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Wip1 abrogation decreases intestinal tumor frequency in APC^{Min/+} mice irrespective of radiation quality

Shubhankar Suman¹, Bo-Hyun Moon¹, Hemang Thakor¹, Albert J Fornace Jr.^{1,2}, and Kamal Datta^{1,*}

¹Department of Biochemistry and Molecular & Cellular Biology and Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC 20057, USA.

²Center of Excellence In Genomic Medicine Research (CEGMR), King Abdulaziz University, Jeddah, Saudi Arabia.

Abstract

Low linear energy transfer (low-LET) γ -ray exposure is a risk factor for colorectal cancer (CRC). Due to high-LET nature, energetic iron ions are expected to pose greater CRC risks to astronauts undertaking long duration space missions beyond low earth orbit (LEO). Wild-type p53-induced phosphatase 1 (Wip1) is important for cellular DNA damage response and its abrogation is reported to inhibit spontaneous intestinal tumorigenesis in APC^{Min/+} mice, a well-studied mouse model of human CRC. However, Wip1 in relation to radiation-induced especially energetic iron ions-induced intestinal tumorigenesis has not been investigated in APC^{Min/+} mice. While we earlier demonstrated greater intestinal tumorigenic potential of energetic iron ions relative to ¹³⁷Cs γ rays, the purpose of the current study was to investigate if Wip1 abrogation could influence radiation quality dependent intestinal tumorigenesis in APC^{Min/+} mice. Intestinal tumor frequency and grade was assessed in APC^{Min/+};Wip1^{-/-} mice and results were compared to those in APC^{Min/+};Wip1^{+/+} mice after exposure to a mean absorbed dose of 2 Gy from ¹³⁷Cs γ rays or 1.6 Gy from 1 GeV/n iron ions. Cellular differentiation and proliferation were also assessed in the intestinal tumors of irradiated and sham irradiated mice. Decreased tumor frequency and lower tumor grade was observed in APC^{Min/+};Wip1^{-/-} relative to APC^{Min/+};Wip1^{+/+} mice. Notably, similar decrease (~6 fold in both groups) in tumor number was observed in sham-, and γ -irradiated APC^{Min/+};Wip1^{-/-} relative to APC^{Min/+};Wip1^{+/+} mice. However, tumorigenesis in energetic iron ions group was reduced ~8 fold in APC^{Min/+};Wip1^{-/-} relative to APC^{Min/+};Wip1^{+/+} mice. Significantly lower proliferation/differentiation index in tumors of energetic iron ions exposed APC^{Min/+};Wip1^{-/-} relative to APC^{Min/+};Wip1^{+/+} mice suggests reduced proliferation and enhanced differentiation as a result of Wip1 abrogation. In conclusion, the current study demonstrates that the absence of Wip1 could block radiation-induced intestinal tumorigenesis irrespective of radiation quality and has implications for developing preventive strategies against tumorigenic potential of radiation exposure on earth and in outer space.

*Corresponding author: Kamal Datta, M.D., Associate Professor, Department of Biochemistry and Molecular & Cellular Biology, Georgetown University, Research Building, Room E518, 3970 Reservoir Rd., NW, Washington, DC 20057, USA. Phone: 202-687-7956, Fax: 202-687-3140, kd257@georgetown.edu.

Introduction

Epidemiological studies conducted on atomic bomb survivor cohort as well as on radiation workers show that development of colorectal cancer (CRC) is a potential long-term health risk of radiation exposure (1–4). Relative to non-exposed controls, radiation exposure has been demonstrated not only to cause greater tumor formation but also to cause adenoma-to-carcinoma transition due to loss of adenomatous polyposis coli (APC) gene function and dysregulation of several other signaling pathways including ATM, p53, Notch, NFkB, TGFβ, p38-MAPK, and Hedgehog (5–7). However, we have very limited *in vivo* quantitative and qualitative data to assess risks of CRC after exposure to heavy ion radiation, which contributes substantially to the effective dose of the galactic cosmic radiation (GCR) ambient in space and is considered densely ionizing high linear energy transfer (high-LET) radiation (8, 9). Recently, we have reported higher intestinal tumor frequency after exposure to energetic iron ions (^{56}Fe) radiation relative to low-LET γ radiation due in part to differential β -catenin pathway activation in $\text{APC}^{\text{Min/+}}$ mice (10). Lack of sufficient *in vivo* molecular data is also an impediment for developing effective prevention strategies against adverse consequences of space radiation. In humans, inactivating mutations in the tumor suppressor APC gene leads to release of β -catenin from the APC- β -catenin-GSK3 β -axin complex in cytoplasm and its subsequent translocation to nucleus causing transcription of downstream target genes such as cMyc and cyclin D1 (5). Mutations leading to loss of function of APC have been associated with familial as well as sporadic CRC, and APC mutant mouse models, considered similar to human CRC initiation and progression, have been extensively studied not only for molecular understanding but also for developing preventive and therapeutic strategies. Specifically, the $\text{APC}^{\text{Min/+}}$ mouse contains a nonsense mutation at codon 850 of the APC gene leading to generation of a truncated protein predisposing these mice to spontaneous intestinal adenoma formation (11, 12).

Wild-type p53-induced phosphatase 1 (Wip1) is an oncogene that has been reported to be overexpressed in a number of malignancies including CRC and is known to crosstalk with other signaling pathways involved in colorectal carcinogenesis [reviewed in (13)]. Wip1 promotes tumorigenesis in part through its known inhibitory effects on p53 apoptotic signaling and related pathways including p38 MAP kinase and ATM as well as through activation of the pro-proliferative hedgehog (HH) signaling (13, 14). Furthermore, Wip1 deletion/suppression has been demonstrated to cause reductions in spontaneous intestinal tumorigenesis in $\text{APC}^{\text{Min/+}}$ mice (15–17). However, the effects of Wip1 deletion on radiation especially heavy ion radiation associated intestinal tumorigenesis have so far not been explored. In this initial report, we have demonstrated that $\text{Wip1}^{-/-}$ in an $\text{APC}^{\text{Min/+}}$ background decreased intestinal tumorigenesis irrespective of radiation quality and has implications for minimizing the long-term risks of radiation toxicity including space radiation.

Materials and methods

Animals and irradiation

For initial breeding male $\text{APC}^{\text{Min/+}}$; $\text{Wip1}^{+/+}$ mice were mated with female $\text{APC}^{+/+}$; $\text{Wip1}^{-/-}$ mice to obtain $\text{APC}^{\text{Min/+}}$; $\text{Wip1}^{+/-}$ mice. Subsequently, male $\text{APC}^{\text{Min/+}}$; $\text{Wip1}^{+/-}$ mice were

mated with female $APC^{+/+};Wip1^{-/-}$ mice to obtain F2 offspring with $APC^{Min/+};Wip1^{-/-}$ genotype mice and genotyping was performed according to protocols described previously (18, 19). All breeding was performed in C57BL/6J genetic background at Georgetown University (GU) animal facility and we used 6 to 8 weeks old male mice for the current study. Mice ($n=20$ for $APC^{Min/+};Wip1^{+/+}$ and $n=25$ for $APC^{Min/+};Wip1^{-/-}$ mice) were exposed either to a mean absorbed dose of 2 Gy from ^{137}Cs γ rays or to a mean absorbed dose of 1.6 Gy from 1 GeV/n iron ions (^{56}Fe) whole body irradiation and control mice were shamirradiated. The energetic iron ions dose of 1.6 Gy was equitoxic to 2 Gy γ rays and the equitoxic dose was calculated using an RBE of lethality for 1 GeV/n iron ions (RBE 1.25 relative to ^{137}Cs γ -ray). The RBE was determined in an earlier LD_{50/30} study in wild type C57BL/6J mice using different doses of ^{137}Cs γ -ray and ^{56}Fe radiation (20). Notably, both the $APC^{Min/+};Wip1^{+/+}$ and $APC^{Min/+};Wip1^{-/-}$ mice are bred into the same C57BL/6J genetic background of the LD_{50/30} mice. For γ radiation (LET: 0.9 keV/ μ m), mice were placed in a pie-shaped transparent plastic container allowing mice easy movement and breathing, placed on a turntable inside a ^{137}Cs irradiator, and irradiated at a dose rate of 0.7 Gy/min. For ^{56}Fe radiation, mice were placed in transparent plastic boxes allowing easy movement and breathing, and twenty such boxes fitted into a styrofoam frame were placed in the ^{56}Fe (energy: 1000 MeV/n; LET: 148 keV/ μ m) beam path at the NASA Space Radiation Laboratory (NSRL) at Brookhaven National Laboratory (BNL). Mice were shipped early morning on the day after irradiation in a temperature-controlled vehicle from BNL animal facility to GU animal facility for a same day delivery. Animal facilities at GU and BNL are Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited facilities and housing conditions were kept similar at both the facilities. The Institutional Animal Care and Use Committee (IACUC) at GU and BNL approved all procedures performed on these mice and we followed guidelines laid down in the Guide for the Care and Use of Laboratory Animals by the Institute of Laboratory Animal Resources, National Research Council, and U.S. National Academy of Sciences for our research. Mice were monitored regularly and any animal with declining health assessed using approved criteria such as hunched posture, ruffled fur, diarrhea, and reduced activity was euthanized by CO₂ asphyxiation and was excluded from the specific study group.

Tumor counting and histopathology

Following 100 to 110 days after radiation exposures, mice were euthanized using CO₂ asphyxiation and the intestinal tract, small and large, was surgically removed, divided into duodenum, jejunum, ileum and colon. The intestinal polyps were counted in each section using a dissecting scope (Leica MZ6, Buffalo Grove, IL) by two observers blinded to the experimental groups. Intestinal tumor samples were fixed overnight in 10% buffered formalin. Fixed tissues were embedded in paraffin and 4 μ m-thick sections were obtained. For histopathological analysis, tissue sections were deparaffinized and hematoxylin and eosin (H&E) stained using standard protocol. Intestinal crypt invasion of muscularis mucosa was used as criteria for invasive carcinomas (21) and a total of 34 tumors in each group were assessed for invasiveness and results expressed as percent of the total number of tumor analyzed.

Alcian blue staining

Alcian blue specifically stains mucin producing goblet cells that represents one of the differentiated cell populations in intestine (10). Fixed and paraffin embedded tumor sections (n = 6 mice per group) were deparaffinized and soaked in 3% glacial acetic acid solution for 3 minute, followed by 30 minute incubation in alcian-blue stain (1% alcian blue in 3% glacial acetic acid; pH 2.5) and after appropriate washing steps, sections were counter stained with Nuclear Fast Red (0.1%; Sigma-Aldrich, St. Louis, MO).

Immunohistochemistry for phospho-histone-H3 (p-H3)

Immunostaining for phospho-histone H3 (p-H3; ser-10; Cat# 09-797; dilution: 1:100; Millipore, Billerica, MA) was performed as per protocol described previously (22) and color developed using SuperPicture TM 3rd Gen IHC detection kit (Cat#87-9673; Invitrogen, Carlsbad, CA) as per manufactures instruction. Proliferation/differentiation index was calculated by taking a ratio of the number of p-H3 and alcian blue positive cells.

Image quantification and statistical analysis

Images were captured using bright field microscopy at a microscopic magnification of 20X and five randomly chosen fields were captured in each section and analyzed using color deconvolution and/or Image-based Tool for Counting Nuclei (ITCN) plug-ins of ImageJ v1.46r software (National Institutes of Health, Bethesda, MD) (23, 24). Statistical significance ($p < 0.05$) between two groups were determined using a two-tailed paired student's t-test and data presented as mean $\pm$ standard error of mean (SEM).

Results

Wip1 abrogation decreased intestinal tumorigenesis in APC^{Min/+} mice irrespective of radiation quality

We observed significantly higher intestinal and colonic tumor frequency (presented as tumor number $\pm$ standard deviation or SD) in ⁵⁶Fe (1.6 Gy) irradiated relative to sham-irradiated APC^{Min/+};Wip1^{+/+} mice (intestinal tumor: 74.4 ± 2.71 , $p < 0.004$; colonic tumor: 3.7 ± 0.66 , $p < 0.02$; Figure 1A and B). In APC^{Min/+};Wip1^{+/+} mice, intestinal tumor frequency was not significantly higher (56.3 ± 2.6 , $p < 0.22$) in γ -irradiated (2 Gy) mice relative to sham-irradiated mice (Figure 1A). However, colonic tumor number was significantly higher (2.1 ± 0.28 , $p < 0.04$) in γ vs. sham irradiated APC^{Min/+};Wip1^{+/+} mice (Figure 1B). Compared to γ radiation (2 Gy), intestinal ($p < 0.01$) and colonic ($p < 0.03$) tumor frequency was significantly higher in ⁵⁶Fe irradiated (1.6 Gy) APC^{Min/+};Wip1^{+/+} mice (Figure 1A and B). In sham-irradiated APC^{Min/+};Wip1^{-/-}, significantly reduced intestinal and colonic tumors were observed relative to sham-irradiated APC^{Min/+};Wip1^{+/+} mice (intestinal tumors in APC^{Min/+};Wip1^{+/+}: 46.8 ± 7.6 , and in APC^{Min/+};Wip1^{-/-}: 7.76 ± 0.67 , $p < 0.00001$; colonic tumors in APC^{Min/+};Wip1^{+/+}: 1.1 ± 0.28 , and in APC^{Min/+};Wip1^{-/-}: 0.2 ± 0.08 , $p < 0.003$) (Figure 1A and B). Compared to irradiated APC^{Min/+};Wip1^{+/+} mice, intestinal tumor frequency was significantly reduced in 2 Gy γ (tumor number in APC^{Min/+};Wip1^{-/-}: 9.0 ± 0.84 , $p < 0.00001$) as well as 1.6 Gy ⁵⁶Fe (tumor number in APC^{Min/+};Wip1^{-/-}: 9.0 ± 1.14 , $p < 0.006$) irradiated APC^{Min/+};Wip1^{-/-} mice. We also observed decreased colonic tumor

formation in APC^{Min/+};Wip1^{-/-} relative to APC^{Min/+};Wip1^{+/+} mice after 2 Gy γ (tumor number in APC^{Min/+};Wip1^{-/-}: 0.28 ± 0.13 , $p < 0.00001$) and 1.6 Gy ⁵⁶Fe (tumor number in APC^{Min/+};Wip1^{-/-}: 0.12 ± 0.06 , $p < 0.00001$) radiation. However, in APC^{Min/+};Wip1^{-/-} mice no significant differences in intestinal and colonic tumor frequency were observed among sham-, γ - and ⁵⁶Fe-irradiated groups (Figure 1A and B). Segmental distribution results of intestinal tumors in duodenum, jejunum, and ileum showed a trend similar to those of the total tumor frequency (Figure 1C, D, and E).

Wip1 deletion attenuates radiation-induced intestinal cell proliferation and differentiation in APC^{Min/+} mice leading to decreased invasive carcinoma

Exposure to 1.6 Gy energetic iron ions (⁵⁶Fe) led to higher percentage of invasive intestinal adenocarcinoma (41% of total tumor count) relative to sham- (4% of total tumor count) and 2 Gy γ - (~6% of total tumor count) irradiation in APC^{Min/+};Wip1^{+/+} mice (Figure 2A). In APC^{Min/+};Wip1^{-/-} mice, however, ⁵⁶Fe radiation exposure caused significantly less adenocarcinoma development (~3% of total tumor count) relative to APC^{Min/+};Wip1^{+/+} mice. Also, all the tumors in sham- and γ -irradiated APC^{Min/+};Wip1^{-/-} mice were adenomas (Figure 2A). We used alcian blue stained goblet cells as a marker of cellular differentiation in intestinal tumors and we show that, unlike in ⁵⁶Fe-irradiated APC^{Min/+};Wip1^{+/+} mice, cellular differentiation in APC^{Min/+};Wip1^{-/-} mice was significantly higher ($p < 0.0008$) and corresponds to sham-irradiated APC^{Min/+};Wip1^{+/+} mice (Figure 2B). Cellular proliferation, unlike in γ and ⁵⁶Fe irradiated APC^{Min/+};Wip1^{+/+} mice, was significantly lower in γ ($p < 0.020$) and ⁵⁶Fe ($p < 0.0007$) irradiated APC^{Min/+};Wip1^{-/-} mice and was similar to sham-irradiated controls (Figure 2C). Proliferation/differentiation index calculated as a ratio of phospho-histone H3 and alcian blue positive cell number in intestinal tumors showed significantly lower value for ⁵⁶Fe-irradiated APC^{Min/+};Wip1^{-/-} mice relative to ⁵⁶Fe-irradiated APC^{Min/+};Wip1^{+/+} mice ($p < 0.001$; Figure 2D).

Discussion

Energetic heavy ion radiation is a major health concern for astronauts engaged in long duration space missions especially beyond low earth orbit (LEO) (9, 25) and developing greater understanding of CRC risks from space radiation exposures through acquisition of *in vivo* data is essential for safe space exploration. Although in the current study, the mean absorbed dose from acute energetic iron ions (⁵⁶Fe) is significantly higher than the doses that astronauts are expected to receive during a long duration space mission, the γ radiation dose of 2 Gy is a fraction commonly used to deliver a planned total dose in fractionated radiotherapy. Nonetheless, we believe ours is the first report on effects of Wip1 abrogation on γ as well as ⁵⁶Fe radiation-induced intestinal tumorigenesis suggesting it as a potential target to reduce risk of radiation carcinogenesis including space radiation carcinogenesis. Considering the importance of solid tumor data for space radiation risk assessment and countermeasure development, we are reporting our initial observations while we undertake studies to delineating underlying molecular events. Wip1 deletion in APC^{Min/+} mice caused a remarkable reduction (~6 fold) in spontaneous as well as radiation-induced (~6 and 8 fold in γ and ⁵⁶Fe irradiated groups respectively) intestinal tumorigenesis. While our results of decreased spontaneous intestinal tumorigenesis after Wip1 abrogation is consistent with

earlier observations without radiation (15–17), an important observation of our study was decreased intestinal tumor frequency in Wip1 deleted APC^{Min/+} mice irrespective of radiation quality. Importantly, Wip1 deletion in APC^{Min/+} mice also significantly reduced invasive carcinoma incidence after ⁵⁶Fe radiation exposure and suggests involvement of Wip1 in radiation-induced intestinal tumor progression. We have earlier reported that ⁵⁶Fe radiation-induced intestinal tumorigenesis was associated with up-regulation of the β -catenin pathway leading to reduced differentiation and higher proliferation of intestinal epithelial cells in APC^{Min/+} mice (10). However, in the current study, Wip1 deletion not only lowered the proliferation/differentiation index in tumors of APC^{Min/+} mice exposed to ⁵⁶Fe radiation but it also restored the index to control level. Although additional molecular analysis is ongoing to understand how Wip1 modulates space radiation-induced intestinal carcinogenesis, deletion of Wip1 is known to activate a robust DNA damage response through ATM-Chk1/2-p53 signaling pathways and proactive DNA damage response is known to suppress carcinogenesis (13, 15, 26). Indeed, γ radiation exposure has been demonstrated to not only enhance DNA damage response but also to reduce spontaneous tumor incidence in Wip1 deleted mice relative to their wild type counterpart (27). In contrast, higher Wip1 expression has been observed in intestinal stem cell (ISC) and Wip1-deficient ISC is susceptible to stress and apoptosis (17). Wip1 deletion has also been demonstrated to block tumor initiation in APC^{Min/+} mice via activated p53 mediated removal of tumorigenic cells (13, 15–17). Activation of cell cycle checkpoints in Wip1 deleted/suppressed cells could provide a basis for efficient DNA repair (28) leading to decreased mutagenesis and thus tumorigenesis in our study. Taken together, our results lead us to speculate that Wip1 is not only important for spontaneous tumorigenesis but is also crucial for radiation-induced tumorigenesis and Wip1 could be a target to effectively block radiation carcinogenesis independent of radiation quality through reduced cell proliferation and enhanced cellular differentiation in intestine.

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Figure 1.

Wip1 abrogation decreased intestinal tumorigenesis in APC^{Min/+} mice. A) Intestinal tumor frequency in APC^{Min/+};Wip1^{+/+} and APC^{Min/+};Wip1^{-/-} mice after exposure to 2 Gy γ and 1.6 Gy ⁵⁶Fe radiation. B) Colonic tumor frequency in APC^{Min/+};Wip1^{+/+} and APC^{Min/+};Wip1^{-/-} mice after exposure to 2 Gy γ and 1.6 Gy ⁵⁶Fe radiation. C) Duodenal tumor frequency in APC^{Min/+};Wip1^{+/+} and APC^{Min/+};Wip1^{-/-} mice after exposure to 2 Gy γ and 1.6 Gy ⁵⁶Fe radiation. D) Jejunal tumor frequency in APC^{Min/+};Wip1^{+/+} and APC^{Min/+};Wip1^{-/-} mice after exposure to 2 Gy γ and 1.6 Gy ⁵⁶Fe radiation. E) Tumor frequency in the ileum of APC^{Min/+};Wip1^{+/+} and APC^{Min/+};Wip1^{-/-} mice after exposure to 2 Gy γ and 1.6 Gy ⁵⁶Fe radiation.

A.

Groups	Adenoma (%)		Adenocarcinoma (%)	
	APC ^{Min/+} ;Wip1 ^{+/+}	APC ^{Min/+} ;Wip1 ^{-/-}	APC ^{Min/+} ;Wip1 ^{+/+}	APC ^{Min/+} ;Wip1 ^{-/-}
Control	95.8	100	4.2	0
γ-ray	94.2	100	5.8	0
⁵⁶Fe	58.9	97.2	41.1	2.8

Figure 2.

Effects of Wip1 deletion on radiation-induced intestinal tumor grade, and intestinal cell differentiation and proliferation. A) Percent of adenoma and invasive adenocarcinoma in intestinal tumors of APC^{Min/+};Wip1^{+/+} and APC^{Min/+};Wip1^{-/-} mice. B) Alcian blue positive cell counts in intestinal tumors of APC^{Min/+};Wip1^{+/+} and APC^{Min/+};Wip1^{-/-} mice exposed to 2 Gy γ and 1.6 Gy ⁵⁶Fe radiation. C) Phospho-histone H3 positive cell counts in intestinal tumors of APC^{Min/+};Wip1^{+/+} and APC^{Min/+};Wip1^{-/-} mice exposed to 2 Gy γ and 1.6 Gy ⁵⁶Fe radiation. D) Cellular proliferation/differentiation index (ratio of p-H3 and alcian blue positive cell counts) was determined in intestinal tumors based on genotype and radiation exposures.