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Bmi-1 confers adaptive radioresistance to KYSE-150R esophageal carcinoma cells

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ABSTRACT

Radiotherapy (RT) is a major modality of cancer treatment. However, tumors often acquire radioresistance, which causes RT to fail. The exact mechanisms by which tumor cells subjected to fractionated irradiation (FIR) develop an adaptive radioresistance are largely unknown. Using the radioresistant KYSE-150R esophageal squamous cell carcinoma (ESCC) model, which was derived from KYSE-150 parental cells using FIR, the role of Bmi-1 in mediating the radioadaptive response of ESCC cells to RT was investigated. The results showed that the level of Bmi-1 expression was significantly higher in KYSE-150R cells than in the KYSE-150 parental cells. Bmi-1 depletion sensitized the KYSE-150R cells to RT mainly through the induction of apoptosis, partly through the induction of senescence. A clonogenic cell survival assay showed that Bmi-1 depletion significantly decreased the radiation survival fraction in KYSE-150R cells. Furthermore, Bmi-1 depletion increased the generation of reactive oxygen species (ROS) and the expression of oxidase genes (Lpo, Noxo1 and Alox15) in KYSE-150R cells exposed to irradiation. DNA repair capacities assessed by γ-H2AX foci formation were also impaired in the Bmi-1 down-regulated KYSE-150R cells. These results suggest that Bmi-1 plays an important role in tumor radioadaptive resistance under FIR and may be a potent molecular target for enhancing the efficacy of fractionated RT.

1. Introduction

Ionizing radiation (IR) is a powerful therapeutic modality. Due to its ability to better preserve normal tissues, fractionated radiotherapy (RT) is widely used in cancer therapy. However, during fractionated RT, surviving tumor cells that repopulate often acquire tumor radioresistance and this has been linked to increased recurrence and treatment failure in many patients [1]. Adaptive resistance to radiation is induced in several tumor cell lines after long-term exposure to fractionated irradiation (FIR). Human HepG2 and Hela cancer cells acquire radioresistance when exposed to FIR of X-rays, and the acquired radioresistance is associated with the overexpression of DNA-PK/AKT/GSK3 beta-mediated cyclin D1 [2]. Human breast cancer cells treated with FIR show enhanced clonogenic survival and NF-κB activation; blocking the NF-κB inhibits the adaptive radioresistance [3]. Esophageal squamous cell carcinoma (ESCC) KYSE-150 treated with FIR shows a higher radioresistance, however, inhibiting the epidermal growth factor receptor signal pathway with cetuximab reverses the radioresistance [4]. Yet, the exact molecular mechanisms through which tumor cells develop an adaptive resistance to therapeutic fractional irradiation are largely unknown.

Recent studies have suggested that polycomb group (PcG) proteins, which have well-established roles in gene regulation, have an important role in the DNA damage response [5]. Among them, the oncogenic Bmi-1 is a very early DNA damage response protein that accumulates at DNA double-strand breaks (DSB) foci and promotes DSB repair [6]. Bmi-1 is required for DNA damage-induced ubiquitination of histone H2A at lysine 119, and the loss of Bmi-1 leads to impaired repair of DNA DSBs by homologous recombination [7]. We have previously reported that Bmi-1 elicits radioprotective effects in normal human keratinocytes by mitigating the genotoxic effects of IR [8]. Bmi-1 is overexpressed in a number of cancers, including ESCC. Targeting Bmi-1 expression in nasopharyngeal carcinoma cells increases their susceptibility to RT through the induction of oxidative stress and apoptosis [9].

Abbreviations: ESCC, esophageal squamous cell carcinoma; RT, radiation therapy; FIR, fractionated irradiation; ROS, reactive oxygen species; SA β-Gal, senescence-associated β-galactosidase; IR, ionizing radiation; DSB, double-strand break; Lpo, lactoperoxidase.

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Bmi-1deficiency in CD133-positive glioblastoma multiforme (GBM) cells severely impairs the DNA DSB response, resulting in increased sensitivity to radiation [10]. Bmi-1 depletion sensitizes head and neck squamous cell cancer-derived ALDH1-positive cells to radiation therapy [11]. Therefore, Bmi-1 is an important determinant of cellular radioresistance and may also regulate the adaptive responses of cancer cells to fractionated RT.

Here, we reveal a new function for Bmi-1, wherein it controls the adaptive radioresistance in cancer cells. Adaptive radioresistant ESCC cells express much higher levels of Bmi-1 than parental ESCC cells. The depletion of Bmi-1 sensitizes radioresistant KYSE-150R ESCC to RT by inducing apoptosis and the generation of reactive oxygen species (ROS), while impairing DNA repair capacities. These findings shed new light on the molecular mechanisms underlying the tumor radioresistance induced by FIR, and suggest that Bmi-1 depletion could be a potential therapeutic approach to conquer radioresistance in ESCC.

2. Materials and methods

2.1. Cell culture and transfection

The parental human ESCC KYSE-150 and radioresistant KYSE-150R cells were gifts from Dr. Shixiu Wu [4]. Cells were cultured in RPMI-1640 (Gibco, Life Technologies Inc., Grand Island, NY) with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37 °C in the presence of 5% CO_2. Bmi-1 Smartpool (siBmi-1), purchased from Dharmacon (Dharmacon, Abgene Ltd., Epsom, UK), was used for the transfection of sub-confluent KYSE-150R cells. The cells were transfected using the Lipofectamine RNAiMAX (Invitrogen Corp., Carlsbad, CA) reverse transfection protocol, according to the manufacturer's instructions. The plates were incubated for 48 h prior to any cell treatments and harvesting. In each experiment, a scrambled pool of siRNA was used as a control. For the irradiation experiments, the cells were irradiated with 1 Gy of X-ray using a high energy linear accelerator (PRIMUS-M, Siemens) at a dose rate of 2 Gy per minute.

2.2. Cell cycle analysis

Cells were trypsinized and stained with propidium iodide staining buffer (Muitisciences Biotech, China) for 30 min and then analyzed by FACS using Modfit software (Becton Dickinson, San Jose, CA).

2.3. Apoptosis assay

Cells were tested with the Annexin V-FITC apoptosis detection kit (BD Biosciences) according to the manufacturer's protocol, and then analyzed by FACSCalibur using Cellquest software (Becton Dickinson, San Jose, CA).

2.4. Senescence-Associated β Galactosidase (SA β-Gal) activity assay

Cells were washed in phosphate buffered saline (PBS) and then fixed in 2% formaldehyde, 0.2% glutaraldehyde solution. The cells were then washed again in PBS and stained with SA β-Gal staining solution (Beyond Biotech, China). The plates were incubated at 37 °C for 16 h.

2.5. Clonogenic assay

The exponential growth cells were plated on six-well plates with 300–10,000 cells/well, based on the dose of radiation necessary to achieve 20–200 colonies/well. One day after cell plating, the plates were irradiated at doses of 0, 2, 5, 8, or 10 Gy. The cells were maintained in culture for an additional 10 days to allow colony formation. Visible colonies consisting of at least 50 cells were stained with 0.5% crystal violet (Sigma) and were then counted. The surviving fraction (SF) and the sensitization enhancement ratio (SER) were estimated.

2.6. Real time PCR

Total RNA was isolated from the cultured cells using the RNeasy Mini kit (Qiagen). Reverse transcription was performed using SuperScript II Reverse Transcriptase (Invitrogen), according to the manufacturer's recommendations. qPCR was performed in triplicate for each sample, as described previously [8].

2.7. Determination of intracellular ROS levels

Cells were stained with CM-H2DCFDA (Invitrogen), a fluorescent marker of intracellular ROS. The fluorescence intensity of CM-H2DCFDA was quantified by FACSCalibur using Cellquest software (Becton Dickinson, San Jose, CA).

2.8. Immunoblotting and immunofluorescence staining

The immunoblotting procedures were the same as in our previous study [8]. Immunofluorescence staining was performed on cells grown on coverslips. Cells were fixed in 3.7% formaldehyde for 15 min before being washed three times in PBS and permeabilized in 0.25% Triton X-100 in PBS for 10 min. Mouse monoclonal anti-phospho-γ-H2AX or Bmi-1, and Alex Fluor®488 or 568 goat anti-mouse IgG (Millipore Inc., Billerica, MA) were used as primary and secondary antibodies, respectively. Images were obtained using a Zeiss LSM 710 confocal microscope system (Carl Zeiss, Germany) and processed with ZEN LE software.

3. Results

3.1. Bmi-1 is overexpressed in adaptive radioresistant ESCC cells

The radiation-resistant cell line KYSE-150R was derived from fractional irradiated KYSE-150 cells (6 weeks of FR with total doses of 21 Gy). We first determined the expression level of Bmi-1 in those cell lines. The Bmi-1 expression level in KYSE-150R was three times higher than in KYSE-150 (Fig. 1A). The Bmi-1 protein expression level assayed by confocal laser scanning microscope (Fig. 1B) and the immunoblotting (Fig. 1C) also confirmed the results of the qPCR. We checked a second radioresistant ESCC cell line, SEG-R, which derived from the fractional irradiation of primary ESCC, the Bmi-1 expression level in SEG-R cells was 1.9 times that of the parental cells (data not shown). These data suggest that Bmi-1 overexpression may be related to ESCC adaptive radioresistance.

3.2. Bmi-1 depletion inhibits KYSE-150R cell growth

To investigate the role of Bmi-1 in the radioresistance of ESCC, we used Smartpool (siBmi-1) to knockdown Bmi-1. SiBmi-1 showed a > 90% reduction in Bmi-1 transcript levels in the KYSE-150R cells, which resulted in an almost undetectable Bmi-1 protein expression when assayed by confocal laser scanning microscope (Fig. 1B) and immunoblotting (Fig. 1C). As loss of Bmi-1 causes derepression of the *Ink4a* and *Arf* genes [12], we first examined the effect of Bmi-1 on cell proliferation. Cell cycle analysis showed that, compared to the control KYSE-150R and KYSE-150 cells, siBmi-1 induced G0/G1-phase arrest resulted in a decreased number of cells in the S-phase (Fig. 1D). Meanwhile, the apoptotic cell

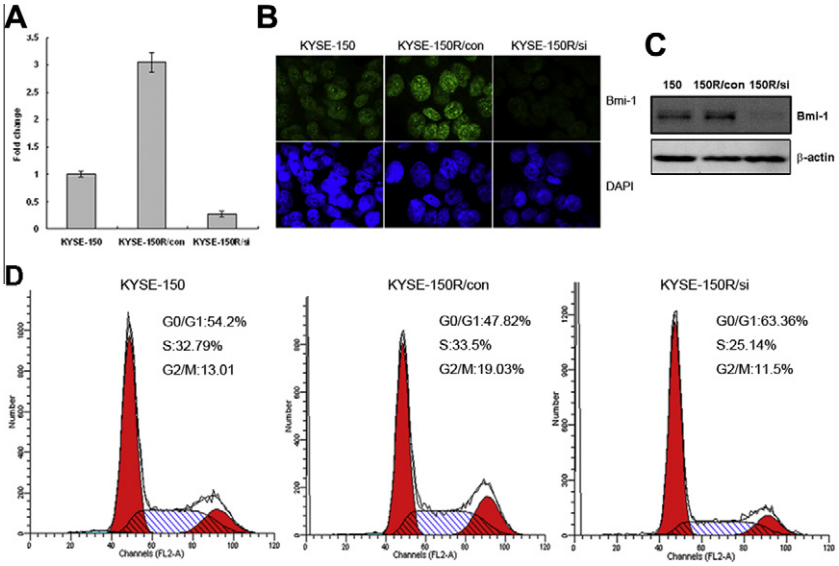

Fig. 1. Bmi-1depletion induces cell cycle arrest in KYSE-150R cells. (A) KYSE-150R cells were transfected with scrambled siRNA (KYSE-150/con) or siBmi-1 (KYSE-150/si). Two days later, the Bmi-1 mRNA expression level was analyzed using real-time PCR and compared with the KYSE-150 parental cells. Error bars, SD. (B) Confocal microscope analysis of the siRNA effect on Bmi-1expression. Green fluorescence indicates Bmi-1 deposition. The nuclei are counterstained with DAPI (blue). (C) Western blot analysis confirmed Bmi-1 depletion in KYSE-150R/si cells, β-actin were included as loading controls. (D) Cell cycle assayed by PI staining. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

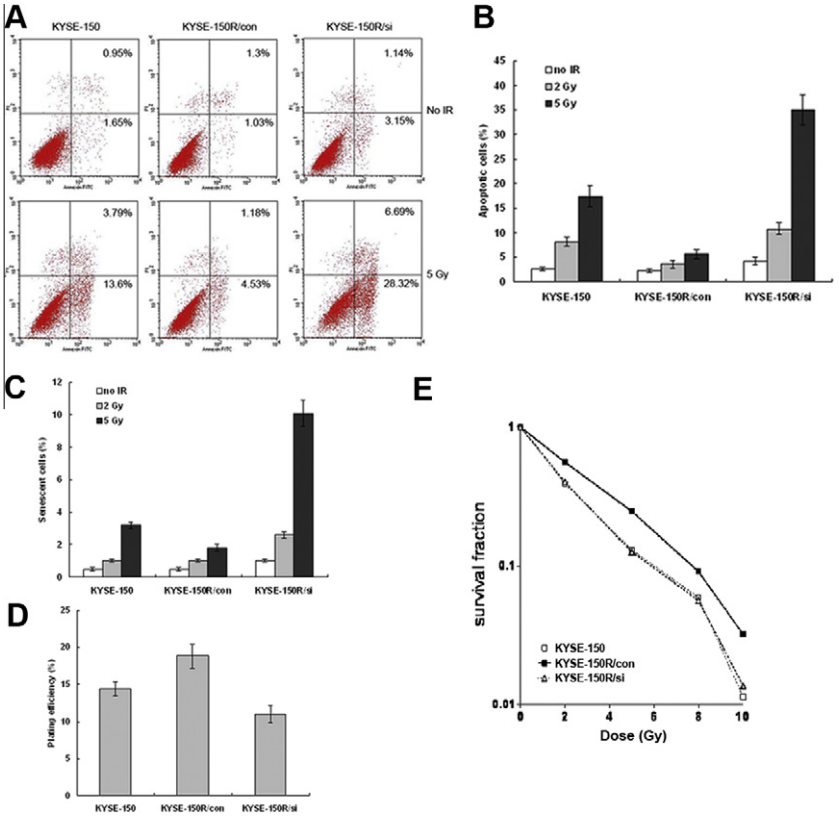

Fig. 2. Bmi-1 depletion reverses the radioresistance of KYSE-150R cells. (A) The tumor cells were exposed to 5 Gy IR and assayed by Annexin V/PI staining 2 days post-irradiation. (B) The apoptotic cell numbers were counted after 2 days of exposure to 2 Gy or 5 Gy IR. Error bars, SD. (C) The tumor cells were exposed to 2 or 5 Gy IR and stained for SA β-Gal, six days post-irradiation. Error bars, SD. (D,E) The tumor cells were cultured in 6 well plates for 24 h, and then irradiated with the indicated doses. After 10 days, the colonies were counted. Plating efficiency (D) was calculated. Surviving fractions (E) were calculated by the number of colonies divided by the number of seeded cells × plating efficiency.

number increased to 4.3% (Fig. 2A) and the amount of senescent cells increased to 1% in the Bmi-1 knockdown KYSE-150R cells (Fig. 2B).

3.3. Bmi-1 depletion sensitizes KYSE-150R cells to IR

To examine if Bmi-1depletion can reverse the radioresistance of the KYSE-150R cell line, we assessed the effect of Bmi-1 on cell death in irradiated KYSE-150R cells. As cell death by apoptosis or senescence may occur following radiation, we first use the Annexin V/PI staining method to check the apoptotic cell number induced by irradiation. The KYSE-150R cells were resistant to IR-induced apoptosis, the apoptotic cells increased from 2.3% to 4.7% after 5 Gy irradiation. KYSE-150 cells were more sensitive to irradiation, they showed 17.4% apoptotic cells post-IR. Bmi-1 depletion significantly increased the apoptotic cell number to 38% in the KYSE-150R cells (Fig. 2A). Similar results were seen in the 2 Gy irradiated KYSE cells (Fig. 2B). We then used SA β-Gal activity assay to measure the percentage of senescent KYSE cells induced by IR. The number of senescent KYSE cells in the untreated samples increased slightly at six days post-irradiation, whereas Bmi-1 depletion increased the ratio to 10% (Fig. 2C). These data demonstrate that the senescent response is relatively weak and apoptosis may be the predominant factor in IR-induced cell death in Bmi-1 depleted KYSE-150R cells. The effect of Bmi-1 on the radiation response of KYSE-150R cells was also evaluated using a clonogenic assay. Bmi-1 depletion reduced the plating efficiency of the treated cells to 59% of the control KYSE-150R cells (Fig. 2D), and increased the radiosensitivity of KYSE-150R, as shown by the marked reduction in clonogenic cell survival rates at 2, 5, 8 and 10 Gy ($P < 0.05$), with a sensitization enhancement ratio (SER) of 1.2 (Fig. 2E).

3.4. Bmi-1 depletion increases ROS generation in KYSE-150R cells exposed to IR

IR elicits genotoxic stress by triggering the production of ROS. We thus investigated the effect of Bmi-1 on ROS generation in KYSE-150R cells. Exposure to 5 Gy of irradiation led to a slightly increased production of intracellular ROS in KYSE-150R cells. Bmi-1 depletion increased the ROS generation 1.9 times, compared to untreated KYSE-150R cells. ROS production under irradiation also notably increased to 2.5 times the amount in the control KYSE-150R cells (Fig. 3A). As NADPH oxidase are major sources of the ROS induced by IR [13], we used RT-qPCR to compare the mRNA levels of several enzymes involved in ROS production, including lactoperoxidase (Lpo), Noxo1 and arachidonate lipoxygenases (Alox15). Bmi-1 depletion significantly increased the base level of Lpo, Alox15, and Noxo1 expression in untreated KYSE-150R cells. Although the Lpo, Alox15, and Noxo1 expression levels were elevated in all the KYSE-150R cells exposed to 2 Gy and 5 Gy irradiation, Bmi-1 depletion induced a much higher expression of those genes than in the control KYSE-150R cells (Fig. 3B, C and D).

3.5. Bmi-1depletion impairs DNA DSB repair in KYSE-150R cells post-IR

As cell survival of radiation exposure can be directly linked with DNA repair, we investigated whether Bmi-1 affected DSB repair in KYSE-150R cells. ESCC cells were exposed to 2 and 5 Gy IR and stained to identify the γ-H2AX foci, which co-localize with DSB *in situ*, at 1, 8 and 24 h post-irradiation (Fig. 4A). In the absence of radiation, the base level of the γ-H2AX signal was much more intense in the Bmi-1-depleted KYSE-150R cells, suggesting decreased genomic stability. 2 Gy irradiation caused a slight increase in γ-H2AX foci formation in KYSE-150R cells, whereas Bmi-1 depletion caused a marked increase in the number of γ-H2AX foci per cell (Fig. 4B). 5 Gy irradiation caused a notable increase in γ-H2AX foci formation in KYSE-150R cells at 1 h post-IR, but the formation diminished at 24 h post-IR, whereas Bmi-1-depleted KYSE-150R cells maintained a high level of γ-H2AX foci formation even at 24 h post-IR (Fig. 4C). These results suggest that Bmi-1 depletion enhances radiation-induced DNA damage and delays DNA repair.

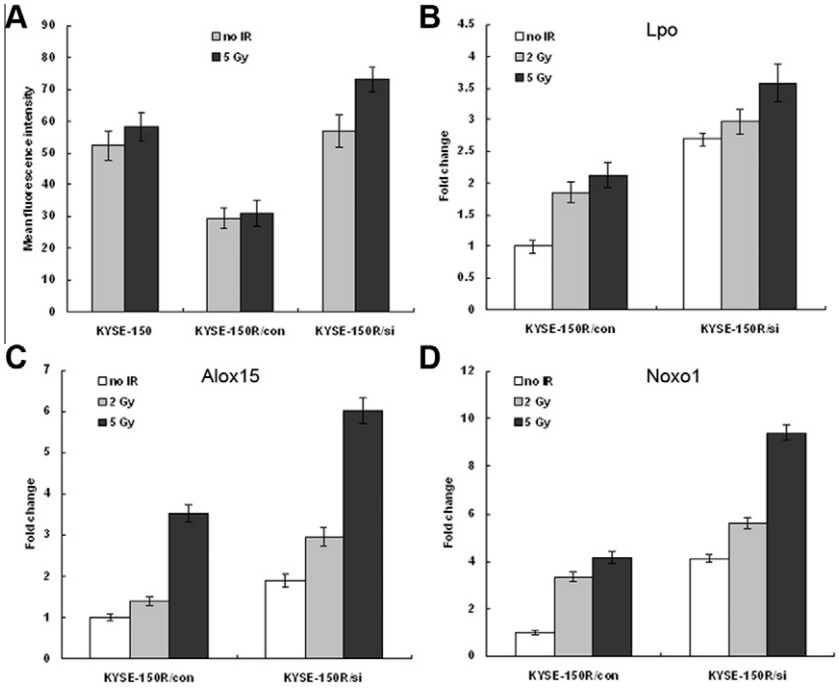

Fig. 3. Bmi-1depletion increases the ROS generation induced by IR in KYSE-150R cells. (A) The tumor cells were exposed to 5 Gy IR and at one day post-irradiation stained with CM-H_2DCFDA, a fluorescent marker of ROS. Fluorescence intensity was measured by flow cytometry. Error bars, SD. (B,C,D) The cells were exposed to 2 or 5 Gy IR, and total RNA was harvested at 1 day post-irradiation. The mRNA levels of Lpo, Noxo1, and Alox15 were measured by RT-qPCR and normalized against GAPDH. Error bars, SD.

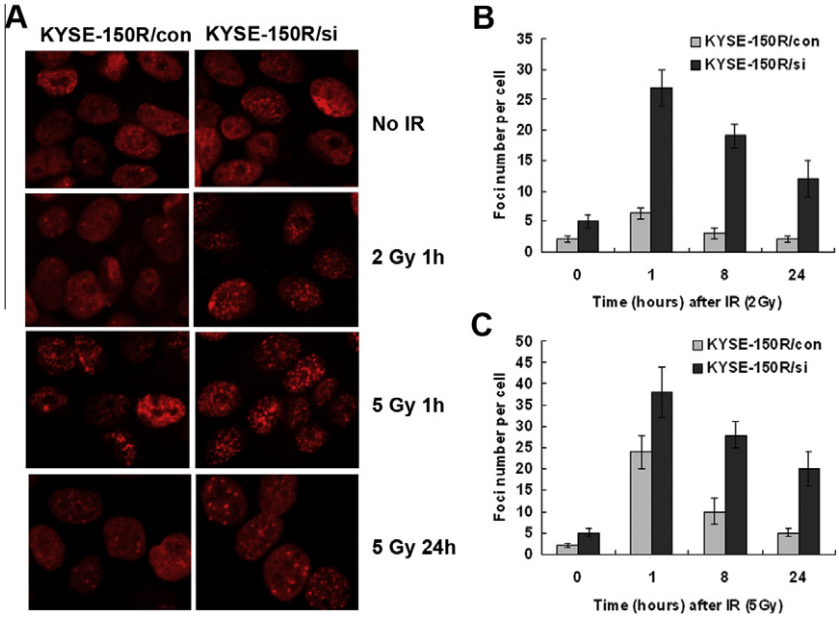

Fig. 4. Bmi-1 depletion impairs the DNA damage response. (A) The tumor cells were irradiated at 2 or 5 Gy and then stained with the γ-H2AX antibody at the indicated times. Representative images were acquired with a Zeiss confocal microscope. (B,C) Bmi-1 depletion affects the kinetic of DNA repair after 2 or 5 Gy irradiation. The number of γ-H2AX foci per cell was counted and calculated for at least 70 cells in each group. Error bars, SD.

4. Discussion

Bmi-1 is a positive regulator of stem cell renewal [14]. Cancer stem cells (CSCs) frequently express high levels of Bmi-1 [15] and have been shown to be radioresistant. Brain CSCs that are associated with tumor radioresistance increase from about 2% to about 8% in control versus irradiated tumors [16]. Breast cancer-initiating cells displaying the marker of breast CSCs (CD24−/low/CD44+) are radioresistant, and the cells with these markers increase after short courses of fractional irradiation [17]. Radioresistant esophageal cancer cell Eca109R, obtained through fractional irradiation from Eca109 cells, carry some CSC-like properties and expressed higher levels of β-catenin, a stem cell marker [18]. Thus, the higher expression of Bmi-1 in KYSE radioresistant cells may be linked to the elevated population of CSC-like cells after fractional irradiation.

Bmi-1 negatively regulates the *Ink4a*/*Arf* locus that encodes two tumor suppressor proteins, $p16^{INK4a}$ and $p19^{ARF}$. $P16^{Ink4a}$ is a cyclin-dependent kinase inhibitor that blocks the activity of Cdk4/6 by preventing its association with cyclin D, this results in Rb hypophosphorylation and cell cycle arrest or senescence [19]. The inhibition of proliferation and G0/G1 arrest in KYSE-150R cells induced by Bmi-1 knockdown may operate through a derepression of $P16^{Ink4a}$. Overexpression of cyclin D1 is responsible for the radioresistance phenotype of long-term FRHepG2 and Hela cells, as this phenotype was completely abrogated when the FR cancer cells were treated by a Cdk4 inhibitor [3]. Thus, the Bmi-1/ $P16^{Ink4a}$/Cyclin D/Cdk4 pathway may be involved in the adaptive radioresistance of KYSE-150R cells.

Our results demonstrate that Bmi-1 depletion dramatically increases the ROS generation and oxidase gene (Lpo, Alox15 and Noxo1) expression in KYSE-150R cells at both the base level and after IR with increased cell death. This is consistent with a recent report showing that Bmi-$1^{-/-}$ mice have impaired mitochondrial function, and a marked increase of both oxidase gene expression and ROS production [20]. Recent studies have shown that oxidase genes, particularly Nox subunits, play critical roles in cellular responses to radiation. X-ray irradiation of HeLa cells causes dose-dependent induction of Gp91 and membrane translocation of p47 to form an active Nox complex, and the resulting ROS production contributes to radiation-induced cell death [13]. Another study reported that UVA irradiation of human keratinocytes activates the Nox1-based NADPH oxidase, which is the major source of ROS in irradiated cells [21]. Our previous study showed that the radioprotective effects of Bmi-1 for human keratinocytes depend, in part, on its ability to repress the Nox subunit genes [8]. Therefore, Bmi-1 may confer adaptive radiorasistance to KYSE-150R cells by silencing various oxidase enzymes.

Studies investigating the kinetics of H2AX dephosphorylation during recovery from DNA damage suggest that γ-H2AX may persist until the DNA repair is substantially complete [22]. The increased persistence of γ-H2AX is related to radiation sensitivity [23]. The level of γ-H2AX foci formation is also markedly increased, and a greater persistence of γ-H2AX is evident in Bmi-1 depleted KYSE-150R cells after irradiation. These data indicate that Bmi-1 mitigates the genotoxic effects of IR by enhancing the repair of damaged DNA in adaptive redioresistant ESCC cells, although the mechanism needs further investigation.

Acknowledgments

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