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Inhibition of microRNA-31-5p protects human colonic epithelial cells against ionizing radiation

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ABSTRACT

MicroRNAs (miRNAs), endogenous non-coding small RNAs, are sensitive to environmental changes, and their differential expression is important for adaptation to the environment. However, application of miRNAs as a clinical prognostic or diagnostic tool remains unproven. In this study we demonstrate a chronic/persistent change of miRNAs from the plasma of a colorectal cancer susceptible mouse model (*CPC;Apc*) about 250 days after exposure to a simulated solar particle event (SPE). Differentially expressed miRNAs were identified compared to unirradiated control mice, including miR-31-5p, which we investigated further. To address the cellular function of miR-31-5p, we transfected a miR-31-5p mimic (sense) or inhibitor (antisense) into immortalized human colonic epithelial cells followed by gamma-irradiation. A miR-31-5p mimic sensitized but a miR-31-5p inhibitor protected colonic epithelial cells against radiation induced killing. We found that the miR-31-5p mimic inhibited the induction of hMLH1 expression after irradiation, whereas the miR-31-5p inhibitor increased the basal level of hMLH1 expression. The miR-31-5p inhibitor failed to modulate radiosensitivity in an hMLH1-deficient HCT116 colon cancer cell line but protected HCT116 3–6 and DLD-1 (both hMLH1-positive) colon cancer cell lines. Our findings demonstrate that miR-31-5p has an important role in radiation responses through regulation of hMLH1 expression. Targeting this pathway could be a promising therapeutic strategy for future personalized anti-cancer radiotherapy.

1. Introduction

Numerous cellular adaptive mechanisms alter gene expression in response to environmental stressors. Only a few studies have shown a mechanistic connection between the response to environmental stress and persistent changes in microRNA (miRNA) expression (Shukla et al., 2008). MiRNAs are a class of small non-coding RNAs that generally negatively regulate gene expression post-transcriptionally by binding to the 3′-untranslated region of the targeted mRNA to inhibit translation (Kim, 2005). It is now widely reported that the expression of miRNAs are altered in several benign and malignant tumors (Ng et al., 2009; Toiyama et al., 2013). Therefore, it is reasonable to assume that miRNAs may potentially regulate intracellular responses to exogenous cytotoxic agents.

Ionizing radiation (IR) is an important modality used in the treatment of solid tumors and is well known to induce acute cytotoxicity. Exposure to IR is associated with DNA damage with subsequent induction of oxidative stress especially in highly proliferating cells such as colonic epithelial transiently amplified stem-like cells. Recent studies have shown that IR also alters a complex cellular response involving the expression of miRNAs both *in vitro* (Josson et al., 2008) and *in vivo* (Ilnytskyy et al., 2008). In addition to detecting miRNAs from tissues, circulating miRNAs in serum/plasma can also be used as biomarkers for assessment of total body irradiation (Cui et al., 2011).

There are well established side effects for individuals undergoing radiotherapy (terrestrial irradiation) as well as space irradiation (e.g. galactic cosmic and solar radiation). Solar particle events (SPEs) pose not only a risk to communications satellites but also a significant risk to humans such as those living under ozone gaps and to astronauts on the international space station. SPEs consist of various energies of protons, typically develop rapidly and may last a few hours to several days, but importantly the occurrence and size of individual events is currently unpredictable (Wilson et al., 1999). Crew members on the international space station could receive doses of 2 Gy or more during a solar flare (SPE) (Parsons and Townsend, 2000; Townsend et al., 1991) and long term consequences of this exposure are unknown. In the present study we conducted an SPE simulation that occurred in August 1972 and then examined for persistent (not acute) alterations in miRNA

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expression level in a genetically engineered mouse model of colon cancer susceptibility.

Alterations in miRNA expression were observed in the plasma of a colorectal cancer mouse model (*CPC;Apc*) (Hinoi et al., 2007) about 250 days after exposure to a SPE simulation to determine chronic/persistent changes in expression of miRNAs. Specifically, significant changes were observed in 4 miRNAs, including miR-31-5p, after exposure to SPE in only female mice. MiR-31-5p is known to regulate expression of human mutL homologue-1 (hMLH1) (Zhong et al., 2013) and inherited mutations in the hMLH1 causes hereditary nonpolyposis colorectal cancer (Papadopoulos et al., 1994) which is one of the familial colorectal cancers (CRC) that account for ~5% of all colorectal cancer patients (Lynch et al., 1996). hMLH1 has an important function in the G2-M cell cycle check point in response to IR (Davis et al., 1998), and a recent study demonstrated that expression of miR-31-5p was significantly decreased in radioresistant oesophageal adenocarcinoma cells and ectopic re-expression of the miR-31-5p significantly re-sensitized these cells to radiation (Lynam-Lennon et al., 2012). These studies implicate that miR-31-5p has a potential function as a modulator of radiosensitivity in cancer cells, however there is no direct evidence how miR-31-5p modulates radiosensitivity and how inhibition of the miR-31-5p affects radiosensitivity in normal cells or cancer cells. To further understand the cellular function(s) of miR-31-5p in radiation responses, a miR-31-5p mimic (sense) or inhibitor (antisense) was transfected in immortalized non-cancerous human colonic epithelial cells (HCECs) or CRC cell lines. We observed that the miR-31-5p mimic sensitized whereas the miR-31-5p inhibitor protected hMLH1-positive normal/cancer cell lines against IR.

2. Materials and methods

2.1. Animals and irradiation

All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center (UTSW) and Brookhaven National Laboratory (BNL). The colorectal cancer mouse model, *CDX2P-NLS Cre;Apc*$^{+/\text{loxP}}$ (*CPC;Apc*) mouse, was kindly provided by Dr. Eric Fearon (Hinoi et al., 2007) and bred and housed in our facilities. Mice, 5 to 8 weeks of age, were shipped to BNL and were acclimated for up to 1 week before irradiation and then returned to UTSW within a week after irradiation (mice that were not irradiated were also shipped to and from BNL). For the 2 hour SPE radiation exposure, mice were housed individually in plastic cuboids boxes (#530C, AMAC Plastic Products, Petaluma, CA). Mice were exposed to total-body irradiation (TBI) with 2.0 Gy of protons. The assembly of 25 animal cubicles was collectively arranged in an array (5×5) within the 20×20 cm beam spot. The average dose rate was about 1.67 cGy/min with various energy ranges. The energy spectrum for the SPE simulation was adapted to mouse dose levels and consisted of 91.67% of the proton dose at energy of 50 MeV/n to 0.14% of the proton dose at 150 MeV/n (Table 1). To assure uniform beam penetration through the mice, the sample assembly was rotated 180° in the vertical axis after completion of every 25% of dose.

Table 1
Energy spectrum for a simulated solar particle event.

Energy (MeV/n)	Dose fraction (%)
50	91.661
60	2.929
70	2.033
80	1.508
90	1.059
100	0.81
110	0.55
120	0.367
130	0.278
140	0.195
150	0.14

2.2. Cells and irradiation

Telomerase immortalized but non-cancerous HCEC CT7s were maintained as described previously (Roig et al., 2010). MLH1-deficient human colon carcinoma (HCT116) cells and MLH1-positive HCT116 3–6 cells (Davis et al., 1998) were grown on basal X-medium (HyClone, Logan, UT) supplemented with 10% cosmic calf serum (HyClone, Logan, UT). Cells were transiently transfected with 80 pmol of miR-31-5p mimic or inhibitor (SABioscience, Qiagen, MD) using 7.5 μl Lipofectamin RNAiMAX Transfection Reagent (Life Technologies, NY) in 6-well plates (reverse transfection). Two days after transfection, cells were exposed to γ-radiation using a 137Cs source at a dose rate of 243.08 cGy/min at the UTSW. Dosimetry of the irradiator was carefully monitored for accuracy by radiation physicists in the Department of Radiation Oncology at UT Southwestern Medical Center. For clonogenic survival assays, cells were trypsinized immediately after exposure to γ-radiation and were seeded in 100-mm dishes in triplicates. Colonies were stained with a mixture of 6.0% (vol/vol) glutaraldehyde and 0.5% crystal violet solution and counted. Colonies were defined as clusters of >50 cells. Plating efficiency was normalized to unirradiated controls.

2.3. MicroRNA arrays and quantitative reverse-transcriptase PCR

Total RNAs including small miRNAs were purified from plasma using the miRNeasy Serum/Plasma Kit (Qiagen, MD). The cDNA was prepared using miScript II Reverse Transcription Kit (Qiagen, MD). The changes of miRNA expression were screened using plasma samples from irradiated *CPC;Apc* mice with or without pretreatment of radioprotector, CDDO-EA ($n = 3$ mice/group). Plasma from unirradiated *CPC;Apc* mice were used as controls. miScript Mouse Serum & Plasma miRNA PCR Array (#MIMM-106Z, Qiagen, MD) was used for miRNA screening, per the manufacturer's protocol and the data was analyzed using the manufacturer's miScript miRNA PCR Array Data Analysis tools (http://pcrdataanalysis.sabiosciences.com/mirna/arrayanalysis.php). Real-time quantitative PCR was performed using miScript SYBR Green PCR Kit (Qiagen, MD) and the LightCycler 480 II (Roche, IN). For further validation of targeted miRNAs, miScript Primer Assays (Qiagen, MD) were used in at least 3 female and 3 male mice in each group.

2.4. DNA repair PCR array

Total RNAs were purified from cells 48 hours after transfection using the miRNeasy Mini Kit (Qiagen, MD). The cDNA was prepared using RT^2 First Strand Kit (Qiagen, MD). The Human DNA Repair RT^2 *Profiler*$^{\text{TM}}$ PCR Array (Qiagen, MD), RT^2 qPCR Master Mixes (Qiagen, MD) and the LightCycler 480 II (Roche, IN) were used for screening, per the manufacturer's protocol.

2.5. γ-H2AX assay

Cells were exposed to 2 Gy of γ-radiation and were fixed using 3.7% paraformaldehyde for 10 min, permeabilized with 0.5% Triton X-100 in PBS on ice for 5 min and incubated with blocking solution (10% goat serum and 3% BSA in PBS containing 0.1% Triton