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ORIGINAL ARTICLE – CANCER RESEARCH

NRF2 orchestrates the redox regulation induced by radiation therapy, sustaining embryonal and alveolar rhabdomyosarcoma cells radioresistance

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Abstract

Purpose Tumor cells generally exhibit higher levels of reactive oxygen species (ROS), however, when stressed, tumor cells can undergo a process of 'Redox Resetting' to acquire a new redox balance with stronger antioxidant systems that enable cancer cells to become resistant to radiation therapy (RT). Here, we describe how RT affects the oxidant/antioxidant balance in human embryonal (RD) and alveolar (RH30) rhabdomyosarcoma (RMS) cell lines, investigating on the molecular mechanisms involved.

Methods Radiations were delivered using an x-6 MV photon linear accelerator and their effects were assessed by vitality and clonogenic assays. The expression of specific antioxidant-enzymes, such as Superoxide Dismutases (SODs), Catalase (CAT) and Glutathione Peroxidases 4 (GPx4), miRNAs (miR-22, -126, -210, -375, -146a, -34a) and the transcription factor NRF2 was analyzed by quantitative polymerase chain reaction (q-PCR) and western blotting. RNA interference experiments were performed to evaluate the role of NRF2.

Results Doses of RT higher than 2 Gy significantly affected RMS clonogenic ability by increasing ROS production. RMS rapidly and efficiently brought back ROS levels by up-regulating the gene expression of antioxidant enzymes, miRNAs as well as of NRF2. Silencing of NRF2 restrained the RMS ability to counteract RT-induced ROS accumulation, antioxidant enzyme and miRNA expression and was able to increase the abundance of γ-H2AX, a biomarker of DNA damage, in RT-treated cells.

Conclusions Taken together, our data suggest the strategic role of oxidant/antioxidant balance in restraining the therapeutic efficiency of RT in RMS treatment and identify NRF2 as a new potential molecular target whose inhibition might represent a novel radiosensitizing therapeutic strategy for RMS clinical management.

Keywords Rhabdomyosarcoma · Radiotherapy · Radioresistance · Reactive oxygen species · Anti-oxidant · NRF2

Introduction

Rhabdomyosarcoma (RMS), the most aggressive, highly malignant and common childhood soft-tissue sarcoma, arises from mesenchymal cells bearing developmental features of skeletal muscle (Arndt et al. 2012). Histologically, the two most common subtypes are embryonal RMS (ERMS) and alveolar RMS (ARMS), respectively, comprising 60% and 25% of all RMS (Newton et al. 1988), with the latter generally associated with a worse outcome (Meza et al. 2006). Treatment of localized RMS provides a multimodality approach based on the use of radiation therapy (RT), combined or not with chemotherapy, performed before or after surgery in localized clinically unresectable or resectable tumors, respectively (Frezza et al. 2018). RT aims to reduce the tumor mass or to eradicate residual tumor cells, especially when the surgical eradication is not complete or limited by the anatomic position, therefore configuring as a prerogative for the multi-modal

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therapy. However, the survival rate of RMS patients has not appreciably improved in the last 15 years (Smith et al. 2014), especially in adults (Wolden and Alektiar 2010), and the treatment failure is commonly due to the ability of RMS cells to become radioresistant. Thus, unraveling the molecular mechanisms responsible for the radioresistance is necessary to set up new radiosensitizing protocols in the RMS clinical management.

RT induces cancer cell death by the direct or indirect action of radiation on the DNA molecules. Indeed, radiation can disrupt the DNA structure through direct molecule breaks or indirectly by increasing the water solvent temperature that leads to the generation of reactive oxygen species (ROS), this causing DNA damage (Desouky et al. 2015). Considering the relative abundance of water in the body, the indirect mechanism of DNA breaks seems to be the preferential one and, therefore, cancer cells often develop antioxidant strategies aimed to counteract ROS production to restrain the RT efficiency (Ciccarese and Ciminale 2017).

ROS levels not only can trigger DNA damage but even induce intracellular responses that help tumor cells to survive to radiation-induced damage (Liou and Storz 2010). An increased threshold of ROS levels has been shown to impact the expression of several antioxidant genes (Liou and Storz 2010) and microRNAs (miRNAs) (Banerjee et al. 2017), a class of ~22-nucleotide small non-coding RNAs that post-transcriptionally regulate gene expression and are frequently deregulated in cancer (Lovat et al. 2011). It is still largely unknown how RT modulates oxidative stress in RMS cells and whether/which genes and miRNAs are involved.

This study describes the in vitro responsiveness of RD (ERMS) and RH30 (ARMS) cell lines to increasing RT doses. Herein we found that both RMS cells efficiently counteract RT-induced ROS accumulation by upregulating the expression of antioxidant-enzymes, such as superoxide dismutases (SOD), catalase (CAT) and glutathione peroxidases 4 (GPx4), and several miRNAs (miR-22, -126, -210, -375, -146a, -34a) (Banerjee et al. 2017). By investigating on the molecular mechanism modulated by RT, we found that radiation increased the expression of NRF2 (nuclear factor erythroid 2-related factor), a transcription factor known to orchestrate the expression of pro- and anti-oxidant proteins, this protecting normal cells and cancer cells from the oxidative stress (Kansanen et al. 2013). NRF2 silencing counteracted the expression of anti-oxidant enzymes and miRNAs and so amplified the RT-mediated toxicity. Altogether these findings indicate, for the first time, a new potential radioresistance-related mechanism based on the ability of RMS cells to overcome RT-induced ROS-mediated toxicity by activating an anti-oxidant response orchestrated by NRF2, which mat represents a potential target for new radiosensitizing therapies in RMS tumors.

Methods and materials

Cell cultures, radiation exposure and clonogenic survival assay

Human RMS cell lines, RD (ERMS) and RH30 (ARMS) were obtained by American Type Culture Collection and maintained in high-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 1% v/v l-glutamine, 100 µg/ml streptomycin and 100 U/ml penicillin and grown at 37 °C in a humidified atmosphere of 5% CO_2. DNA profiling using the GenePrint 10 System (Promega Corporation, Madison, WI, USA) was carried out to authenticate cells, by comparing the DNA profile of our cell cultures with those found in GenBank (Faggi et al. 2015).

For clonogenic survival, exponentially growing cells (70% confluence) were cultured in regular medium and then irradiated at room temperature (rt) with increasing doses of radiation (0–5 Gy) using an X-ray linear accelerator (dose rate of 200 cGy/min). Non-irradiated controls were handled identically to the irradiated cells, except for the radiation exposure. After treatment, cells were re-seeded into 6-well plates (2000 cells/well) in triplicate; 14 days later, the medium was removed and colonies were fixed with methanol: acetic acid (10:1, v/v), stained with crystal violet and photographed. Colony quantification was performed as previously described (Megiorni et al. 2017; Marampon et al. 2018).

Mitochondrial superoxide anion ($\cdot O_2^-$) production

RMS cell lines were seeded in 6-well plates at a starting number of 6×10^4 cells/well for 24 h in DMEM with 10% FBS and, then, irradiated. Immediately and 12 h after radiation exposure, flow cytometry analysis was performed. Medium was discarded, and cells were incubated in Hank's Balanced Salt Solution (HBSS) (Sigma-Aldrich, Milan, Italy) and MitoSOX Red (Thermo Fisher Scientific, Milan, Italy) for 15 min at 37 °C in dark, to evaluate mitochondrial superoxide anion ($\cdot O_2^-$) production. MitoSOX Red was dissolved in DMSO at the final concentration of 5 µM. Cells were then harvested by trypsin, collected into cytometry tubes and centrifuged at 1500 rpm for 10 min. Besides, 1×10^4 cells per assay were resuspended in saline solution and analyzed by flow cytometry. Data were analyzed with CellQuest software (Becton Dickinson) and results were represented as median of fluorescence (AU) (Cheleschi et al. 2017).

RNA isolation and qPCR

Twenty-four hours after radiation exposure total RNA was extracted by using TriPure Isolation Reagent according to the manufacturer's instructions (Euroclone, Italy). The concentration, purity, and integrity of RNA were evaluated as already described (Cheleschi et al. 2017). Reverse transcription for target genes (SOD, CAT and GPx4) was performed by using QuantiTect Reverse Transcription Kit (Qiagen, Hilde, Germany), whilst miRNAs (miR-22, -126, -210, -375, -146a and -34a) were retro-transcribed by using the cDNA miScript PCR Reverse Transcription (Qiagen, Hilde, Germany), according to the manufacturer's instructions. Target genes and miRNAs were analyzed by real-time PCR (qPCR), as already described (Pelosi et al. 2017). For data analysis, the C_t values in each sample and the efficiencies of the primer set were calculated using LinReg Software and then converted into relative quantities (RQ) and normalized according to the Pfaffl model. Normalization was carried out using, as housekeeping genes, HPRT-1 for mRNA targets and SNORD-25 for miRNAs.

Protein extraction and western blotting

Total extracts were prepared with RIPA buffer and protein concentration was determined using the BCA protein assay kit (ThermoFisher Scientific). Western blot experiments were performed as already described (Scicchitano et al. 2018) by using the following primary antibodies: H2AX (C-20), NRF2 (A10) and GAPDH (0411) by Santa Cruz Biotechnology; phospho-H2AX (Ser139) (2577) by Cell Signaling Technology (Danvers, MA, USA). Appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology) were used for 1 h at rt. Protein signals were detected by using Western Bright ECL kit (Advansta, Menlo Park, CA, USA), according to the manufacturer's instructions, and visualized by ChemiDoc XRS+ (Bio-Rad, Hercules, CA, USA). Densitometry was performed to quantify changes in protein expression using the Image Lab 5.1 software (Bio-Rad).

siRNA transfection

Small interfering RNAs (siRNAs) were transfected as already described (Marampon et al. 2016). Briefly, RMS cells were seeded at 50–60% confluence in 6-well plates. Small interfering RNA (siRNA) against human NRF2 ($NRF2^{siRNA}$, sc-37030 by Santa Cruz Biotechnology, Dallas, TX, USA) or siRNA negative control (CTR^{siRNA}, sc-37007 by Santa Cruz Biotechnology) were combined with RNAiMAX (Invitrogen) and used at 60 nM final concentration; $NRF2^{siRNA}$ is a pool of three target specific 19–25 nt-siRNAs designed to specifically knock down the targeted genes.

Statistical analysis

The results were expressed as the mean ± SD of three independent experiments, each performed in triplicate. Data normal distribution was confirmed by Shapiro–Wilk, D'Agostino and Pearson and Kolmogorov–Smirnov tests. Real-time PCR experiments were evaluated by one-way (ANOVA) with a Tukey's post hoc test using $2^{-\Delta\Delta C_t}$ values for each sample. Flow cytometry data were analyzed by ANOVA with a Bonferroni post hoc test. All analyses were performed using the SAS System (SAS Institute Inc., Cary, NC, USA) and GraphPad Prism 6.1. A statistically significant effect was indicated by a p value < 0.05.

Results

RMS cells quickly and efficiently brought back to lower than basal levels the reactive oxygen species (ROS) release induced by increasing dose of radiation

Clonogenic potential of RMS cells was assessed by treating RD and RH30 cells with a single dose of radiation equal to (2 Gy), lower (0.5–1 Gy) or higher (3–5 Gy) than the conventional dose used in clinical practice (Fig. 1). As showed in Fig. 1a, dose of RT equal to 2 Gy or higher, significantly reduced the ability of RMS cell lines to form colonies (2, 3 and 5 Gy vs. 0 Gy), reaching the maximum efficiency at 5 Gy both in RD and RH30 cells (5 Gy vs. 0.5–3 Gy) (Fig. 1a). Mitochondrial ROS production was assessed 5 min (Fig. 1b) and 12 h (Fig. 1c) after RT, by measuring the superoxide anion production. ROS production was significantly increased 5 min after irradiation ≥ 2 Gy both in RD and RH30 cells (Fig. 1b), whilst 12 h later resulted significantly lower than in non-irradiated RMS cells, without no statistically significant difference between the different RT doses (Fig. 1c). These data indicate that RMS cells rapidly and efficiently counteracted the ROS accumulation induced by RT.

RT induces the expression of anti-oxidant-related enzymes and miRNAs

Gene expression of specific antioxidant enzymes, such as superoxide dismutase (SOD-2), catalase (CAT), glutathione peroxidase (GPx-4), (Fig. 2) and ROS-related miRNAs, such as miR-22, miR-126, miR-210, miR-146a, miR-34a, and miR-375 (Fig. 3), was assessed by q-PCR, 12 h after treating

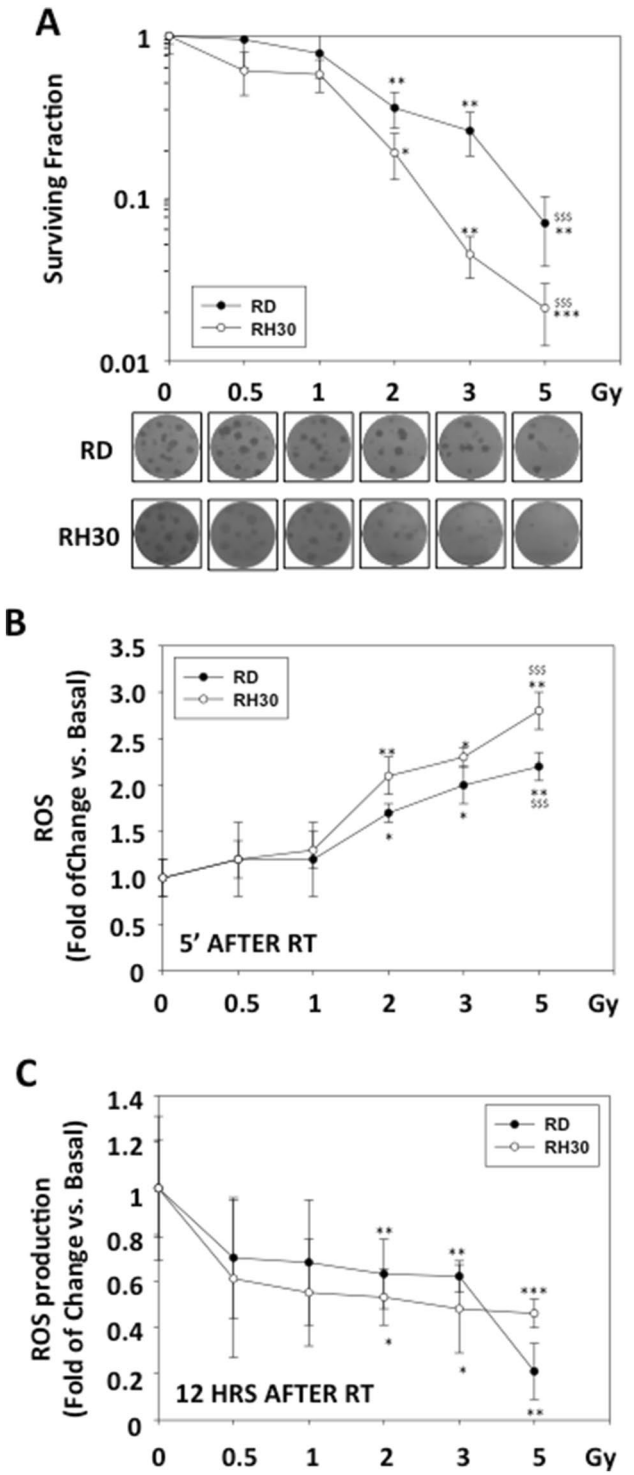

Fig. 1 RT affects RMS clonogenic ability modulating mitochondrial superoxide anion production. Human embryonal (RD) and alveolar (RH30) rhabdomyosarcoma cell lines were treated on not with different doses of radiation (0.5, 1, 2, 3 and 5 Gy). **a** Clonogenic assay; staining was performed 14 days later RT. **b, c** Mitochondrial superoxide anion production was assessed by MitoSox Red staining, 5 min (**a**) or 12 h (**b**) after RT. Data were expressed as fold of change vs. untreated cells (0 Gy). Single results are representative of three different experiments performed in triplicate. Statistical analyses: $*p<0.05$, $**p<0.01$, $***p<0.001$ vs. 0 Gy

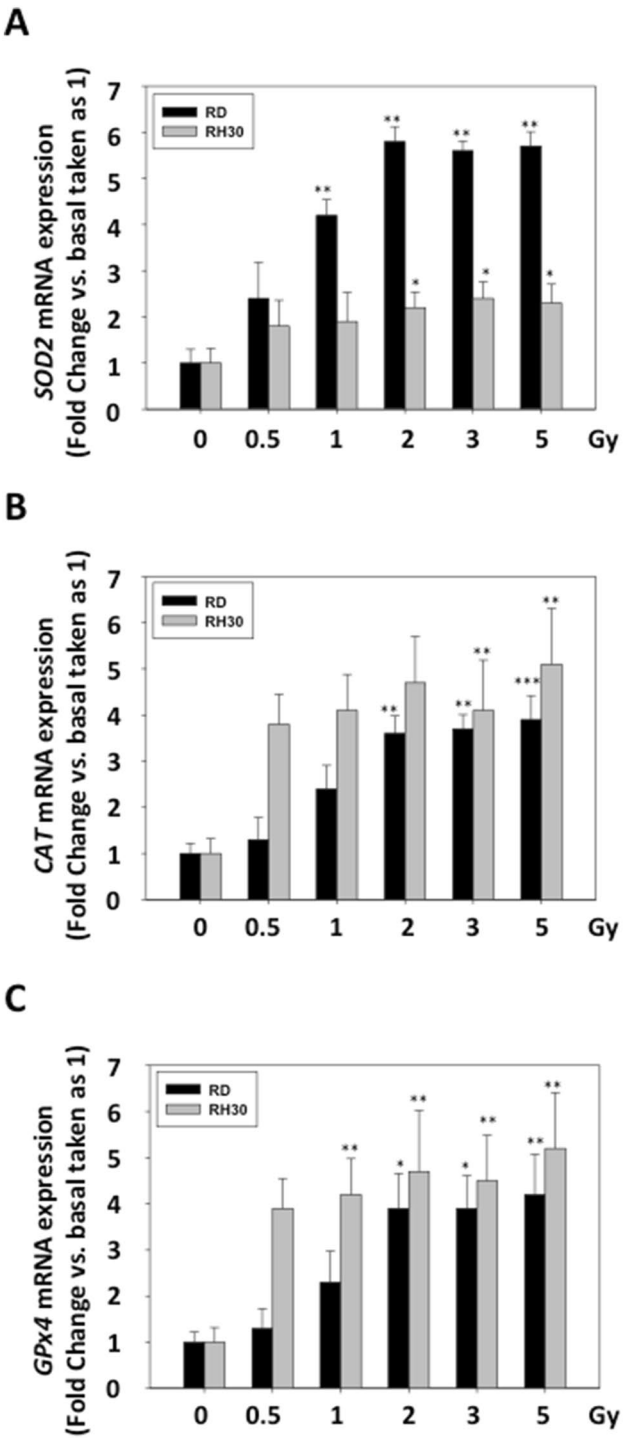

Fig. 2 RT induces gene expression on antioxidant enzymes. Human embryonal (RD) and alveolar (RH30) rhabdomyosarcoma cell lines were treated on not with different doses of radiation (0.5, 1, 2, 3 and 5 Gy) and gene expression of antioxidant enzymes superoxide dismutase (SOD-2), catalase (CAT), glutathione peroxidase (GPx)-4 and nuclear factor erythroid 2 p45-related factor (NRF2) was investigated by real-time PCR, 12 h after RT. The gene expression was referenced to the ratio of the value of interest and basal conditions. The value of basal conditions was reported equal to 1. Single results are representative of three different experiments performed in triplicate. Statistical analyses: $*p<0.05$, $**p<0.01$, $***p<0.001$ vs. 0 Gy

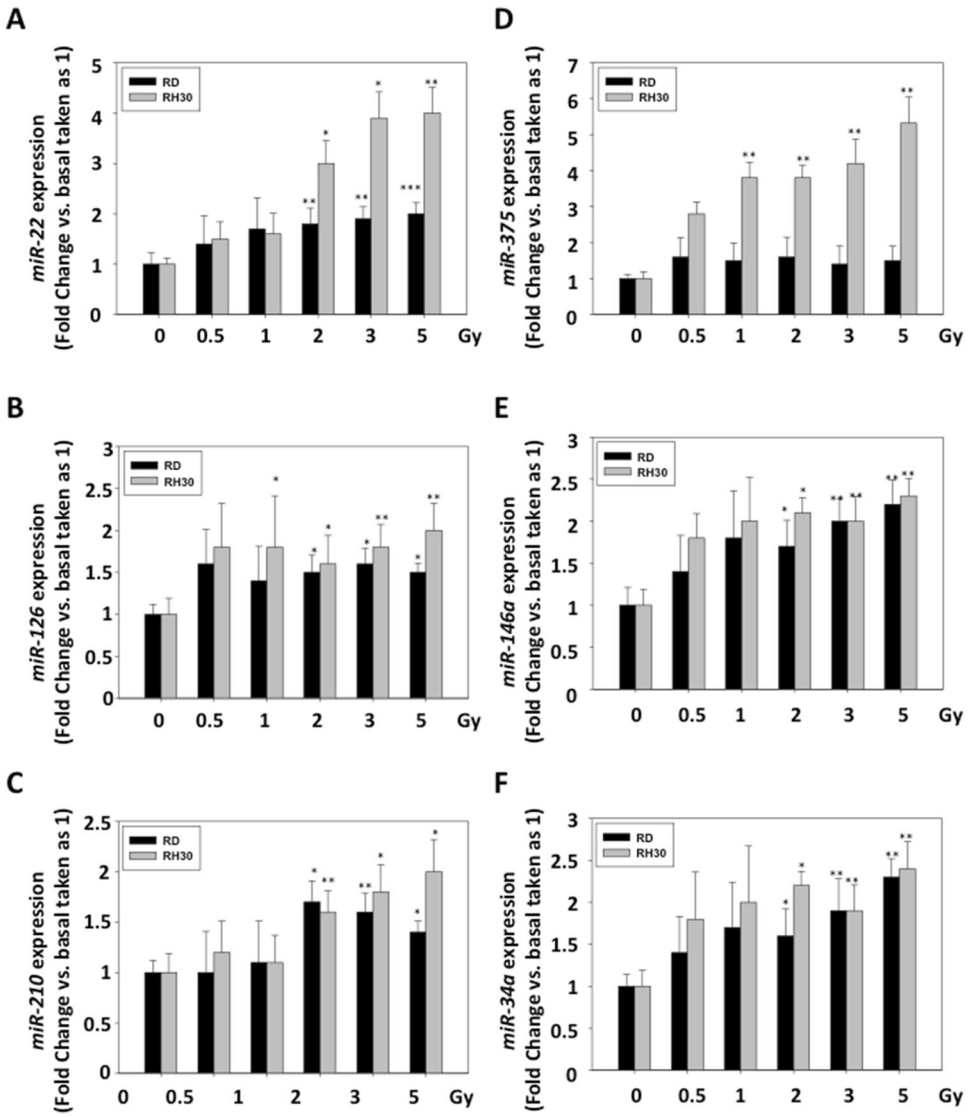

Fig. 3 RT induces gene expression on antioxidant miRNAs. Human embryonal (RD) and alveolar (RH30) rhabdomyosarcoma cell lines were treated on not with different doses of radiation (0.5, 1, 2, 3 and 5 Gy) and gene expression of miR-22, miR-126, miR-210, miR-375, miR-146α and miR-34α was investigated by real-time PCR, 12 h after RT. The gene expression was referenced to the ratio of the value of interest and basal conditions. The value of basal conditions was reported equal to 1. Single results are representative of three different experiments performed in triplicate. Statistical analyses: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs. 0 Gy

cells with increasing doses of RT. Radiation therapy significantly up-regulated gene expression of SOD-2 (Fig. 2a), CAT (Fig. 2b), GPx4 (Fig. 2c), miR-22 (Fig. 3a), miR-126 (Fig. 3b), miR-210 (Fig. 3c), miR-146a (Fig. 3e) and miR-34a (Fig. 3f) both in RD and RH30 cells, whilst miR-375 over expression (Fig. 3d) was observed in RH30 but not in RD cells. No correlation between RT doses and gene expression was described for any investigated target (Figs. 2, 3). Altogether, these evidence indicate that RMS respond to irradiation-induced oxidative stress by inducing the expression of anti-oxidant-related enzymes and miRNAs.

Antioxidant response activated by RT in RMS cell lines is orchestrated by the expression of NRF2 whose silencing radiosensitizes RMS cells by increasing irradiation-induced DNA damage

Gene expression levels of NRF2, known to protect against oxidative damage by positively regulating the expression of antioxidant genes (Menegon et al. 2016), were investigated. RT doses, ranging from 2 to 5 Gy, significantly increased NRF2 gene expression both at transcript (Fig. 4a) and protein level (Fig. 4b) in RD and RH30 cell lines. NRF2

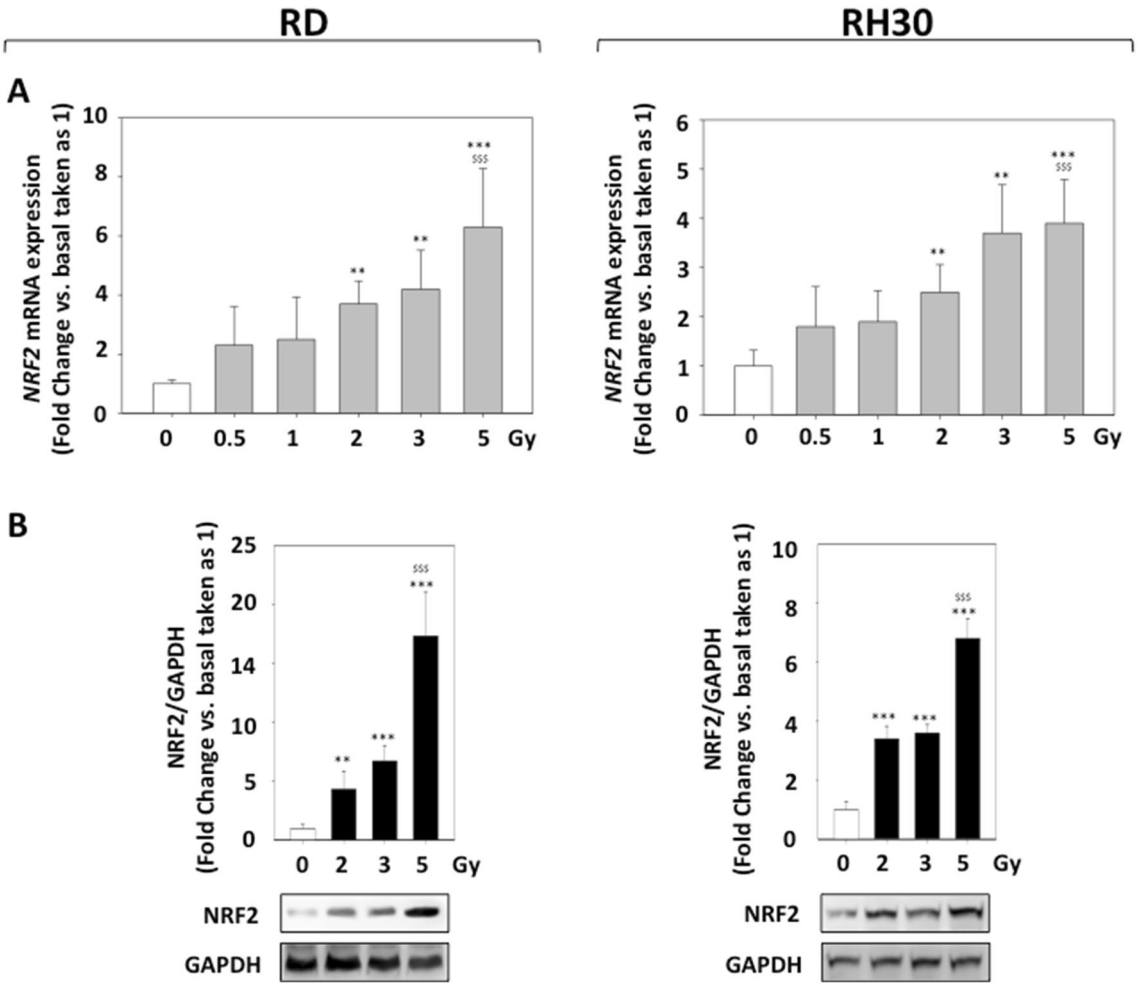

Fig. 4 RT induces Nrf2 gene expression and protein accumulation. Human embryonal (RD) and alveolar (RH30) rhabdomyosarcoma cell lines were treated on not with different doses of radiation (0.5, 1, 2, 3 and 5 Gy). 12 h after RT, Nrf2 **a** gene and **b** protein expression were investigated by real-time PCR or western blot, respectively. The gene and protein expression were referenced to the ratio of the value of interest and basal conditions. The value of basal conditions was reported equal to 1. Single results are representative of three different experiments performed in triplicate. Statistical analyses: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs. 0 Gy, $^{\$}p<0.05$, $^{\$\$}p<0.01$, $^{\$\$\$}p<0.001$ vs. 2 Gy, $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs. 3 Gy

mRNA and protein expression was not dose-dependent (Fig. 4a, b); however, its expression increase was higher in 5 Gy than in 3, 2, 1, 0.5 Gy treated cells (Fig. 4a, b). The role of NRF2 on RMS anti-oxidant responsiveness to RT was investigated by transfecting RMS cells with specific siRNAs directed against NRF2 ($NRF2^{siRNA}$) or a sequence against the *C. elegans* (CTR^{siRNA}), used as a negative control (Fig. 5). Western blotting analysis at 72 h after transfection showed that NRF2 protein levels were specifically reduced in $NRF2^{siRNA}$-transfected cells than in mocked controls (Fig. 5a). At this time, RMS cells were irradiated with a single dose of 2 Gy and mitochondrial ROS production (Fig. 5b) as well as SOD-2, CAT, GPx-4 gene expression (Fig. 5c) were assessed. Specifically, 12 h after RT, ROS levels dropped down in CTR^{siRNA} samples and remained at high levels in NRF2-depleted cells ($NRF2^{siRNA}$ + RT vs. CTR^{siRNA} + RT, 12 h after RT), this suggesting that NRF2 silencing was able to counteract the ability of RD and RH30 tumor cells to bring back ROS levels (Fig. 5b). In accordance with this evidence, the expression of SOD-2, CAT and GPx-4 genes, able to detoxify cells from ROS accumulation, was significantly downregulated after RT in $NRF2^{siRNA}$ transfected cells (Fig. 5c, $NRF2^{siRNA}$ + RT vs. CTR^{siRNA} + RT). To investigate the effects of NRF2 silencing in radiosentizing RD and RH30 cells, control- and NRF2 -siRNA transfected cells were irradiated at 2 Gy and colony formation assay was performed after treatment with ionizing radiation. As shown in Fig. 6a, a significant reduction in the number of cell colonies were observed in $NRF2^{siRNA}$ + RT cells compared to control-siRNA transfected cells. We assessed whether NRF2 silencing may sensitize RMS cells to ionizing radiation by promoting the DNA damage. To this purpose,

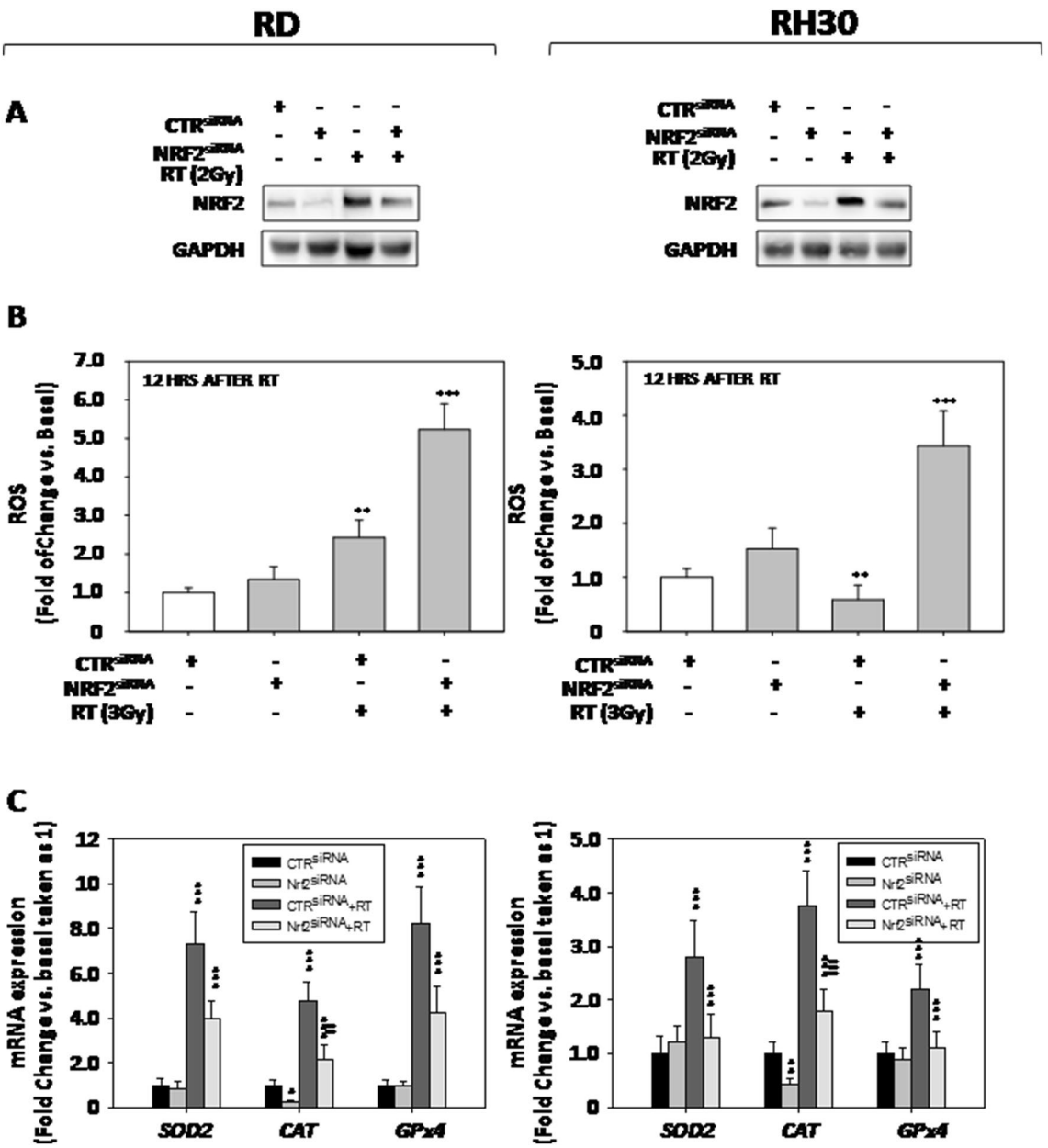

Fig. 5 Nrf2 knocking down by RNA interfering affects RMS ability to bring back RT-induced ROS accumulation and anti-oxidant gene expression. Silencing Nrf2 was performed in RD and RH30 cells ($Nrf2^{siRNA}$); control cells were obtained by transfecting cells with non-targeting control siRNA (CTR^{siRNA}). Seventy-two hours after transfection cells were treated (+) or not (−) with 2 Gy of RT. Twelve hours after RT, **a** cell lysate was collected and Nrf2 protein expression levels measured by Western blotting. Images show representative Western blots of three independent experiments; GAPDH was used as loading control. **b** Mitochondrial superoxide anion production was assessed by MitoSox Red staining. **c** SOD-2, CAT and GPx-4 gene expression were investigated by real-time PCR. The value of basal conditions was reported equal to 1. Single results are representative of three different experiments performed in triplicate. Statistical analyses: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs. CRT^{siRNA}, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs. $CRT^{siRNA}+RT$

control- and $NRF2^{siRNA}$ transfected cells were irradiated with a single dose of 2 Gy and cell lysates were processed 4 and 12 h after RT. The analysis of the abundance of γ-H2AX levels, a biomarker for DNA double-strand breaks, showed that after 4 h from RT, silencing of NRF2 did not significantly increased the RT-induced upregulation of γ-H2AX levels (Fig. 6b, 4 h), whilst restrained the ability of RMS cells to restore to basal levels γ-H2AX at 12 h after RT (Fig. 6b, $NRF2^{siRNA}+RT$ vs. $CTR^{siRNA}+RT$). Altogether, these findings indicate that NRF2 orchestrates the activation of the anti-oxidant program that preserved RMS from RT-induced DNA damage.

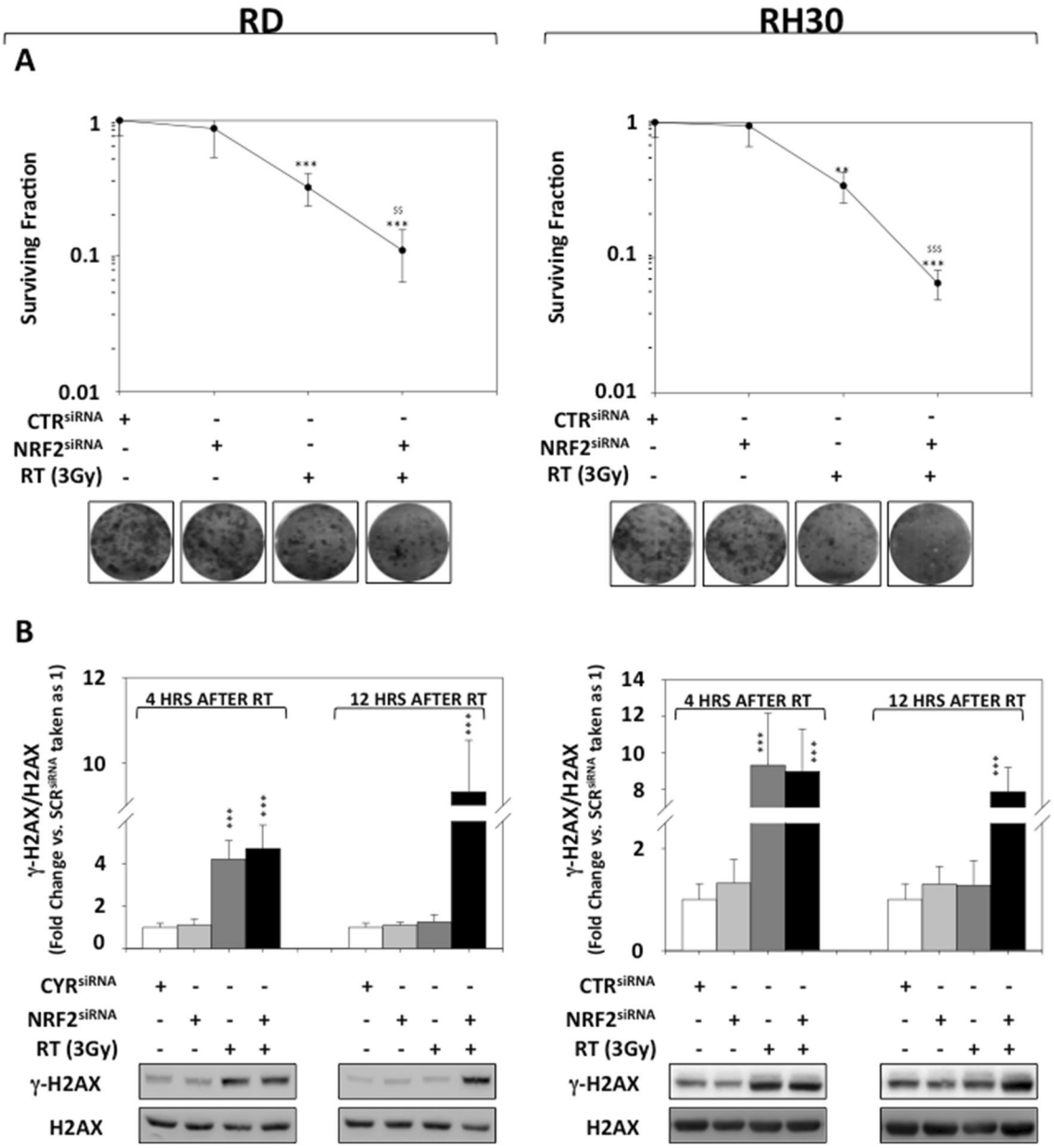

Fig. 6 Nrf2 knocking down by RNA interfering radiosensitizes RMS cells by improving RT-induced DNA double-strand break damages. Silencing Nrf2 was performed in RD and RH30 cells ($Nrf2^{siRNA}$); control cells were obtained by transfecting cells with non-targeting control siRNA (CTR^{siRNA}). Seventy-two hours after transfection cells were treated (+) or not (−) with 2 Gy of RT. **a** Clonogenic assay. Staining was performed 14 days later RT. **b** Cell lysate was collected and phosphorylation of H2AX (γ-H2AX) levels measured by Western blotting. Images show representative Western blots of three independent experiments; H2AX was used as loading control. Data were expressed as fold of change vs. CTR^{siRNA}. Single results are representative of three different experiments performed in triplicate. Statistical analyses: $*p<0.05$, $**p<0.01$, $***p<0.001$ vs. CTR^{siRNA}

Discussion

RMS, a rare and lethal neoplastic disease of mesenchymal origin, is the third most common extracranial malignant solid tumor in pediatric patients, accounting for approximately 5% of all pediatric cancers and about one-half of all soft tissue sarcomas (Arndt et al. 2012; Newton et al. 1988; Meza et al. 2006). RMS provides a complex therapeutic management and whether the radical excision is still considered the standard of care for RMS, frequently the anatomic site limits the complete tumor resection and adjuvant or neoadjuvant radiotherapy (RT), with or without chemotherapy becomes necessary (Frezza et al. 2018).

The multimodal approach has significantly increased the cure rates. In this regard, advanced RT techniques using conformal treatment with intensity-modulated radiotherapy and proton therapy, particularly advantageous for the treatment of sites close to critical structures, such as the head and neck and genitourinary system, have been shown to play a key role in RMS treatment. However, many patients frequently experience local recurrences, this indicating the high intrinsic radioresistance of RMS cells (Frezza et al. 2018; Smith et al. 2014; Wolden and Alektiar 2010). RT kills cancer cells by exploiting the ability of ionizing radiation to induce irreparable damages in cell structures, firstly in DNA molecules. Damages occur directly or, more frequently, through the accumulation of reactive oxygen species (ROSs), which are produced by the radiolysis of water (Desouky et al. 2015; Ciccarese and Ciminale 2017). Thus ROS, known to sustain cancer cells transformed phenotype (Sosa et al. 2013), when in excess, can promote detrimental effects (Trachootham et al. 2009), this suggesting the existence of a ROS concentration threshold, typical for each type of tumor, below or above which ROS levels promote or counteract tumor phenotype (Liou and Storz 2010). Low levels of ROS have been shown to stimulate cancer cells proliferation (Storz 2005) while higher levels are able to induce growth arrest, senescence and apoptosis (Szatrowski and Nathan 1991), which tumor cells try to counteract by expressing increased levels of antioxidant proteins able to detoxify from ROS. So, further considering that targeting oxidative stress has been indicated as a strategic therapeutic pathway in treating in RMS (Chen et al. 2013; Fanzani and Poli 2017), we supposed that RMS radioresistance could depend by the ability of these cells to counteract RT-induced ROS accumulation. The present in vitro study supports this hypothesis showing that radioresistance could really depend by the ability of RMS cells to upregulate the expression of several antioxidant molecules by the activation of specific antioxidant-related transcription factors to protect DNA from ROS-induced lethal damages.

Conventional RT treatment for RMS is performed by treating patients with a total dose up to 60 Gy divided in fraction of 2 Gy/day (5 fractions/week), in combination with radiosensitizing chemotherapies (von Mehren et al. 2018); however, doses have been tested such as hypofractionated RT combined to chemotherapy in which a dose of 5 Gy/days (5 fractions/week) has been used for a total dose of 25 Gy (Spencer et al. 2017). Herein, we found that RT significantly affected the ability of RMS to form colony, a well-known test for evaluating radiosensitivity (Williams et al. 2008), and increased ROS production starting from 2 Gy of treatment. Particularly, whether no significant differences were described between the doses of 2 and 3 Gy, 5 Gy of treatment induced the maximum toxic effect by promoting the highest accumulation of ROS. To investigate whether and how RMS cells were able to activate an anti-oxidant response, ROS levels were further assessed 12 h after RT, because usually the production of ROS after RT persists for at least 24 h after RT (Szatrowski and Nathan 1991; Azzam et al. 2012). Surprisingly, we found that already after 12 h from RT, RMS restored ROS to lower levels than those in non-irradiated cells with no significant differences between the dose used. Thus, the collected data indicate that whether increasing dose of RT were able to induce an increased production of ROS, RMS cells prevented the prolonged accumulation in a dose-independent manner. These results seem to suggest that RMS cells are particularly sensitive to increased ROS accumulation and that their antioxidant response is always maximal. Such an efficient response may indicate that tumor cells are able to activate an equally efficient antioxidant response.

Detoxification from ROS is a complex process that involves the participation of different molecular mechanisms, consisting of enzymatic [Catalase (CAT), Glutathione peroxidase (GPx4), Superoxide dismutase (SOD2)] and non-enzymatic (Vitamins, Glutathione, miRNAs) components (Azzam et al. 2012; Copin et al. 2000; Mates and Sanchez-Jimenez 2000). Oxidative stress, caused by increased free radical generation and/or decreased antioxidant levels in the target cells and tissues, has been suggested to play an important role in carcinogenesis and that the production of ROS combined to a decreased antioxidant enzyme level may be characteristic of tumor cells (Moldogazieva et al. 2018). However, several cancer types have been found to have elevated levels of antioxidant enzymes, particularly SOD2 (Oberley and Oberley 1997) and the activation of antioxidant systems can directly inhibit the antitumor activity of some anticancer agent (Alexandre et al. 2006; Llobet et al. 2008) and radiation therapy (Bairati et al. 2005). To date, the levels of these antioxidant enzymes in RMS cells have not been evaluated. However, it has been shown that cancer cells not expressing antioxidant enzymes, upon drug treatment, can over-express them by triggering a process named 'Redox Resetting'. Redox Resetting permits cancer cells to acquire a new redox balance with higher levels of ROS accumulation/resistance and stronger antioxidant systems (Liu et al. 2016). Herein, the evidence that RT upregulated SOD2, CAT and GPx4 in both RMS cell lines suggests that the 'Redox Resetting' may be one of the mechanisms through which RMS tumors acquire their radioresistance. In support of this hypothesis, we investigated whether RT was able to activate other anti-oxidant non-enzymes mechanisms, focusing the attention on the expression of microRNAs (miRNAs).

miRNAs are small regulatory RNA molecules that binding the mRNA of target genes, induces its degradation participating in the post-transcriptional regulation of gene expression (van Kouwenhove et al. 2011). miRNAs control several physiological processes (Li et al. 2009) and their

up- or down-expression was found in human cancers (Zhang et al. 2007), including RMS (Romania et al. 2012; Smolle et al. 2017) and shown to actively participate in tumor onset, progression and chemo- or radiotherapy-therapy resistance (Drusco and Croce 2017; Kasinski and Slack 2011). In this context, several miRNAs have been shown to sustain cancer cells radioresistance by preventing the DNA damages trough the activation of the anti-oxidant status (Banerjee et al. 2017; Fabrizio et al. 2018; Lan et al. 2018) beyond that trough the promotion of the repair of damaged DNA (Hu and Gatti 2011). Recent evidences indicate that miR-22 (Tang et al. 2018), -126 (Yang et al. 2017), -210 (Magenta et al. 2013), -146a (Wan and Li 2018) act as anti-oxidants whilst miR-375 (Guo et al. 2018) and -34a (Baker et al. 2016) as positive oxidative stress mediators. Herein, we found that after irradiation both RD and RH30 cells up-regulated the expression of miR-22, -126, -210 and -146α and accordingly with their anti-oxidant role, we suppose that these miRNAs are expressed to negative control the RT-induced toxic accumulation by ROS. We noticed also the up-regulation of the pro-oxidant miR-375 and miR-34α; however, we noted that miR-375 was not up-regulated in RD cells that clonogenic assay show to be more resistant to radiation than RH30. It remains to demonstrate whether the up-regulation of miR-22, -126, -210 and -146α directly participate in reducing RT-induced accumulation and whether RD cells prevent RT toxicity by restraining miR-375 expression.

Antioxidant response is orchestrated by the nuclear factor erythroid 2-related factor (NRF2) and the Kelch ECH associating protein 1 (Keap1), the major regulators of cytoprotective responses to stresses caused by ROS (Kansanen et al. 2013). NRF2 promotes the expression of antioxidant genes by preventing ROS-mediated cellular damage whilst Keap1 induces NRF2 degradation by the ubiquitin proteasome pathway (Itoh et al. 1999). Physiologically, NRF2 levels are very low and are increased upon oxidative stress whilst the aberrant NRF2 expression and activation protects malignant cancer cells against ROS accumulation induced by chemo- and radiation-therapy. Ionizing radiation activates the NRF2 antioxidant response that in turn restrains the ROS mediated toxicity induced by RT (McDonald et al. 2010; Zhao et al. 2016; Zhou et al. 2013). Herein, accordingly with the literature, we show that the key role of NRF2 in sustaining RMS radioresistance. Indeed, we found that the mRNA and protein expression of NRF2 resulted upregulated by RT and that NRF2 knocking-down, by transfecting cells with specific siRNAs, counteracted the ability of RMS cells to bring back the RT-induced ROS levels and to promote the expression of the anti-oxidant genes. Furthermore, according to what shown in non small cell lung cancer (Zhao et al. 2016), silencing NRF2 radio sensibilized RMS cells and promoted the accumulation of γ-H2aX, marker of DNA double-strand break (Kuo and Yang 2008), as a possible consequence of ROS accumulation. We do not know whether silencing NRF2 also affect the molecular mechanisms responsible for DNA damaged repair and experiments in this regard are ongoing.

Different mechanisms have been reported to be involved in NRF2 activation in cancer cells (Taguchi et al. 2011). Somatic mutations within the NRF2 or KEAP1 genes, epigenetic silencing of the KEAP1 gene, the accumulation of KEAP1 interacting proteins that block NRF2 binding to KEAP1 and KEAP1 modifications by oncometabolites, have been shown to lead to NRF2 accumulation (Taguchi and Yamamoto 2017). Furthermore, NRF2 is phosphorylated by several signal transduction pathways which can alter the interactions between Nrf2 and KEAP1 affecting NRF2 localization, protein degradation, and DNA binding (Taguchi and Yamamoto 2017). Je et al. (2012) found the tissue expressions of NRF2 in 93% of the tissue sarcomas analyzed, indicating that NRF2 signaling might be activated in most sarcomas. However, they showed that loss of KEAP1 expression was in 24% of the sarcomas, whereas neither NRF2 nor KEAP1 somatic gene mutation was seen in the sarcomas and data collected suggest that the pro-oncogenic action of NRF2 in sarcomas could be due to mechanisms independent by the simple protein accumulation. Embryonal and alveolar rhabdomyosarcoma onset, progression and therapy resistance have been respectively shown to be sustained by RAS/MEKs/ERKs (Ciccarelli et al. 2016; Marampon et al. 2011) and PI3K/AKT pathways (Jothi et al. 2012; Barr 2001), known to regulate NRF2 nuclear localization in several cancer types (Taguchi and Yamamoto 2017). Herein we have not investigated on the molecular mechanisms responsible of the RT-induced NRF2 accumulation, however, we strongly suppose a potential role for these pathways as well as the effects of RT on KEAP1 expression, intracellular distribution and interaction with NRF2 will be object of future investigations.

Characterizing the response of the tumor cells to RT is essential to understand the mechanisms through which they develop radioresistance, so determining the failure of the anti-cancer treatment itself. We have previously clarified how different signals and molecular mechanisms determine the radioresistance of RMS (Ciccarelli et al. 2016; Giannattasio et al. 2018; Gravina et al. 2016; Megiorni et al. 2017; Marampon et al. 2011); however, many other mechanisms remain to be understood such as the one herein investigated. The results of this study demonstrate that RMS can overcome RT treatment by resetting the redox status through the activation of the NRF2-regulated pathways and therefore NRF2 targeting could represent an efficient strategy to radiosensitize and kill RMS cells.

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Compliance with ethical standards

Conflict of interest The other authors declare that they have no competing interests.

Ethical approval Studies on animal models were not performed. This article does not contain any studies with human participants performed by any of the authors.

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