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Fractionated irradiation promotes radioresistance and decreases oxidative stress by increasing Nrf2 of ALDH-positive nasopharyngeal cancer stem cells

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Abstract

Radiotherapy is widely regarded as the primary therapeutic modality for nasopharyngeal cancer (NPC). Studies have shown that cancer cells with high resistance to radiation, known as radioresistant cancer cells, may cause residual illness, which in turn might contribute to the occurrence of cancer recurrence and metastasis. It has been shown that cancer stem-like cells (CSCs) exhibit resistance to radiation therapy. In the present study, fractionated doses of radiation-induced epithelial-mesenchymal transition (EMT) and ALDH+ CSCs phenotype of NPC tumor spheroids. Furthermore, it has been shown that cells with elevated ALDH activity have increased resistance to the effects of fractionated irradiation. Nuclear factor erythroid-2-related factor 2 (Nrf2) plays a pivotal role in regulating cellular antioxidant systems. A large body of evidence suggests that Nrf2 plays a significant role in the development of radioresistance in cancer. The authors' research revealed that the application of fractionated irradiation resulted in a decline in Nrf2-dependent reactive oxygen species (ROS) levels, thereby mitigating DNA damage in ALDH+ stem-like NPC cells. In addition, immunofluorescence analysis revealed that subsequent to the process of fractionated irradiation of ALDH+ cells, activated Nrf2 was predominantly localized inside the nucleus. Immunofluorescent analysis also revealed that the presence of the nuclear Nrf2+/NQO1+/ALDH1+ axis might potentially serve as an indicator of poor prognosis and resistance to radiotherapy in patients with NPC. Thus, the authors' findings strongly suggest that the radioresistance of ALDH-positive NPC CSCs to fractionated irradiation is regulated by nuclear Nrf2 accumulation. Nrf2 exerts its effects through the downstream effector NQO1/ALDH1, which depends on ROS attenuation.

Keywords: ALDH, cancer stem cells, fractionated irradiation, nasopharyngeal cancer, nuclear factor erythroid-2-related factor 2, reactive oxygen species

Introduction

Radiotherapy is the predominant and pivotal therapeutic method for the treatment of nasopharyngeal carcinoma (NPC)[1]. The locoregional control rate of NPC has shown significant enhancement over the course of the last ten years. Despite notable advancements in the locoregional control rates of NPC, local recurrence remains a substantial contributor to both mortality and morbidity, raising concerns. Effective therapy for local failure in the advanced stages of NPC continues to pose a significant problem[2]. Over the course of recent decades, extensive radiobiological investigations have yielded substantial data supporting the notion that the phenotype of cancer stem-like cells (CSCs) and the inherent radiosensitivity of these cells significantly impact the potential for successful radiotherapy treatment[3]. Recurrence and metastasis may be attributed to residual illness, which is a consequence of the radioresistant nature of CSCs. This phenomenon has been observed in both experimental and clinical settings[4]. Fractionated irradiation (IR) has the potential to induce epithelial-mesenchymal transition (EMT) and augment

HIGHLIGHT

- Our research focused on evaluating the CSCs-related signaling of nasopharyngeal cancer (NPC) radioresistance. We suggested that the radioresistance of ALDH-positive NPC CSCs to fractionated irradiation is regulated by the accumulation of nuclear Nrf2. Nrf2 exerts these effects through the downstream effectors NQO1/ALDH1, which depends on ROS attenuation. Our results provide evidence supporting the Nrf2/NQO1/ALDH1 axis as a potential signaling pathway for CSCs and radioresistance of NPC.

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stemness-like characteristics in cancer cells[5]. The therapeutic levels of IR on CSCs in terms of HPV status of patients. CSCs proportions of tumor populations are not fixed but subject to change in response to IR at therapeutic dose levels[6].

Nuclear factor erythroid-2-related factor 2 (Nrf2) serves as a pivotal regulator of antioxidant defense systems, thus exerting significant control over redox equilibrium[7]. In a state of homeostasis, the degradation of Nrf2 occurs via its interaction with Kelch-like ECH-associated protein 1 (KEAP1), an adaptor protein associated with Cullin 3 E3 ubiquitin ligase, leading to its subsequent degradation through the proteasome[8]. Oxidative and xenobiotic stressors induce the creation of an oxidizing cellular milieu, resulting in conformational alterations in KEAP1. This conformational shift subsequently triggers the release of Nrf2, leading to its accumulation and subsequent translocation into the nucleus[9]. Previous research has shown that heightened levels of Nrf2 play a pivotal role in governing the resilience of CSC-enriched breast tumors to chemoradiation[10]. Additionally, activation of Nrf2-associated antioxidant genes, such as NAD(P)H quinone oxidoreductase 1 (NQO1) and heme oxygenase 1 (HO1), contributes to the resistance of various cancer cells to radiation therapy[11]. Activation of these genes induces resistance to xenobiotic and oxidative stressors. The adaptive radioresistance observed in CSCs may be attributed, at least in part, to the presence of reduced amounts of reactive oxygen species (ROS) and an improved defensive mechanism against ROS[12]. Hence, the exploration of the underlying processes and the mitigation of low levels of ROS in NPC CSCs might potentially serve as a valuable approach to enhance the efficacy of radiation treatment. Further studies are required to explore the involvement of Nrf2 in radioresistance and ROS buildup in NPC CSCs. The technique of sphere production may be used as a targeted approach to enhance the enrichment of CSC subpopulations with increased functional capabilities. Hence, the suspension culture method may effectively sustain the undifferentiated state of CSCs, thereby allowing their enrichment.

In this study, we observed that fractionated doses of radiation selectively increased the CSCs population of ALDH + tumorspheres. High ALDH activity displays radioresistance upon exposure to fractionated irradiation, which is regulated by Nrf2. Nrf2 suppression abrogated the radioresistance of ALDH + cancer stem-like NPC cells by inhibiting ROS accumulation. Thus, our findings revealed that the Nrf2/NQO1/ALDH1 axis represents a plausible signaling pathway associated with CSCs and the development of radioresistance in NPC. This finding has significant therapeutic relevance as it may provide possible implications for the treatment of NPC.

Materials and methods

Cell culture and irradiation

The cell lines used in this study were acquired from the Shanghai Cell Biology Institute, which is affiliated with the Chinese Academy of Sciences. NPC cell lines CNE2 and SUNE1 were grown in Dulbecco's Modified Eagle Medium (DMEM) (Hyclone) supplemented with 10% fetal bovine serum (FBS) (Hyclone) and 100 U/ml penicillin/streptomycin (Gibco). Cells were cultivated under controlled conditions of humidified air containing 5% carbon dioxide at a temperature of 37°C. The cells were then exposed to the specified doses of radiation using a 250 kV orthovoltage X-ray machine and a linear accelerator (Infinity). Tumor cells were harvested after 24 h and irradiated in 0 Gy (control group), 6 Gy (a sole dose), or 2 Gy × 3 fractions (3 fractions of 2 Gy each).

Tumor spheroid formation assay

Cells that had been exposed to radiation (at a concentration of 1000 cells/ml) were cultured in serum-free medium known as Ham's F-12 (Gibco). This medium was supplemented with B27 at a ratio of 1:50 (also manufactured by Gibco), as well as 20 ng/ml of epidermal growth factor (EGF) from Invitrogen, located in Grand Island, NY, USA, and 20 ng/mL of fibroblast growth factor (FGF). The tumorspheres were observed and enumerated using a microscope after a 72-h culture period.

ALDEFLUOR assay by fluorescence-activated cell sorting (FACS)

The ALDEFLUOR test (Stem Cell Technologies, Inc.) was used to detect ALDH activity, which was subsequently analyzed using FACS. The cells were suspended in ALDEFLUOR assay buffer, which is composed of an ALDH substrate, and then incubated for 40 min at 37°C. As a negative control, an aliquot of cells was treated with a particular ALDH inhibitor, diethylaminobenzaldehyde (DEAB), at a concentration of 50 mM. The FACS analysis was conducted with a FACSAria flow cytometer manufactured by BD Biosciences. Data analysis was conducted using FlowJo 7.6.3, developed by FlowJo LLC, located in Ashland, Oregon, United States.

Cell immunofluorescence

We investigated the localization of DNA damage using γ-histone 2AX (γ-H2AX) as a marker of DNA damage in ALDH + spherical cells. The cells were cultivated on the surface of cover slides and treated with a 4% paraformaldehyde solution for fixation. Following rehydration in PBS, the fixed cells were incubated with primary antibodies against γ-H2AX and Nrf2 (obtained from Abcam). This incubation was performed either at room temperature for 1 h or at a temperature of 4°C overnight. Secondary antibodies, specifically Alexa594-conjugated goat anti-mouse IgG (Invitrogen) or Alexa488-conjugated goat anti-rabbit IgG (Invitrogen), were used in the experiment. The nuclei were subjected with 4',6-diamidino-2-phenylindole (DAPI). Sections were analyzed using a confocal microscopy (Olympus-FV1000).

Western blot analysis

Total protein was extracted from the cells using cell lysis buffer. The proteins were placed onto a gel with a 10% concentration of SDS-PAGE and then separated. Following separation, proteins were deposited onto polyvinylidene difluoride (PVDF) membranes. Following the addition of 50 g/l non-fat milk in TBST (20 mmol/l Tris-HCl, 137 mmol/l NaCl, 1 g/l Tween 20, pH 7.6) for 2 h at room temperature, the membranes were incubated overnight at 4°C with primary antibodies against E-cadherin, Vimentin, Nrf2, NQO1 and ALDH1 (Abcam). The membranes were then subjected to a 1-h incubation period with HRP-conjugated secondary antibodies (Invitrogen). Membranes were visualized using an ECL-Plus detection system (Bio-Rad).

Lentiviral constructs and infection of NPC cells

The pLKO.1 lentiviral shRNA vector and control shRNA targeting green fluorescent protein (GFP) were acquired from Sigma. The targeting sequence of Nrf2 has been previously reported[13]. The sense and antisense oligonucleotides were subjected to annealing and subsequent ligation into the pLKO.1 lentiviral vector. Subsequently, the viruses were packaged in 293T cells. The process of viral generation and subsequent infection of target cells was conducted in accordance with a previously established methodology[14].

Detection of intracellular ROS

The intracellular ROS levels were quantified using H2DCFH-DA (Invitrogen) as an indicator. Cells were treated with DMSO or SAL before exposure to IR. Subsequently, the cells were treated with a solution containing 10 μM H2DCFH-DA for 20–30 min. The cells were then rinsed with PBS before trypsinization. Following detachment, the cells were collected, subjected to two rounds of washing, and resuspended in 500 μl of PBS). The fluorescence was detected using a FACSAria flow cytometer (BD Biosciences).

The use of tissue microarray (TMA) and multiplex immunofluorescence techniques

TMA was procured from Outdo Biotech Company, located in Shanghai, China, and was accompanied by clinical information. The arrays included 110 cases of malignant nasopharyngeal tissues, including a range of grades and stages. The most recent follow-up was 31 March 2017. Written informed consent was obtained from all the patients. Multiplex immunofluorescence was performed in accordance with a previously documented protocol. Immunofluorescence analysis was performed on one paraffin section. The TMA specimen underwent a baking process at 63°C for 1 h. A fully automated LEICAST5020 dyeing machine manufactured by LEICA was used to deparaffinize the specimen and facilitate antigen retrieval. Subsequently, a commercially available hydrogen peroxidase solution was used for 10 min to eliminate endogenous peroxidase. Subsequently, the microarray was blocked for 10 min, followed by incubation with one of the following antibodies: Nrf2 (1:200, Abcam), NQO1 (1:200, Abcam), and ALDH1 (1:200, Abcam) for 1 h to facilitate the immunofluorescence staining process. Following the washing of TMA with TBST, TMA was incubated for 10 min with the secondary antibody (K8000, DAKO). Subsequently, opal dye diluent was introduced and incubated at room temperature for 10 min. The antibody complex was eliminated using microwave treatment. The subsequent markers were counterstained, and this process was continued iteratively until all indications were evaluated. The slides were counterstained using DAPI for 5 min, after which they were enclosed with an antifade mounting medium.

Statistical analysis

Statistical significance was set at P less than 0.05. Data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 19.0, and the resulting means ± SD are shown. Differences across groups were assessed using either Student's t-test or one-way analysis of variance (ANOVA). Immunofluorescent staining data were examined using the χ^2 test, and the findings are summarized in graphs. Survival probability estimates were computed using the Kaplan–Meier technique.

Results

Fractionated doses of radiation selectively increase the nasopharyngeal CSC population

In-vitro generation of nasopharyngeal CSCs populations has been achieved by the formation of tumorspheres under culture conditions that are devoid of serum[15]. Stem-like cancer cells are enriched in NPC tumorspheres. ALDH has been identified as a possible marker of NPC CSCs[16]. ALDH plays a crucial role in detoxification by facilitating the oxidation of aldehydes present in the cellular environment. The current investigation included the enrichment of NPC CSCs populations from spheroids of CNE2 and SUNE1. The ALDEFLUOR test was used to evaluate ALDH enzymatic activity. Fractionated IR with 2 Gy × 3 fractions of X-rays increased about three fold of the population of ALDH + cells in the CNE2 and SUNE1 spheroids. In contrast, ALDH + cells were notably decreased by 40–50% upon exposure to an acute dose (6 Gy) of IR compared to the parental tumorsphere cells (Fig. 1A). Tumorsphere formation efficiency (TFE) in CNE2 cells increased upon exposure to fractionated doses of IR. In contrast, TFE was decreased in CNE2 cells exposed to acute doses of IR (Fig. 1B). Taken together, the findings of this study indicate that administration of fractionated doses of radiation leads to the emergence of CSCs in NPC spheroids. This phenomenon is likely to play a role in the development of resistance to radiation therapy and subsequent recurrence of tumors.

NPC cells with high ALDH + activity display EMT upon exposure to fractionated IR, which is regulated by Nrf2

These findings suggest that fractionated IR has the potential to induce EMT and augment stemness-like characteristics in cancer cells. In the course of our investigation, we discovered that fractionated irradiation led to EMT initiation. This was shown by a 50% decrease in the expression of the epithelial marker E-cadherin and a 65% increase in the expression of the mesenchymal marker vimentin inside tumorspheres enriched with CSCs. We also detected the typical Nrf2 target gene, NQO1. CNE2 tumorspheres received fractionated IR with 2 Gy × 3 fractions of X-ray, resulting in an ~50–60% increase in the protein expression of Nrf2 and NQO1 relative to spheroids treated with 0 Gy X-ray (sham IR). Meanwhile, acute 6 Gy X-ray IR caused an ~50–70% decrease in the protein levels of Nrf2 and NQO1 compared to spheroids treated with 0 Gy X-ray (sham IR) (Fig. 2A-B). Confocal microscopy indicated that in ALDH + tumorsphere cells treated with 0 Gy X-ray, the distribution of activated Nrf2 was mostly observed in close proximity to the cell membrane. Following fractionated IR of ALDH + cells at 2 Gy × 3 fractions, active Nrf2 was mainly located in the nucleus rather than around the cell membrane. Meanwhile, it was difficult to examine nuclear Nrf2, which was irradiated by an acute 6 Gy X-ray (Fig. 2C). These results revealed that the activation of Nrf2 plays an important role in the radioresistance of ALDH + stem-like NPC cells.

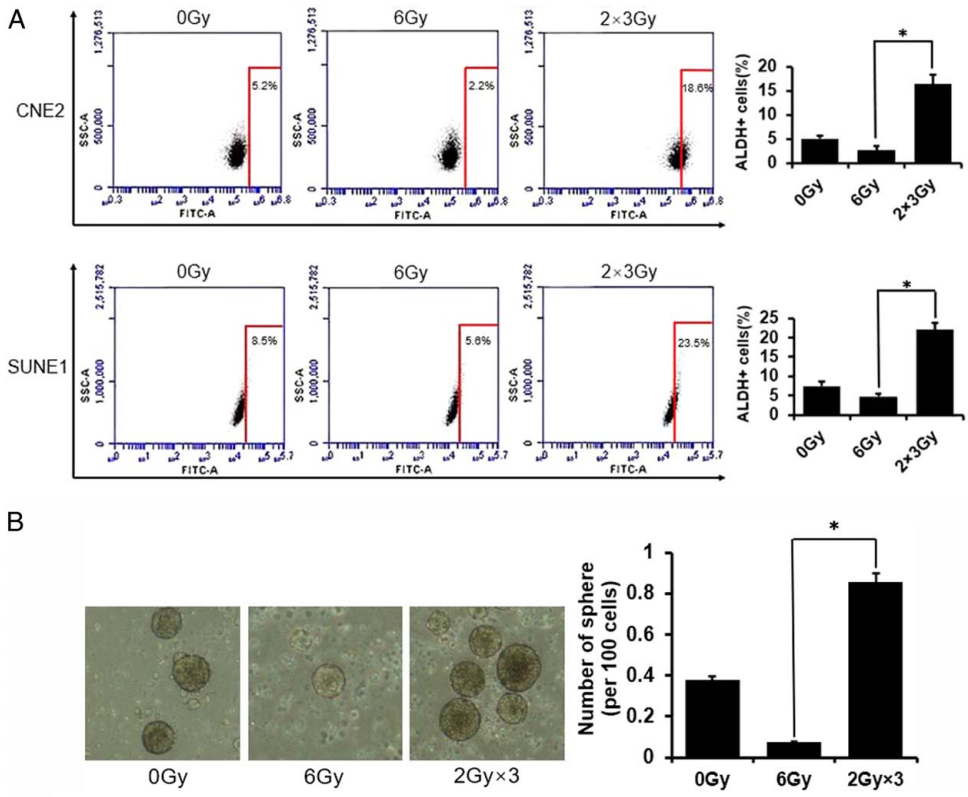

Figure 1. Fractionated doses of radiation induce the cancer stem-like cell (CSC) phenotype of nasopharyngeal cancer (NPC) spherical cells. (A) NPC CSC population was identified in CNE2 and SUNE1 cells irradiated with a fractionated and acute dose of IR by assessing ALDH activity. The error bars in the graph indicate the standard error derived from three separate and distinct studies. (B) Tumorsphere formation of irradiated CNE2 cells cultivated in serum-free medium for 72 h. Data were shown as mean ± SD, $^*P < 0.01$.

Increased radioresistance of ALDH + NPC CSCs is caused by Nrf2-dependent ROS and leads to DNA damage

To determine whether the increase of Nrf2 protein levels was induced by fractionated irradiation in ALDH-enriched NPC cells, lentivirus-mediated shRNA was used to induce Nrf2 knockdown in CNE2 cells. The expression levels of Nrf2 and ALDH1 in shNrf2 sphere cells were not significantly changed after treatment with acute 6 Gy X-ray IR. Nrf2 and ALDH1 protein expression was significantly upregulated by fractionated IR treatment in shNrf2 sphere cells (Fig. 3A). In the current study, intracellular levels of ROS were quantified after exposure to IR using the fluorescent indicator H2DCFH-DA. The quantities of ROS were found to be significantly greater in CNE2 cells lacking the Nrf2 protein than in parental tumorsphere cells. Nrf2 depletion increased about 4-fold of ROS generation in tumorsphere cells that received fractionated IR compared to sham irradiation. A further increase in ROS generation was observed in shNrf2 sphere cells that received acute irradiation (Fig. 3B). To discern disparities in radiation-induced DNA damage among ALDH + sphere cells, we sorted ALDH + cells from shNrf2 sphere cells. The results of immunostaining analysis revealed that ALDH + shNrf2/CNE2 cells exhibited a higher number of γ-H2AX foci than the control NC/CNE2 cells after fractionated IR. Furthermore, ALDH + shNrf2/CNE2 cells had an ~80% increase in γ-H2AX foci after acute IR compared to fractionated IR (Fig. 3C). Fractionated IR increased the level of Nrf2-dependent ROS and alleviated DNA damage in ALDH + sphere cells. Thus, the silencing of Nrf2 attenuates the radioresistance of ALDH + cancer stem-like NPC cells through elevated ROS generation.

Expression of nuclear Nrf2, NQO1, and ALDH1 are correlated and can predict the prognosis of radiotherapy in human NPC samples

The findings of our tests provide evidence that the radioresistance seen in ALDH + NPC CSCs is attributed to a decrease in ROS, a process that is controlled by Nrf2, as shown *in vitro*. Positive nuclear immunostaining for Nrf2 was observed in 38 of the 110 tumors (34.5%). Twenty-five of the 38 (65.8%) specimens with positive nuclear Nrf2 staining displayed both NQO1 and ALDH1 immunoreactivity. Of the 72 nucleus Nrf2 negative specimens, 48 (66.7%) did not show NQO1 or ALDH1 staining (Fig. 4A). Statistical analysis of the obtained findings revealed a significant association between the presence of nuclear Nrf2 and ALDH1 ($P < 0.01$) (Fig. 4B). Interestingly, there was a significant correlation between NQO1 and ALDH1 immunoreactivity ($P < 0.01$) (Fig. 4B). Radiotherapy is considered to be the primary therapeutic modality for NPC. Nuclear NRF2 + and NQO1 + /ALDH1 + cells indicates CSCs-like cells in NPC specimen. Those exhibiting nuclear Nrf2 – /NQO1 – /ALDH1 – expression had a significantly improved prognosis compared to those exhibiting nuclear Nrf2 + /NQO1 + /ALDH1 + expression ($P = 0.017$)

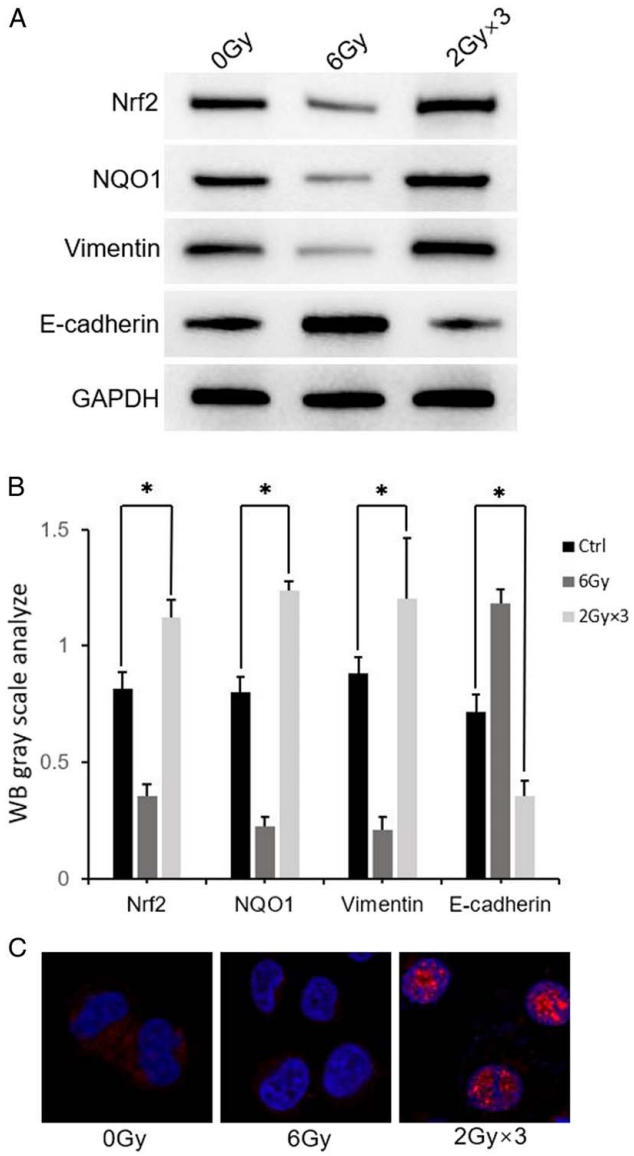

Figure 2. ALDH+ nasopharyngeal cancer stem-like cells display epithelial-mesenchymal transition upon exposure to fractionated IR, which is regulated by nuclear factor erythroid-2-related factor 2 (Nrf2). (A) CNE2 sphere cells were treated with 0 Gy, 6 Gy × 1 fraction, or 2 Gy × 3 fractions, respectively. Western blot analysis of the expression of E-cadherin, Vimentin, Nrf2, and NAD(P)H quinone oxidoreductase 1 (NQO1). (B) Quantitative detection of the expression of E-cadherin, Vimentin, Nrf2, and NQO1. (C) Immunofluorescence of Nrf2 in ALDH+ CNE2 cells after 0 Gy, 6 Gy × 1 fraction, or 2 Gy × 3 fractions IR. The expression of Nrf2 is seen using a red color, whereas the nuclei are stained using 4',6-diamidino-2-phenylindole, resulting in a blue coloration. The acquisition of images was performed using confocal microscopy. Data were shown as mean ± SD, $^*P < 0.01$.

(Fig. 4C). Therefore, there is a correlation between the expression of nuclear Nrf2, NQO1, and ALDH1.

Discussion

The current research has confirmed that CSCs play a significant role in therapeutic resistance, carcinogenesis, and tumor recurrence. Radiation exposure has the potential to CSCs apoptosis via ROS production and the infliction of DNA damage[17]. Nevertheless, The findings of our study indicate that the administration of fractionated doses of IR resulted in augmentation of the NPC TEF and the population of ALDH+ CSCs. Importantly, ALDH-positive cells showed significantly increased radioresistance when exposed to fractionated IR compared to a single high dose of IR in the present study. It is hypothesized that a higher single dose of radiation is expected to have a stronger effect in eradicating CSCs than fractionated IR. The observation of a greater increase in CSCs following fractionated IR supports the notion that the expansion of CSCs induced by IR is a result of the adaptation of NPC cells accompanied by the acquisition of stem cell properties and resistance to anticancer therapy.

Nrf2 is a nuclear transcription factor that protects cellular integrity by assimilating signals of cellular stress, orchestrating several transcriptional programs, and participating in a range of cellular phenomena including proliferation, differentiation, migration, apoptosis, and angiogenesis. A growing body of data supports the involvement of Nrf2 in the development of resistance to radiation treatment in cancer cells. The downregulation of Nrf2 results in increased sensitivity to IR in some cancer cell lines[18,19]. The aforementioned research has provided evidence that the continuous activation of Nrf2 and the subsequent regulation of Nrf2-associated genes are responsible for the development of neoplastic radioresistance to anticancer medicines[20]. In the present study, we found that NPC tumorspheres that received fractionated IR exhibited a significant increase in the levels of Nrf2 and the NRF2 target gene NQO1. Furthermore, we directly examined the nuclei of the ALDH+ cells. After subjecting ALDH+ cells to fractionated IR, it was shown that Nrf2 underwent translocation to the nucleus. The findings presented in this study provide evidence that the accumulation of nuclear Nrf2 is correlated with the maintenance of stem cell properties and increased resistance to radiation in NPC patients.

Molecular targeting of Nrf2 has been suggested as a promising strategy for overcoming chemoradioresistance in tumors[21]. Nevertheless, the number of natural compounds identified as Nrf2 inhibitors is limited. In our study, the expression levels of Nrf2 and ALDH1 were significantly upregulated after treatment with fractionated irradiation rather than high-dose irradiation in Nrf2-deficient CNE2 tumorspheres. Therefore, our data support the hypothesis that CSCs radioresistance induced by fractionated irradiation is associated with the inhibition of Nrf2. The transcriptional control of several detoxification enzymes by Nrf2 protects cells from damage caused by oxidative stress[22]. The transcription factor Nrf2 plays a significant role in the cellular defense system against ROS formation, which is a crucial process contributing to radioresistance[23]. In several cancer cell types, it has been shown to play a significant role of ROS production[24]. Elevated ROS levels may result in substantial DNA damage, apoptosis, and activation of oxidative stress pathways[25]. The findings of our study indicate that IR plays a significant role in the production of ROS in shNrf2/CNE2 cells. Additionally, our findings revealed that CNE2 cells lacking Nrf2 exhibited significantly elevated levels of ROS compared to control parental cells. ROS levels and DNA damage in shNrf2/CNE2 cells dramatically increased after exposure to acute irradiation. Our results indicate that NPC radioresistance and CSCs are regulated by the Nrf2 antioxidant pathway, which leads to the attenuation of the defense system against ROS. Notably, fractionated IR, rather than acute IR, can induce NPC CSCs and radioresistance, which is regulated by Nrf2. A study conducted by Kim *et al.*[26]

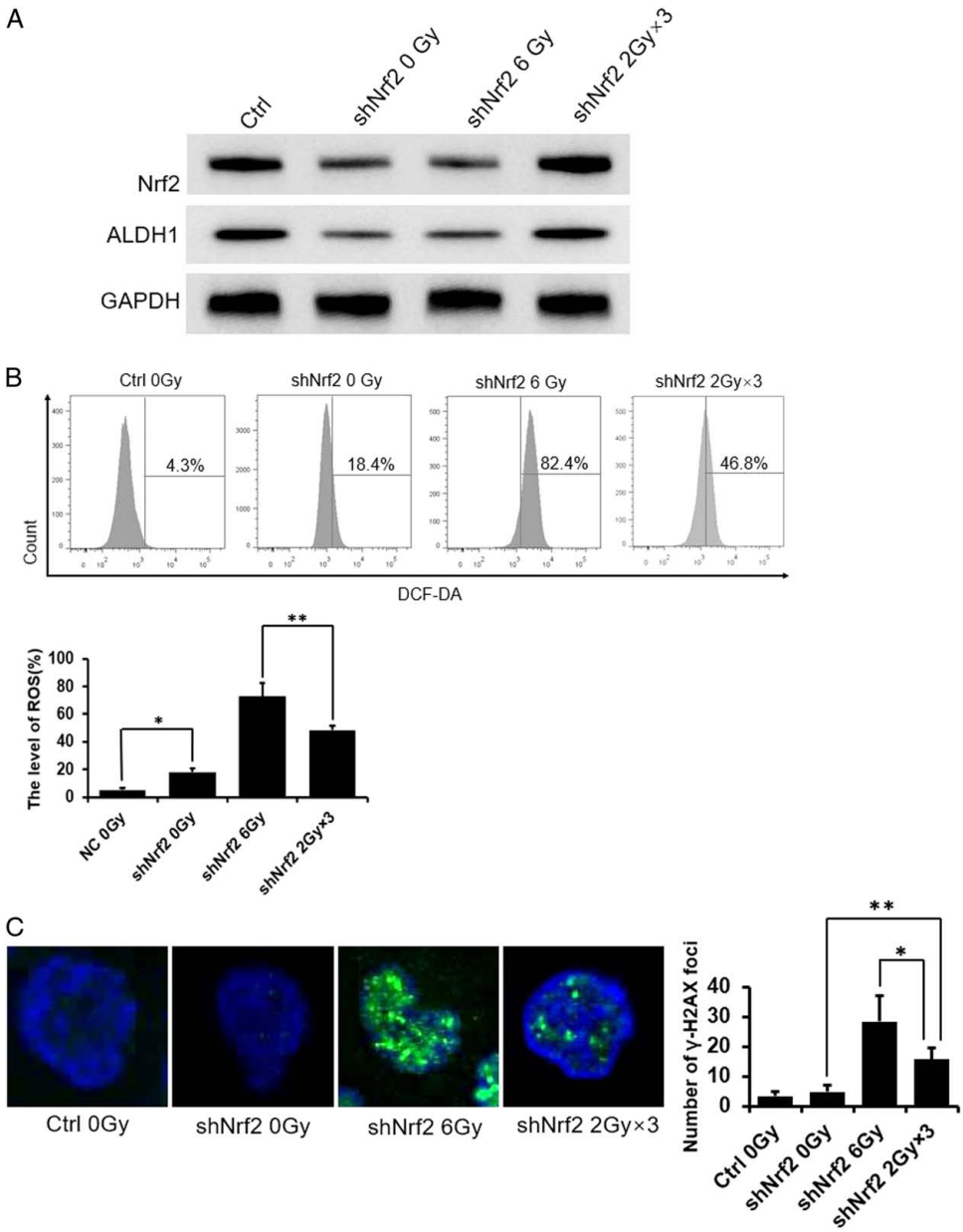

Figure 3. Radioresistance of ALDH + nasopharyngeal cancer stem-like cells is caused by nuclear factor erythroid-2-related factor 2 (Nrf2)-dependent reactive oxygen species (ROS) and leads to DNA damage. (A) shNrf2/CNE2 sphere cells were treated with 0 Gy, 6 Gy × 1 fraction or 2 Gy × 3 fractions X-ray, respectively. Western blot analysis of the expression of Nrf2 and ALDH1. (B) The levels of ROS were detected using the fluorescent probe H2DCFH-DA in the field of fluorescence-activated cell sorting. Cells that had been exposed to IR were seen to exhibit signs of IR following a 24-h treatment period. The bar chart indicates the level of ROS. (C) shNrf2/CNE2 ALDH + sphere cells were sorted directly onto glass slides following IR at 0 Gy, 6 Gy × 1 fraction, or 2 Gy × 3 fractions and immunostained with anti-γ-H2AX (Green). Nuclei were stained with 4',6-diamidino-2-phenylindole (Blue). The error bars in the graph depict the standard error derived from three distinct and separate experimental trials. Data were shown as mean ± SD, $^{*}P < 0.01$, $^{**}P < 0.01$.

showed that fractionated IR induced EMT signaling pathways through counter-regulation of ROS-scavenging and ROS-generating systems. This transition boosts the migratory, invasive, and stemness-like characteristics of the cells. These findings shed light on potential causes of radioresistance in cancer cells. In addition, Zhang *et al.*[27] reported that the fractionated IR-induced esophageal squamous cancer cell line KYSE-150R developed EMT-like changes that showed relative quiescence and significant propensity for spherical expansion and carcinogenesis, indicating the presence of stemness-like features. The current study observed an elevation in CSCs, namely, the ALDH + population and vimentin, which are suggestive of EMT. Additionally, a reduction in the expression of the mesenchymal marker E-cadherin was observed. These findings suggest that fractionated doses of IR contribute to the augmentation of CSCs with a mesenchymal phenotype. Numerous lines of evidence have shown that cancer cells that have completed EMT are prone to exhibit stemness-like characteristics across various types of

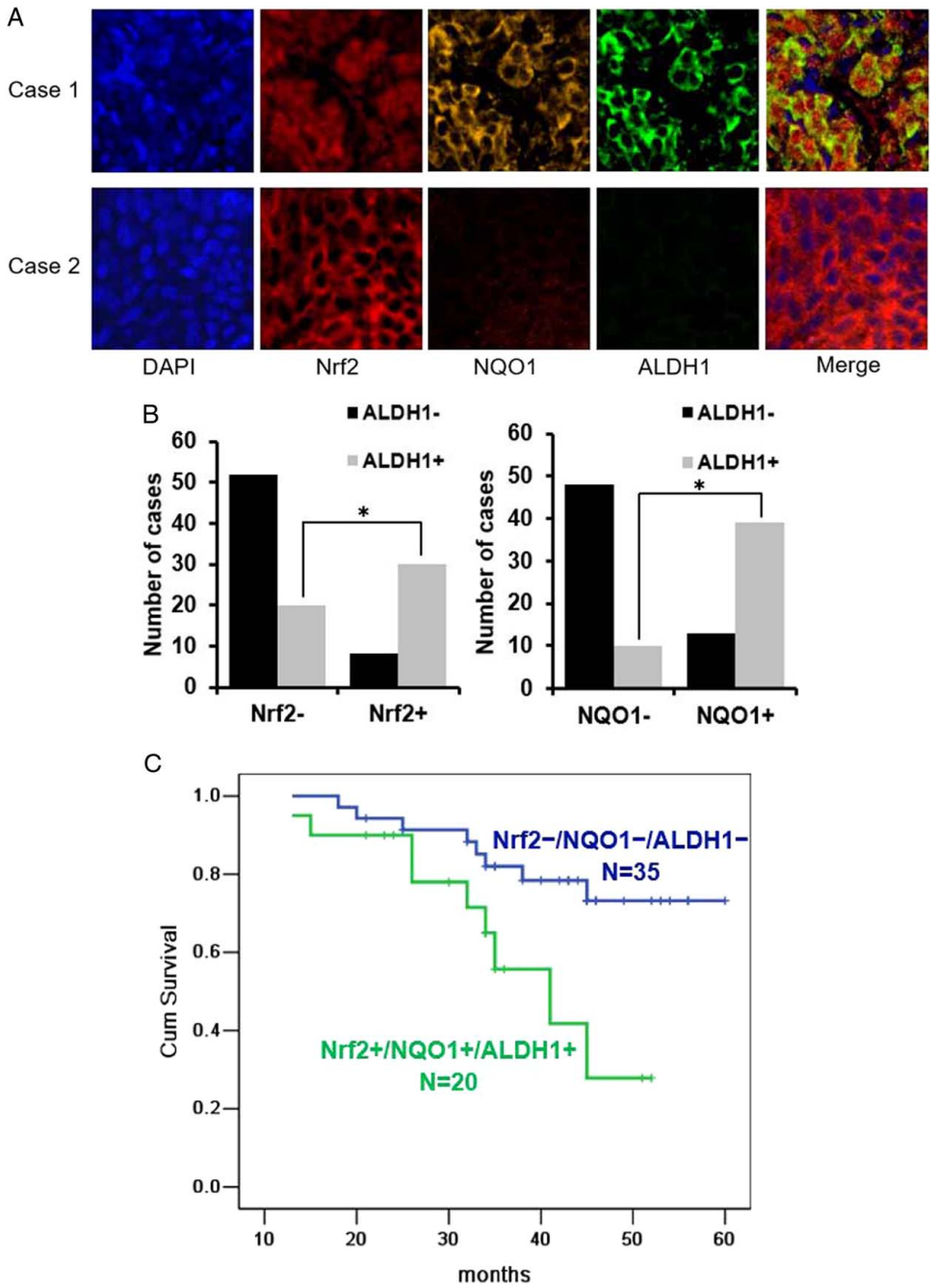

Figure 4. The expression levels of nuclear factor erythroid-2-related factor 2 (Nrf2), NAD(P)H quinone oxidoreductase 1 (NQO1), and ALDH1 exhibit a significant correlation and possess the potential to serve as prognostic indicators for radiotherapy outcomes in nasopharyngeal cancer (NPC) specimens. (A) Multiplex immunofluorescent labeling was performed on representative instances from 110 NPC specimens in TMA, targeting nuclear Nrf2, NQO1 and ALDH1. Photographs were captured with a magnification level of 200 times. (B) Graphs illustrating the results of a chi-squared analysis conducted on immunohistochemical staining data for Nrf2 compared to ALDH1, denoted by an * indicating $P < 0.01$. Additionally, the analysis was performed on NQO1 compared to ALDH1, indicated by an * $P <$ 0.01. (C) The overall survival of patients with NPC who had radiation was analyzed using the Kaplan–Meier method. The study was based on the expression of nucleus Nrf2 + /NQO1 + /ALDH1 + in a group of 20 patients and nucleus Nrf2 – /NQO1 – /ALDH1 – in a group of 35 patients. The statistical analysis revealed a significant difference in overall survival between the two groups, with a p value of 0.017.

cancer[28]. However, the effects of high doses of acute IR on cancer cells are quite different. According to a previous report, murine hematopoietic stem and progenitor cells exhibit activation of the p53-dependent DNA damage response following exposure to high IR doses. This activation gives rise to various potential consequences, such as the induction of apoptosis through the upregulation of p53 upregulated mediator of apoptosis, the activation of the granulocyte colony-stimulating factor (G-CSF)/Stat3/BATF pathway leading to differentiation, or the occurrence of long-term senescence accompanied by persistent

overproduction of ROS[29]. Moreover, research conducted with elevated levels of IR has shown that the induction of autophagy by rapamycin may effectively shield cancer cells from the damaging effects of IR both *ex vivo* and *in vivo*[30].

In the current study, it was shown that augmented levels of nuclear Nrf2, as well as heightened expression of NQO1 and ALDH1, were significantly correlated with worse prognosis among individuals diagnosed with NPC who received radiation. The correlation between the expression of NQO1 and ALDH1 was clearly shown in the nuclear compartment, but no such correlation was observed in the membranous or cytoplasmic compartments of Nrf2. Of the total sample size of 110, 85 patients had undergone radiotherapy with a total dose of 60–70 Gy, administered in fractions of 2 Gy each. In this study, we analyzed the survival rate of patients based on the expression levels of nuclear Nrf2, NQO1, and ALDH1. In this study, we put forward the hypothesized that the Nrf2 + /NQO1 + /ALDH1 + axis plays a significant role in the development of CSCs and acquisition of radioresistance in individuals diagnosed with NPC.

In summary, the findings of our study indicate that Nrf2 plays a pivotal role in the modulation of CSCs and the resistance of NPC to radiotherapy. NPCs possess characteristics similar to those of CSCs, and exhibit resistance to fractionated IR. The actions of Nrf2 are mediated through the downstream effector NQO1/ALDH1. It is worth mentioning that the activation of nuclear Nrf2 plays a crucial role in the regulation of CSC phenotypes and the ability of NPC cells to resist the effects of radiation. The findings of our study provide preclinical evidence for the possible role of the Nrf2/NQO1/ALDH1 axis as a signaling pathway in the context of CSCs and the development of radioresistance in NPC.

Ethical approval

This study was conducted in accordance with the Declaration of Helsinki. The study protocols were approved by the National Human Genetic Resources Sharing Service Platform (no. 2005DKA21300). The patients and their families provided written informed consent for the use of the specimens in the present study.

Consent

The patients and their families provided written informed consent for the use of the specimens in the present study.

Source of funding

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Author contribution

G.D. and Z.Y. performed FACS and immunofluorescence staining. X.D. conducted western blot analysis. L.H. and M.L. prepared the lentiviral constructs and subsequently infected NPC cells. L.L. performed the IR. Y.X. conducted a statistical study. The research was developed and the article was written by G.Z. The funding acquisition come from G.Z. The final paper was reviewed and endorsed by all authors.

Conflicts of interest disclosure

The authors declare that they have no competing financial interests or personal relationships that could have influenced the work reported in this study.

Research registration unique identifying number (UIN)

No. 2005DKA21300.

Guarantor

All authors.

Data availability statement

We confirm that any datasets generated during and analyzed during the current study are publicly available.

Provenance and peer review

Yes.

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