradicating CSCs has not been identified despite research efforts to overcome tumor radioresistance. The adenosine monophosphate-activated protein kinase (AMPK) is responsible for transmitting nuclear DNA damage signals to the mitochondria, which in turn generate adenosine triphosphate to execute a DNA damage response. Disruption of this mitochondria-mediated genomic defense mechanism may be an effective strategy to enhance the cytotoxic efficacy of RT. Here, we investigated the potential efficacy of the pan-AMPK inhibitor dorsomorphin (Dor) in preventing CSC radioresistance. Radioresistant cancer stem-like cells were derived from the human liver cancer cell line HepG2 (HepG2 82FR-31NR). The radiosensitizing effect of Dor was then examined in HepG2 82FR-31NR cell cultures by clonogenic assays. Low-dose Dor markedly suppressed the recovery of HepG2 cancer stem-like cells after radiation but had little effect on normal fibroblast proliferation and survival, whether applied alone or in combination with radiation. In conclusion, this study strongly suggests that Dor treatment can radiosensitize cancer stem-like cells at doses that have no significant cytotoxic effects on normal human fibroblasts.

Keywords: tumor radioresistance; cancer stem cell; AMPK; mitochondria; radiation

INTRODUCTION

Cancer is a leading causes of premature death worldwide, and both the disease and existing treatments drastically impair quality of life [1]. Radiotherapy (RT) is used to selectively kill cancer cells, often in combination with other therapies such surgical excision, chemotherapy, and more recently immunotherapy [2]. However, tumor radioresistance frequently arises, resulting in tumor recurrence [3] and limiting further RT efficacy [4]. Furthermore, RT has undesirable side effects such as erythema and fibrosis, so the use of RT must be limited [5]. Tumors contain cancer stem cells (CSC) that have self-renewal capacity and preserve the hierarchical organization of cancer cells. The residual CSCs after RT drive tumor resistance and recurrence. We previously established radioresistant CSCs from the human liver cancer cell line HepG2. HepG2-derived cancer stem-like cells (HepG2 82FR-31NR) exhibited high tumorigenic potential after transplantation into nude mice and upregulated the expression of the molecular markers of CSCs such as CD133, cytokeratin14 and ABC transporters [6]. CD133-positive cells were <10% before isolation in parental cells, but increased to around 90% in HepG2 82FR-31NR cells [6]. Since HepG2 82FR-31NR cells are a heterogeneous mixture of CSC and non-CSC cells, these cells are referred to as cancer stem-like cells. However, the underlying signaling mechanisms associated with tumor radioresistance are not fully understood.

Mitochondria are major determinants of eukaryotic cell fate (survival or death) under stress. Mitochondria produce adenosine triphosphate (ATP) through oxidative phosphorylation, which provides the chemical energy required for damage repair [7]. However, severe mitochondrial damage causes outer membrane permeabilization and cytochrome c leakage, which ultimately activates caspase-dependent cell death pathways [8, 9]. Therefore, targeting mitochondrial regulation of cell death signaling is a promising therapeutic approach to eradicating cancer cells [10].

The adenosine monophosphate-activated protein kinase (AMPK) signaling pathway acts as a cellular energy sensor to maintain energy homeostasis, suggesting therapeutic potential as an adjunct treatment for cancer eradication [11]. Indeed, the AMPK activator metformin has been investigated as a treatment for certain types of cancer [12]. Metformin has been shown to improve tumor response to RT in patients with cancer and diabetes [13]. However, AMPK signaling is associated with the survival of some cancers, suggesting that AMPK inhibition instead may have therapeutic potential [14]. Activation of AMPK by phosphorylation in response to decreased ATP levels promotes mitochondrial biosynthesis [15, 16]. We have previously reported that irradiation (IR) reduces ATP levels due to enhanced consumption for the DNA damage responses (DDR) and induced AMPK phosphorylation in human cells [13]. Therefore, AMPK inhibitors can suppress DDR and so may enhance the therapeutic cytotoxic effect of radiation on cancer cells. Here we examined if AMPK inhibition can prevent the development of chemoresistance in culture and in mouse xenograft tumors.

MATERIALS AND METHODS

Cells and chemicals

The HepG2 was obtained from the Cell Resource Center for Biomedical Research, Tohoku University. Radioresistant HepG2-derived cancer stem-like cells (HepG2 82FR-31NR) were isolated by repeated fractionated radiation as described [6].

Both HepG2 and HepG2 82FR-31NR cells were grown in RPMI1640 medium (NacalaiTesque, Kyoto, Japan) supplemented with 5% heat-inactivated fetal calf serum (FCS). The normal human fibroblast line TIG-3 was obtained from the Human Science Foundation, and maintained in alpha minimum essential medium (Nacalai Tesque) supplemented with 10% heat-inactivated FCS. TIG-3 cells were used for experiments after 23 population doublings. The AMPK inhibitor dorsomorphin (Dor) was obtained from MedChemExpress and dissolved in DMSO for cell application.

Irradiation

Cells were irradiated using an AB-160 X-Ray Irradiation System (Acro-Bio, Tokyo, Japan) at 0.9 Gy/min as confirmed by a dosimeter.

Antibodies

The expression levels and distributions of target proteins were determined using the following primary antibodies: phospho (p)-AMPK (Thr172) (Cell Signaling Technology #2535), AMPK (Cell Signaling Technology #2532S), and β-tubulin (Proteintech 10 068-1-ap). For immunocytochemistry, antibody binding was detected by goat antirabbit IgG Alexa Fluor 488 (Invitrogen #A-11034) and antimouse IgG Alexa Fluor 647 (Invitrogen #A21236). A rabbit horseradish peroxidase-conjugated IgG (GE Healthcare, NA934-1ML) was used for Western blot detection of protein expression.

Western blotting

Cells were lysed in ice-cold RIPA buffer and total protein concentrations measured by the Bradford method. Protein samples (20 μg per gel lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto poly(vinylidene) fluoride membranes for immunoblotting. Protein bands were then visualized using Chemi-Lumi One Western blotting substrate (Nacalai Tesque), and band intensities quantified using Image Lab software (Bio-Rad, Hercules, CA). β-tubulin was used as an internal gel-loading control. All protein levels are expressed relative to the values obtained from corresponding nonirradiated cells (the negative control condition).

Cell proliferation assays in the presence of Dor and IR

Cells were seeded in 60-mm dishes (Falcon) at 2.0×10^5/dish and incubated overnight, treated with the indicated concentration of Dor for 2 h and irradiated as indicated in the continued presence of Dor for 24 h. Cells were rinsed with PBS(−) for drug wash-out and then in fresh medium for 2 days. The number of cells was counted at the end of incubation using a hemocytometer and an optical microscope.

Clonogenic assays

Cells were seeded in T25 Flask (Thermo Scientific) at 1.0×10^5/dish and incubated overnight, treated with the indicated concentration of Dor for 2 h and irradiated as indicated in the continued presence of Dor for 24 h. Cells were rinsed with PBS(−) for drug wash-out and then in fresh medium. Clonogenic survivals of the cells were assayed by seeding 10^4 cells per 60-mm dish, and the dishes were incubated for 7–10 days until colonies were visible. They were fixed with ethanol for 30 min and stained with Giemsa solution (Merck, Pennsylvania, PA, USA). The colonies of more than 50 cells were counted under a light microscope.

Apoptosis assays

Apoptotic cells were detected using an annexin V-FITC apoptosis detection kit (Bio Vision, Mountain View, CA, USA) according to the manufacturer's instructions. Cells were incubated with Dor for 2 h before IR, irradiated and incubated in the continued presence of Dor for 48 h. Cells were then stained with annexin V-FITC. Positively stained cells were detected by a fluorescence-activated cell sorter (FACScan; Becton Dickinson, Oxford, UK).

RNA interference

AMPK-targeted siRNAs (AMPK siRNA-α1/α2:sc-45 312, AMPK siRNA-α1:sc-29 673, AMPK siRNA-α2:sc-38 923) and control siRNAs (control siRNA-A:sc-37 007, control siRNA-B:sc-44 230, control siRNA-C:sc-44 231) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Cells were transfected with these siRNAs overnight using Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA).

Statistical analyses

Error bars represent the standard deviation of at least three independent experiments (separately seeded and treated cultures or individually treated mice). Differences between two groups for the analysis of RNA interference and apoptotic cells were evaluated by independent samples *t*-tests and differences among more than two groups by ANOVA followed by post hoc Dunnett's tests for pair-wise comparisons. All statistical calculations were performed using Microsoft Excel.

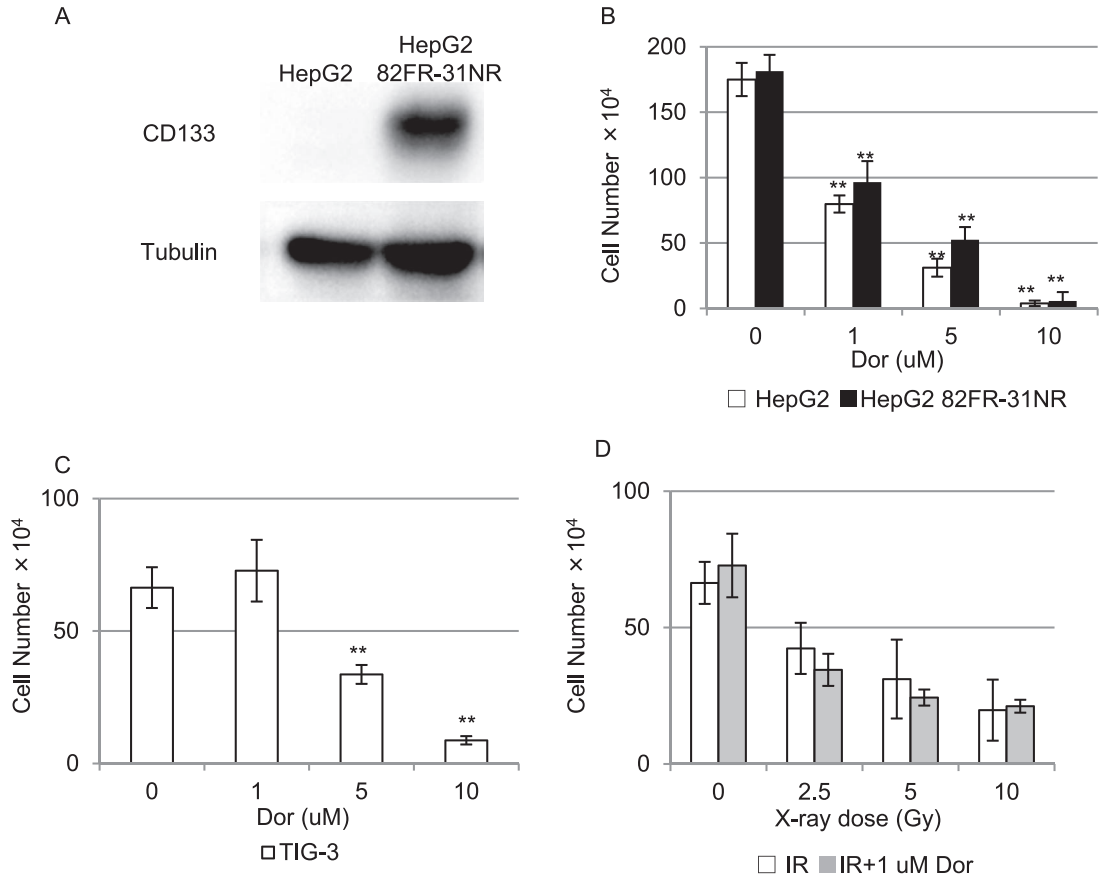

Fig. 1. Selective reduction of human cancer cell proliferation by Dor. (A) CD133 expression of HepG2 and HepG2 82FR-31NR cells. Total numbers of HepG2 and HepG2 82FR-31NR cells (B) and TIG-3 cells (C) remaining after Dor treatment at the indicated doses. The asterisk indicates a significant decrease in cell number compared to nontreated control cells. (D) Total numbers of TIG-3 cells remaining after pretreatment with Dor and IR.

Statistical significance is indicated in figures by an asterisk as follows: $^{*}P < 0.05$, $^{**}P < 0.01$.

RESULTS

Dor selectively inhibited cancer cell proliferation

The CSC-like properties of HepG2 82FR-31NR cells were confirmed by CD133 expression in Fig. 1A. Dor (or compound C) is effective in blocking AMPK activation [17]. Ideally, the blockade of AMPK activation should be higher in target (cancer) cells than in nontarget (noncancer) cells, since side effects of anticancer drugs are a critical determinant of patient acceptance. Therefore, we first investigated the effects of Dor on AMPK in normal cells as well as in CSCs. Normal human fibroblast cells are difficult to form colonies. Therefore, we used cell proliferation assay to examine the effect of an AMPK inhibitor on radiation sensitivity. The assay revealed that Dor alone dose-dependently suppressed the proliferation of HepG2 cells and HepG2 cancer stem-like cells (HepG2 82FR-31NR) (Fig. 1B and C). In contrast, 1 μM Dor did not reduce the total number of normal fibroblasts (Fig. 1C). This finding indicates that a low dose of Dor can selectively suppress the proliferation of cancer cells. IR alone dose-dependently suppressed the proliferation of TIG-3 cells, while 1 μM Dor did not increase the radiosensitivity of normal fibroblasts (Fig. 1D).

Dor inhibited AMPK activation (phosphorylation)

AMPK activation (phosphorylation) was compared to untreated controls by Western blotting using AMPK and p-AMPK antibodies (Fig. 2A). Cells were treated with 1 μM Dor for 2 h and then irradiated with 5 Gy. Lysate samples were prepared after 3 h of Dor treatment. IR increased AMPK phosphorylation in HepG2 cells and HepG2 82FR-31NR cells. Dor treatment attenuated radiation-induced p-AMPK expression in HepG2 82FR-31NR cells, while it had no effect on AMPK activation in parental cells (Fig. 2B). Thus, 1 μM Dor appears to preferentially suppress AMPK signaling in the targeted cancer stem-like cells.

Dor treatment increased the radiosensitivity of cancer stem-like cells

To evaluate whether Dor can enhance radiosensitivity, cells were treated with IR alone or the combination and cell survival was compared using a clonogenic assay. HepG2 82FR-31NR cells exhibited

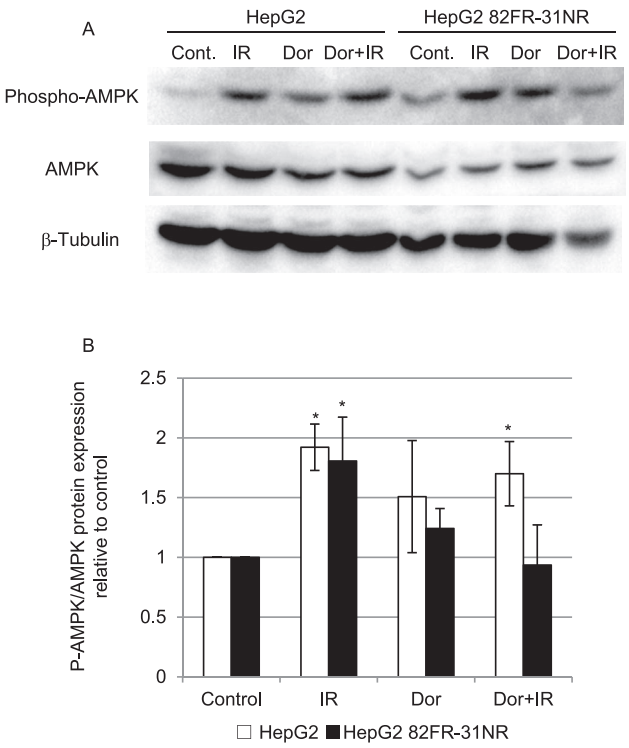

Fig. 2. Inactivation of AMPK by Dor. Cells were treated with 1 μM Dor for 2 h and then irradiated with 5 Gy. (A) Western blotting bands from HepG2 and HepG2 82FR-31NR cell lysates. (B) p-AMPK band intensities were shown relative to the corresponding total AMPK band intensities. Protein expression levels were normalized to the values of untreated control cells. The asterisk indicates a significant increase in phosphorylation levels compared to untreated control cells.

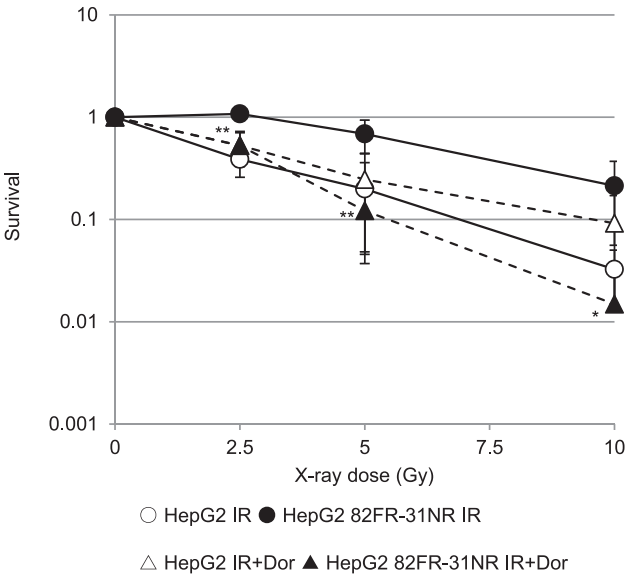

Fig. 3. Dor treatment exacerbated the toxicity of IR on cancer cells. Survival of HepG2 and HepG2 82FR-31NR cells after combined treatment with Dor and IR. The asterisk indicates a significant decrease in cell survival compared to cells treated with radiation alone.

a radioresistant phenotype compared to the corresponding parental cells (Fig. 3). Notably, 1 μM of Dor treatment prior to IR enhanced the radiosensitization of HepG2 82FR-31NR cells (Fig. 3). However, Dor did not increase the radiosensitivity of parental HepG2 cells (Fig. 3). Radiation alone is sufficient to kill the parent cells, whereas 5 Gy X-ray IR did not induce apoptosis in HepG2 82FR-31NR cells (Fig. 4). However, Dor treatment enhanced apoptosis of 5-Gy irradiated HepG2 82FR-31NR cells (Fig. 4) compared to IR alone but not in parental cells.

Knockdown of AMPK enhanced cancer stem-like cells radiosensitivity

To further support the radiosensitizing effects of Dor and exclude potential non-target pharmacological effects, we investigated the effect of genetic AMPK inhibition. As expected, transfection of a targeted siRNA reduced AMPK expression levels compared to cells transfected with a control siRNA (Fig. 5A and B). Beclin-1 is as an important downstream target of AMPK in mediating autophagy (Fig. 5A) [18]. Transfection of an AMPK siRNA also reduced Beclin-1 protein expression. Radiosensitivity of HepG2 82FR-31NR cells further enhanced by transfection with AMPK siRNA (Fig. 5C).

Several different constructs for control siRNAs and AMPK siRNAs were used to confirm the effect of genetic AMPK inhibition on radiosensitizing HepG2 82FR-31NR cells (Supplementary Fig. 1).

DISCUSSION

The AMPK plays a critical role in the regulation of cell proliferation, autophagy and metabolism as a metabolic stress sensor [17]. The AMPK pathway is also associated with DDRs. In response to ionizing radiation, ATM activates AMPK to protect cells from apoptosis by regulating the cell cycle checkpoint and survival [15, 19]. Chemical inhibition of AMPK signaling has been reported to induce apoptosis of many cancer cell types [20, 21]. One such AMPK inhibitor, Dor, has been shown to inactivate AMPK and radiosensitize colon cancer cells [19]. Conversely, however, preradiation treatment with Dor was also reported to enhance the radioresistance of human lung cancer cells [15]. Therefore, the precise effects of AMPK signaling on the radioresistance of tumors remain to be elucidated. Here, we report that Dor can overcome tumor cell radioresistance and is especially effective against radioresistant cancer stem-like cells. The non-target effects (side effects) of anticancer drugs are a major factor leading to discontinuation, so we examined the effect of Dor on normal cells and found relatively selective activity on CSCs at lower doses. Specifically, 1 μM Dor treatment alone inhibited the proliferation, and reduced the survival rates of liver cancer stem-like cells but had little effect on fibroblast proliferation or survival, even when combined with radiation. Thus, Dor is unlikely to exacerbate the side effects of RT. Furthermore, AMPK-targeted siRNA-mediated knockdown increased the cytotoxicity of

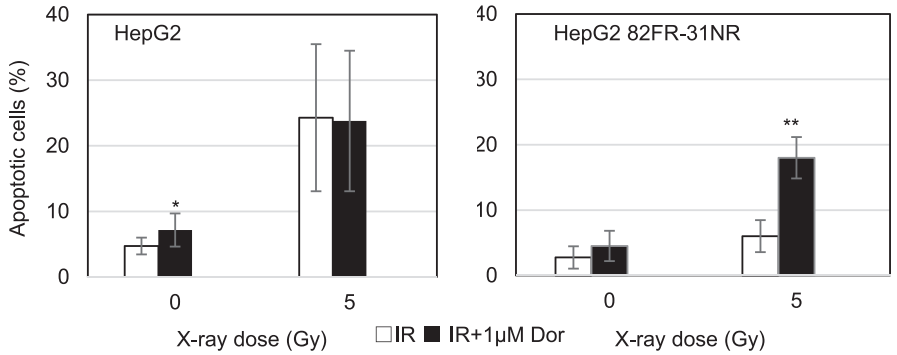

Fig. 4. Induction of apoptosis after IR and Dor treatment. Number of apoptotic cells following 5-Gy IR with or without Dor treatment at the indicated doses in HepG2 and HepG2 82FR-31NR cells. The asterisk indicates a significant increase in the number of apoptotic cells compared to IR alone.

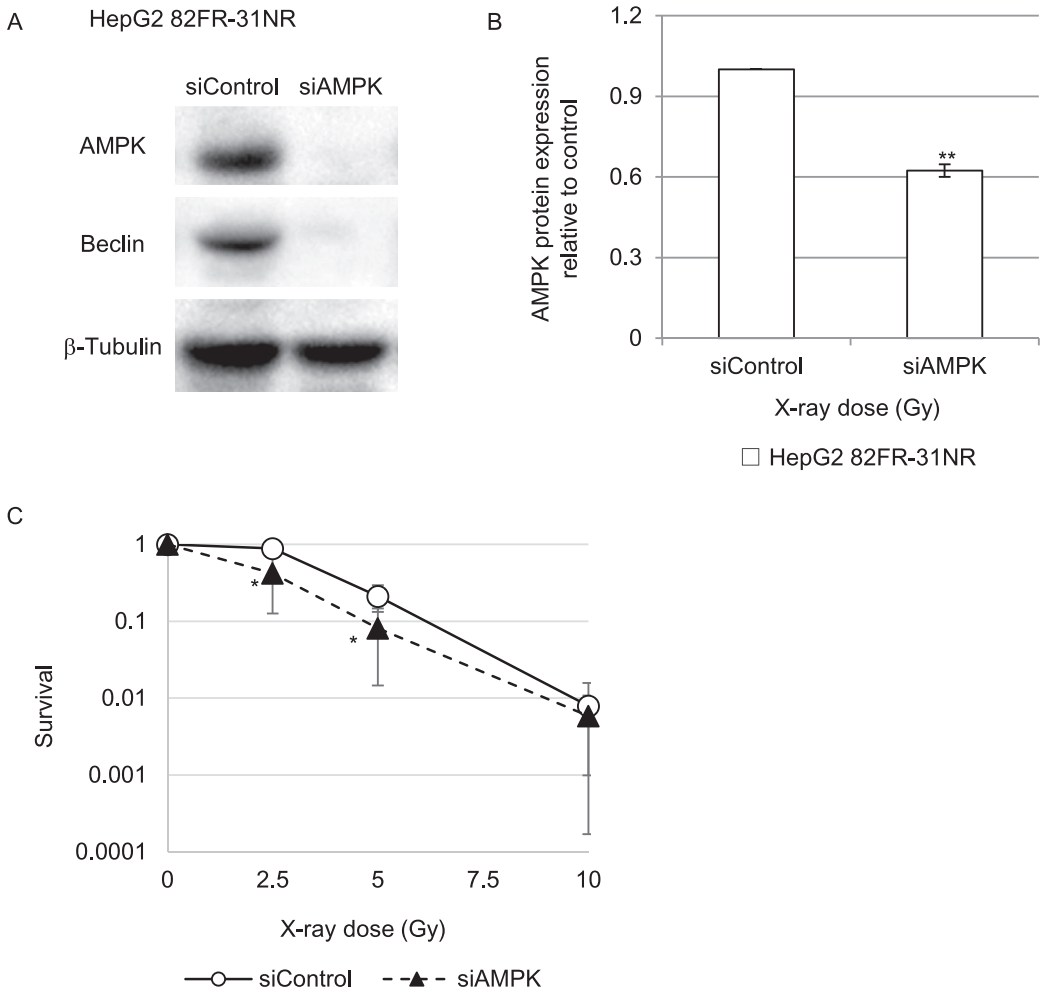

Fig. 5. Downregulation of AMPK protein expression by AMPK siRNA transfection increased the radiosensitivity of HepG2 CSCs. (A) Western blotting bands of AMPK, Beclin and β-tubulin (the gel loading control) from HepG2 CSC cell lysates. (B) Expression of AMPK in cells transfected with targeted siRNA, AMPK siRNA-α1/α2 (siAMPK) relative to cells transfected with control siRNA-A (siControl). The asterisk indicates a significant decrease in AMPK expression level compared to cells transfected with siControl. (C) Survival of HepG2 CSCs transfected with siControl or siAMPK. The asterisk indicates a significant decrease in cell survival compared to cells transfected with siControl.

radiation in cancer stem-like cells. Because cancer stem-like cells are highly sensitive to Dor treatment, radiation resistance is suppressed by combined treatment with radiation and Dor. Repeated fractionated IR activates the AKT/cyclin D1 survival pathway, which confers radioresistance through efficient DNA repair of radiation-induced DNA damage in 82FR-31NR cells. An AKT inhibitor, API-2, or cyclin D1 siRNA resulted in a loss of efficient DNA repair and radiosensitization of 82FR-31NR cells. AMPK also has an effect on DNA damage repair [22]. Thus, we successfully suppressed the radioresistance of cancer stem-like cells by inhibiting AMPK signaling pharmacologically and through genetic suppression.

Chemotherapy-induced DNA damage activates AMPK which in some cancer types enhances the cytotoxicity of the chemotherapeutic agent. For instance, etoposide, cisplatin and doxorubicin have been shown to induce ATM-AMPK signaling in cancer cells which in turn enhanced chemosensitivity [23–25]. Thus, AMPK signaling is regarded as a promising adjunct target for chemotherapy, although the direction of modulation required may vary among cancer types.

This study has several limitations, including the examination of only one CSC type. The effects of Dor on the radiosensitivity of other cancer cell types remains to be examined. As mentioned, Dor has multiple targets other than AMPK and was reported to suppress cancer cell proliferation independently of AMPK inhibition [20]. Therefore, further studies are necessary to identify more selective AMPK inhibitors for clinical use. Solid tumors contain multiple heterotypic cell types, including cancer cells and surrounding tumor stromal cells. Therefore, future studies should also evaluate Dor efficacy in immunocompromised mice transplanted with intact patient-derived tumor tissue.

Mitochondria determine the fate of cancer cells under treatment by regulating apoptosis and the DDR, so agents that can enhance mitochondria-dependent apoptosis are potentially valuable anticancer treatments. We show that the AMPK inhibitor Dor can reduce cancer cell proliferation and enhance cancer cell death under IR by promoting apoptosis without substantially reducing the proliferation or survival of noncancerous cells. Selective disruption of mitochondria function could be a promising therapeutic strategy to eradicate radioresistant cancer stem-like cells.

SUPPLEMENTARY DATA

Supplementary data is available at *Journal of Radiation Research* online.

CONFLICT OF INTEREST

None declared.

DATA AVAILABILITY

The data presented in this study are available on request to the corresponding author.

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