RESEARCH PAPER

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Dietary flaxseed modulates the miRNA profile in irradiated and non-irradiated murine lungs

A novel mechanism of tissue radioprotection by flaxseed

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Keywords: flaxseed, miRNA, radiation pneumonopathy, lung fibrosis, antioxidant, lung injury, ROS, inflammation, mouse model, miR-34a, miR-29c, miR-150

Abbreviations: FS, flaxseed; ROS, reactive oxygen species; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; SARRP, small animal radiation research platform; SEM, standard error means; TLDA, TaqMan Low Density Array; XRT, X-ray treatment; miRNA, microRNA

Introduction: Dietary flaxseed (FS) displays antioxidant and anti-inflammatory properties in preclinical models of lung disease including radiation-induced pneumonopathy, however the mechanisms of lung radioprotection are incompletely understood. MicroRNAs (miRNAs) are short oligonucleotides that act as important posttranscriptional regulators of diverse networks including inflammatory response networks. Responses of miRNA profiles to diet and radiation exposure have been reported, but the potential contribution of miRNAs to diet-related radioprotection has never been tested.

Methods: In this exploratory pilot study, mice were fed 10% FS or a 0% FS isocaloric control diet and exposed to a single-fraction 13.5 Gy thoracic X-ray radiation treatment (XRT). Lung RNA was extracted 48 h post-XRT and small RNAs profiled by OpenArray.

Results: FS significantly modulated expression of multiple miRNAs, including 7 with $P < 0.001$. miR-150 was downregulated approximately 2.9-fold in the FS groups and is disproportionately integrated into immune response-related networks. Although few miRNAs were significantly changed by radiation, interaction between diet and radiation was observed. For example, miR-29c was greatly downregulated in the FS/Control group (10- to 50-fold) but slightly upregulated in the FS/radiation group. Compared with FS/control, the FS/radiation group experienced a 50% decrease of the p53-responsive miR-34a, which regulates senescence- and apoptosis-related factors.

Conclusions: FS induced significant changes in lung miRNA profile suggesting that modulation of small RNA by dietary supplements may represent a novel strategy to prevent adverse side-effects of thoracic radiotherapy. This pilot study provides insight into a potential mechanism of flaxseed's radioprotection and provides a useful model-system to further explore and optimize such small RNA-based therapies.

Introduction

The effectiveness of radiotherapy for thoracic malignancies is limited by the low tolerance of normal lung parenchyma to ionizing radiation.[1,2] Clinically significant radiation-induced lung injury occurs in 30% of patients irradiated for lung cancer[3] and 10–15% of other thoracic oncology patients.[4] A greater proportion of patients have subclinical adverse effects from radiation of the lung, identifiable by imaging and/or physiological testing.[5] Highly reactive oxygen species (ROS) and reactive nitrogen species (RNS) are induced in large quantities by radiation therapy (XRT) and have been implicated in this form of lung injury.[6]

There is currently no known effective pharmacologic therapy for the prevention of acute or chronic radiation pneumonopathy. To date, the only means to avoid life-threatening radiation pneumonopathy is to modify the irradiation technique to minimize the volume of normal lung that receives a significant radiation dose. A safe and effective biologic radioprotector would thus be extremely useful. Preclinical data suggest that antioxidant molecules and/or enzymes might offer protection of the lung.[7-9] Our group has shown that systemic administration of polyethylene glycol conjugated antioxidant enzymes at the time of XRT alters several early biomarkers of lung injury, decreases apoptosis, and ameliorates late pulmonary fibrosis in a murine model.[10] Although encouraging, this potential therapeutic is far from

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human clinical trials, and a safe, more accessible radioprotector is therefore needed.

Our group has identified dietary flaxseed (FS) as a potential radioprotector against radiation-induced lung injury in a murine model of thoracic XRT. FS is a non-toxic whole grain composed of high concentrations of omega-3 fatty acids and lignans. While omega-3 fatty acids reduce inflammation, lignans possess potent antioxidant properties. Specifically, secoisolariciresinol diglucoside (SDG) is the most abundant lignan present in FS and was shown in vitro to have direct hydroxyl radical scavenging properties and to inhibit lipid peroxidation[11-13] as verified recently by our group in the context of oxidative lung injury.[14,15] We confirmed the cell and tissue protective effects of the lignan precursor SDG on the abrogation of radiation-induced ROS generation in vitro. In addition, we provided evidence of radioprotection of FS and of dietary SDG formulations. Importantly, both FS and SDG formulations protected normal tissue damage from radiation and inflammation, while not decreasing lung tumor response to XRT using an orthotopic model.

In this pilot study, we investigated another potential mechanism of FS-induced radioprotection by examining modulation of microRNAs (miRNAs) in the lung in response to FS diet and radiation. miRNAs are short (approximately 22 nucleotides) RNA molecules which contribute to posttranscriptional gene regulation. As biomarkers, miRNAs hold tremendous clinical promise as diagnostics, prognostics, and predictors of response to therapy. The normal miRNA profile of a given cell or tissue type is often dysregulated in cancers and in response to inflammation. Beyond "bystander" effects, miRNAs may also be intimately involved in pathogenesis and thus represent targets of molecular therapies: expression enhancement strategies that replace a missing miRNA or antagomir approaches that block the actions of a specific miRNA. The latter has proven successful, for example, in a phase 2 trial of miraversen, an antisense inhibitor of the liver-enriched miR-122 that functions in the replication cycle of Hepatitis C virus. The ability to modulate endogenous miRNAs by dietary interventions would be a new and exciting therapeutic option.

Results

In this pilot study, we pre-fed mice 10% FS diet as compared with an isocaloric control for 3 wk prior to a single dose radiation challenge to the thorax[16,17] and evaluated lung tissues 48 h post-exposure (**Fig. 1**). The time post radiation exposure was based on previous studies where we determined that acute, damage-response genes are upregulated in lung tissues and abrogated by the FS diet.[18] The dose of FS and the duration of pre-feeding was selected based on the kinetics of expression of antioxidant and cytoprotective genes[15] which we determined to increase over control and reach a plateau by 2–3 wk on the diet. Comparisons were made between irradiated and non-irradiated lung tissues from flaxseed- or control diet-fed mice with respect to their miRNA profile.

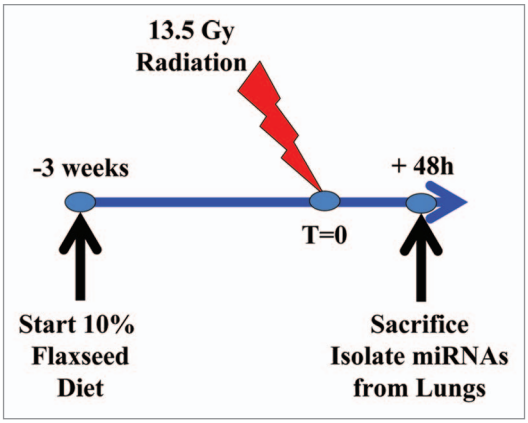

Figure 1. Thoracic radiation model and experimental plan. Mice are subjected to a single fraction thoracic radiation (13.5 Gy). Mice (n = 3 per group) were fed with 0% or 10% FS diet initiated prior (–3 wk) to single fraction X-ray radiation treatment (XRT). Mice were sacrificed at 48 h post-XRT and lung miRNA profile evaluated.

miRNA profiling: evaluation and quality control

miRNA profiling was conducted using the medium-throughput rodent miRNA "OpenArray" platform from Life Technologies. This platform includes TaqMan probes for 750 rodent miRNAs along with several small RNA control features. In contrast with the predecessor "TaqMan Low Density Array" (TLDA) platform, which requires the use of two 384-well PCR cards with 1 μL reaction vessels to profile one sample, OpenArray reactions are performed in 30 nL hydrophobic through-hole reaction vessels. Three thousand and seventy-four of these vessels are contained in each array, and three samples may be profiled per array. Four arrays may be processed simultaneously, allowing a 24-fold increase in throughput over the TLDA platform. At the same time, the ~30-fold decrease in reaction volume may result in lower sensitivity and greater variability, particularly at the low end of the abundance spectrum. Accordingly, we tested six samples in technical duplicate during different instrument runs to assess reproducibility. With the exception of several minor loading problems that were occasionally observed from sample to sample—a variable and small percentage of wells that were not filled and thus unreliable—the majority of features that were consistently detected in each duplicate sample were separated by 0.5 cycles or less. Only features that exceeded a minimum "amplification score" (assigned by the manufacturer's software) were included in subsequent analysis. Most detected miRNAs amplified before 28 PCR cycles.

The number of features—miRNA or other small RNA—detected in each lung sample ranged from 294 to 326. Animals on a flaxseed diet and no radiation exposure had slightly fewer detected features (294 to 309) than were found in samples from other experimental groups (321 to 326). 274 miRNAs were consistently detected in all samples, along with all structural small RNA controls, while a negative control feature (a plant miRNA) was not detected. Unsupervised hierarchical clustering roughly recapitulated the experimental groupings (**Fig. 2**).

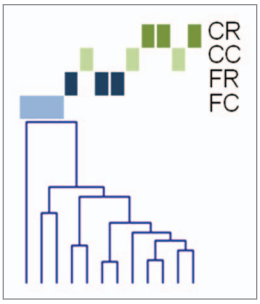

Figure 2. Unsupervised hierarchical clustering of miRNAs reveals relationship of groups. Experimental groups were largely recapitulated by unsupervised hierarchical clustering of all results from OpenArray miRNA profiling. Clustering was performed by Pearson correlation with average linkage. CC, control diet, no radiation; CR, control diet, radiation; FC, flaxseed, no radiation; FR, flaxseed, radiation.

miRNA profile in lung tissues of flaxseed-fed mice

miRNAs and other features with expression differences between experimental conditions (as determined by two-way analysis of variance with a *P* value cutoff of 0.05) were used to hierarchically cluster samples. More than 20 features appeared to distinguish FS-fed mice from those on a control diet (**Fig. 3**). In contrast, few features were differentially expressed between irradiated and unexposed mice (**Fig. 4**). Several of these features were also detected in fewer than 100% of samples (**Fig. 4**), emphasizing a lack of reliable differences.

qPCR validation of miRNA profile in lung tissues of irradiated flaxseed-fed mice

Selected miRNAs were assayed using individual qRT-PCR hydrolysis probe-based tests. These reactions differ from those performed with the OpenArray platform in that reverse transcription is conducted for only one to five miRNAs at a time, in contrast with the hundreds of miRNAs in the OpenArray system. Furthermore, there is no pre-amplification step. Because individual assays are less expensive, technical repeats and larger numbers of controls can be performed. Individual miRNA assays largely validated the profiling results. As indicated by the arrays, there was little modulation of the examined miRNAs in response to radiation, but significant differences were associated with FS diet.

Specifically, individual qPCR assays confirmed the response to diet of miRs-34a, -142-3p, and -150. miRs-142-3p and -150, which were significantly downmodulated by 2- to 4-fold in animals with FS diet, irrespective of radiation exposure (**Fig. 5**). In contrast, miR-34a was significantly upregulated by approximately 2-fold. One miRNA, miR-143, had a nominally significant *P* value for a slightfold-downregulation (**Fig. 5**). While this finding was of potential interest, the magnitude of downregulation and the small number of repeats reduced confidence in the result.

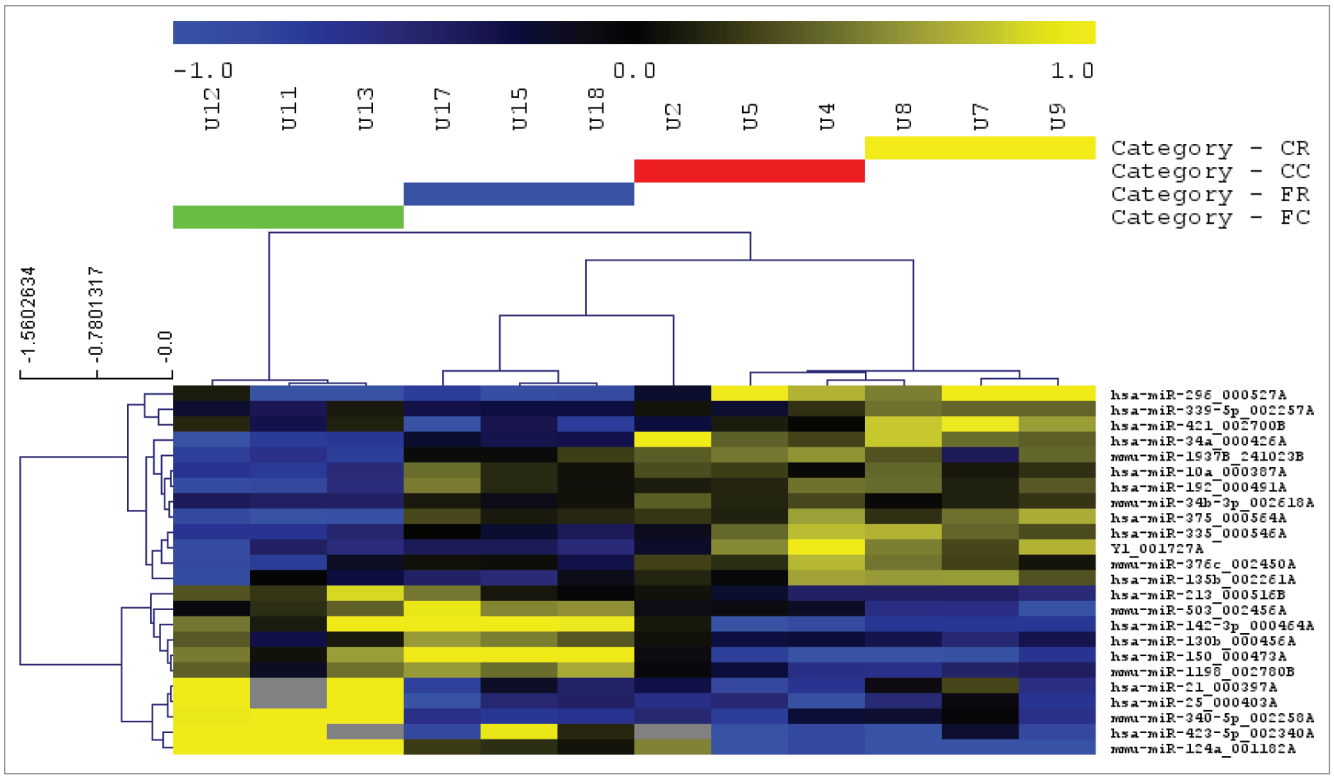

Figure 3. Heatmap of miRNA profile in response to dietary flaxseed administration in mice Significantly differentially expressed, diet-associated miRNAs as identified by two-way ANOVA ($P < 0.05$) were used to cluster samples and features (Euclidean distance, average linkage). The expression heatmap legend (top) indicates relative abundance of median-centered expression values on a log2 scale. Yellow indicates reduced expression and blue indicates increased expression of the selected miRNAs for the given diets. In the heatmap, gray indicates failed detection. CC, control diet, no radiation; CR, control diet, radiation; FC, flaxseed, no radiation; FR, flaxseed, radiation.

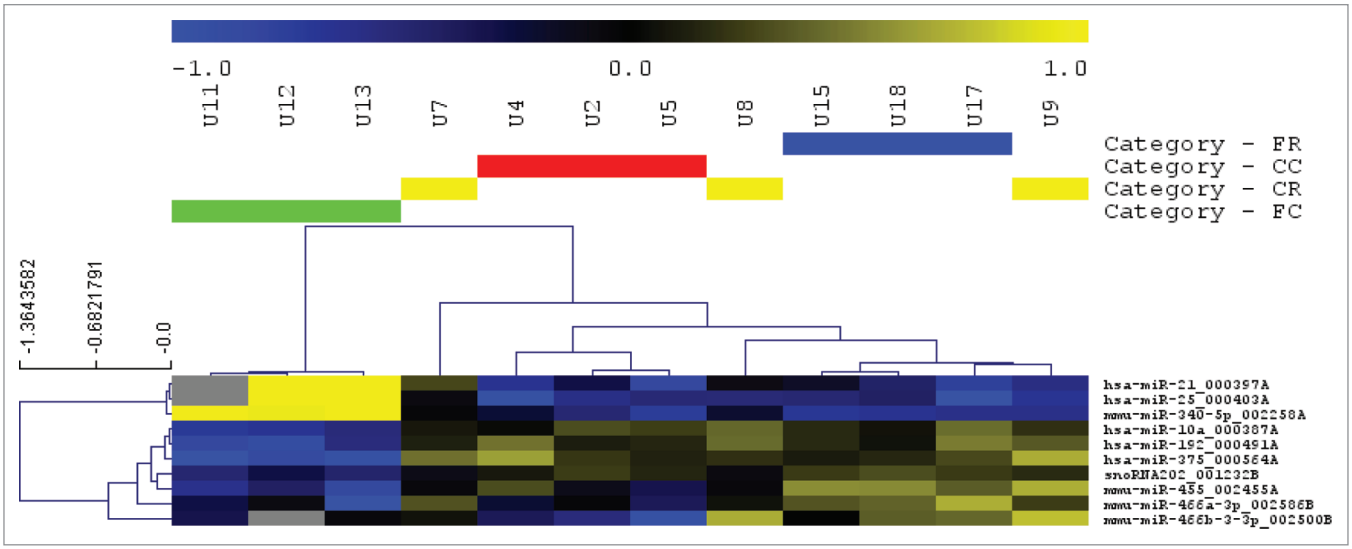

Figure 4. Heatmap of miRNA profile in irradiated mouse lungs. Significantly differentially expressed, diet-associated miRNAs as identified by two-way ANOVA ($P < 0.05$) were used to cluster samples and features (Euclidean distance, average linkage). The expression heatmap legend (top) indicates relative abundance of median-centered expression values on a log2 scale. Yellow indicates reduced expression and blue indicates increased expression of the selected miRNAs for the given diets. In the heatmap, gray indicates failed detection. There was comparatively little response of lung miRNA expression to radiation when apparent radiation-associated changes were identified. CC, control diet, no radiation; CR, control diet, radiation; FC, flaxseed, no radiation; FR, flaxseed, radiation.

Comparison/correlation with previous gene expression results[18]

To determine whether flaxseed-associated miR-34a upregulation might contribute to downregulation of genes in murine lung tissue, we re-examined our previously published gene profiling data of murine lung tissue fed 0 or 10% FS[18] to find genes in common between these data and a list of miR-34a targets generated by the stringent TargetScan algorithm.[19] We note several inverse relationships between the upregulated miR-34a and downregulated genes, such as Pdgfra (downregulated –1.70 fold), Ubp1 (–1.57), Fgfr1 (–1.79), Bcl2 (–1.73), and Fbxo10 (–1.83).[18] Downregulation of Fgfr1 has been confirmed by qPCR.[18] These observations provide insight into miRNA-specific effects on lung gene expression after feeding a 10% flaxseed diet. Further investigation of the direct or indirect nature of miR-34a mediated regulation of these and other genes is warranted.

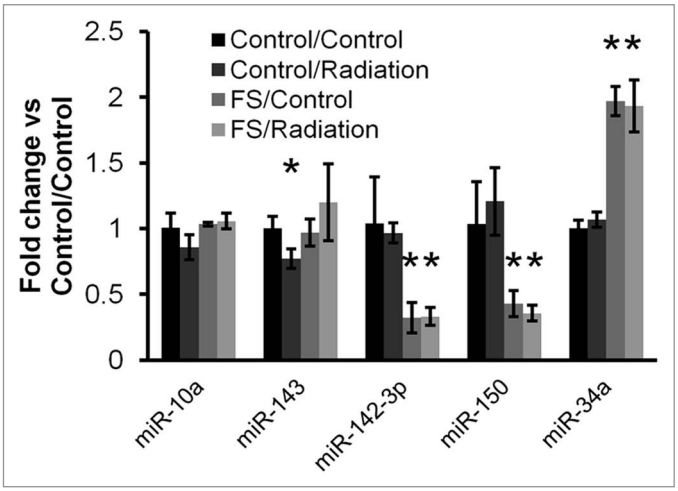

Figure 5. qRT-PCR confirmation: flaxseed alters the "set point" of several miRNAs. Mature miRNAs and two structural small RNA controls (U6 and sno-135) were quantitated by qRT-PCR for samples from the four indicated groups. Data were processed by relative Cq method, with normalization to the geometric mean of all assayed RNAs. Kruskal–Wallis tests were performed with the Dunn post-hoc test. Statistical tests were done with relative Cq-level data before transformation to fold change relative to the control/control condition (control diet and no radiation). Shown are the resulting fold change values for five mature miRNAs. Indicated comparisons (*) were significant at α of 0.05.

Discussion

miRNAs play significant roles in maintaining cellular function and tissue homeostasis, however, few reports have investigated diet-induced changes of the miRNA profile in normal tissue. Although just a pilot study, our approach integrates multiple innovations, beginning with novel uses of a mouse model of radiosensitization/radioprotection to investigate newly discovered, small RNA-mediated mechanisms of the effects of flaxseed diet. OpenArray is a state-of-the-art medium-throughput system that has a 24-fold improvement in throughput over the previous TaqMan low density array platform.[20] Our findings are the first to report on miRNA changes in normal lung tissue of FS-fed mice in the presence or absence of radiation. Robust decreases in miR-142-3p and miR-150 and increases in miR-34a levels were detected by array and confirmed by qPCR. Previous studies have reported miRNA profile alterations of lung cancer cells exposed to ionizing radiation (for example, ref. 21). Our results show at 48 h post-radiation exposure few changes in irradiated, non-malignant lung as compared with non-irradiated

lung. Examination of earlier or later time points or even different radiation doses might identify a different miRNA profile. An exciting finding of the study was the change of the miRNA profile by FS feeding which changed the "set point" of expression of several miRNAs in the lung, with or without radiation exposure. Importantly, this is supported by previous work with the model, showing differential regulation of several target genes of the upregulated miR-34a.[18]

Involvement of miRNAs in pathogenesis of lung disease

The identities and direction of regulation of FS-associated miRNAs are consistent with contributions to the previously reported effects of FS. The lung is highly susceptible to changes in gene expression that result from environmental exposures.[22] miRNAs fulfill a modulatory role in lung pathogenesis.[23] Significant loss of miRNAs associates with increased pulmonary inflammation.[24] Noxious challenges, such as radiation exposure, sepsis, and environmental pollutants alter the expression of certain miRNAs and are correlated with specific lung disease processes.[25] Modulating miRNA levels prior to exposure, as we have done here through a dietary intervention, might well moderate the damage of environmental insults.

Downregulation of miRs-142-3p and -150 by FS

miR-142-3p appears to have important functions in lung development, inflammation, and carcinogenesis. Contributions to lung development and functioning are supported by high levels of expression in the human adult lung and significant differential expression in lung of neonate and adult mice.[26,27] miR-142-3p has also been implicated in inflammatory responses,[28,29] with significant upregulation at 3 and 6 h post-exposure in an lipopolysaccharide (LPS)-induced model.[29] Bronchoalveolar lavage (BAL) fluid revealed an increase in polymorphonuclear (PMN) cell content. In light of previous reports that FS reduced BAL fluid white blood cell count and PMN content in a mouse model of radiation pneumonitis,[17,30] our findings suggest a beneficial role of FS in reducing lung inflammation by reducing miR-142-3p. Of additional interest are associations of both of FS-downregulated miRNAs—miRs-142-3p and -150—and cancer: a positive correlation of miR-142-3p and lung carcinogenesis,[31,32] as well as a role of increased miR-150 through deregulation of cell cycle control.[33]

FS increases miR-34a in lung; role in cell-cycle control and apoptosis

miR-34a, increased 2-fold by FS in this study, is significantly downregulated or undetectable in many cancers, including human lung cancer, and microarray analyses have identified a role for miR-34 family members in response to radiation damage.[34,35] miR-34a exerts its effects through modulation of cell cycle control and apoptosis.[36,37] Transactivated by p53, miR-34a declines with p53 dysregulation. Targets of miR-34a are disproportionately involved in cell cycle control.[37,38] Interestingly, a recent report also implicates miR-150 as a suppressive agent in the p53/miR-34a regulatory network, with lower miR-150 activity associated with increased levels of miR-34a,[39] similar to what we report here. The effects of miR-34a on the p53 network have made this miRNA an attractive therapeutic target in cancers.[40,41] FS-enhanced lung levels of miR-34a may boost tissue defenses in response to radiation damage, cell cycle arrest and apoptotic cell death as shown in our earlier work.[17]

Flaxseed and other botanicals alter microRNA profile

Modulation of miRNAs by other botanicals and polyphenols has been the subject of notable investigations. Izzotti and coworkers were the first to report on miRNA regulation by chemopreventive agents in cigarette-exposed rat lung tissue.[42] miRNAs can be modulated by botanicals in lung cancer.[43-46] The effects of curcumin on a human retinal pigment epithelial cell line, ARPE-19, included downregulation of 20 and upregulation of 9 miRNAs.[47] Effects of resveratrol on miRNA have also been studied.[48] miR-34a was modulated by resveratrol in A549 human non-small cell lung cancer (NSCLC) cells.[49] This treatment reduced the expression of oncogenic miRNAs and induced pro-apoptotic miRNAs in colon cancer cells.[50,51] Two flavonoids, rhamnetin and cirsiliol, upregulated p53-dependent miR-34a expression in NSCLC cells, resulting in radiosensitization through increased apoptosis.[52] Our study adds to the accumulating evidence of natural product-mediated miRNA control.

Downstream effects

Additional work is needed to identify the various targets of the miRNAs we found in this study, but several promising candidates of the upregulated miR-34a should be mentioned. A common theme in the miR-34a literature has been the reliable, multiply-verified targeting of Bcl2 by miR-34a,[53-55] both directly and indirectly. Bcl2 was also downregulated in our model.[18] Additionally, we found that miR-34a expression is negatively associated with FGFR1 following FS feeding. As a cellular receptor implicated in multiple cell signaling pathways, FGFR1 represents another potential target for inhibition in the treatment of malignancies that result via its cell signaling pathway.[56]

Taken together, our results as well as numerous reports in the literature suggest that FS-modulated miRNAs, miRs-34a, 142-3p, and -150, may play different but complementary, roles in tumor suppression and cancer radiosensitization in addition to contributing to salutary effects of dietary FS in protecting normal tissue. The therapeutic potential of miR-34a in particular is illustrated by MRX34, an injectable, liposome-formulated miR-34a mimic currently being evaluated in a phase I clinical trial in patients with liver cancer and liver metastases (trial NCT01829971).[41] As results from our study and others show, modulation of miRNA levels by natural products such as FS may prove to be an additional avenue to explore in a variety of diseases linked to cell proliferation and apoptosis. We encourage and look forward to additional work in this area.

Materials and Methods

Animals

Our studies used female C57/BL6 mice, a strain well characterized in the field of pulmonary radioprotection.[10,16,17,30,57] Mice were obtained from Charles River and irradiated at 6–8 wk of age under animal protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of

Pennsylvania. Animals were housed in conventional cages under standardized conditions with controlled temperature and humidity and a 12:12-h day–night light cycle. Animals had free access to water and formulated study diets. For this study we used $n = 3$ mice for each irradiated (XRT, 13.5 Gy) or control group on test diets (0% FS, 10% FS).

Diet composition and dietary treatments

Two diets were used for this study, all based on a semi-purified AIN-93G diet which was modified to contain the test ingredient as previously described.[16] Importantly, control (no test ingredient added) and experimental diets were isocaloric, isonitrogenous, and contained equal amounts of dietary lipid and carbohydrate. Mice were maintained on control (0% FS) or 10% FS-supplemented diet given ad libitum for three weeks prior to XRT and were maintained on the respective diets until conclusion of the study and tissue harvest.

Radiation procedure

The Small Animal Radiation Research Platform (SARRP), (Xstrahl) was used to irradiate animals with a custom-made beam collimator. This system uses a Varian model NDI-225–22 kV X-ray tube mounted on a gantry that rotates between 0 and 120 degrees. The custom collimator creates a 12.5 cm circular field with well-defined borders and with animals arranged in a circular, "head in" arrangement using a single central shield which provides uniform irradiation to the thoracic portion of multiple mice simultaneously. This set-up consists of a single, anterior 225 kV, 15 mA X-ray beam with a 0.15 mm Cu at an SSD of 35 cm that is designed to accurately reproduce the internal radiation dose distribution in mice that were used in previous studies.[16-18,30,57] The dosimetry and shielding of this system has been tested extensively.[28] The dose of radiation is a single fraction delivered via single AP (anterior-posterior) approach. The dose used is 13.5 Gy (roughly corresponding to LD_{50}) as described in our previous work.[16,30,57] For quality assurance, thermoluminescent dosimeters are placed over selected mice, to verify correct dose administration.

RNA isolation

RNA was isolated and purified from mouse lung tissue 48 h post-radiation exposure (including non-irradiated controls) using a modified Trizol protocol with column clean-up. Twenty to thirty milligrams of frozen tissue was placed into 1 mL Trizol (Invitrogen) in 2 mL screw-cap tubes, and Lysing matrix D (MP Biomedicals) was added. Tissue was disrupted using a desktop bead beater for 2 times 30 s, with 5 min on ice interspersed. Tubes were spun to collect beads and homogenate, and 200 μL chloroform was added. Tubes were firmly capped and shaken vigorously for 1 min, then centrifuged at 12 000 × g in an Eppendorf C2415 centrifuge for 15 min at 4 °C. The aqueous phase was transferred to a new tube, with care taken not to disturb the interphase. One hundred percent ethanol was added in a volume at least 1.25 times that of the recovered aqueous phase for each tube. Sample was then applied to columns from a mirVana miRNA isolation kit (Ambion) to obtain total RNA. Washing and elution was done per the manufacturer's protocol. RNA concentration and purity was analyzed by NanoDrop. RNA was stored at −80 °C.

QuantStudio OpenArray profiling

Profiling was conducted as described previously.[20] One hundred nanograms of RNA per sample was reverse transcribed using the RT primers of rodent-specific primer pools A and B (Life Technologies). cDNA was pre-amplified for 12 cycles using Rodent Pre-Amp primer pools A and B, also per manufacturer's protocol. Pre-amplified product was diluted 1:40 with 0.1× TE, pH 8.0. After mixing 1:1 with TaqMan OpenArray master mix, sample was loaded onto OpenArray plates by robot and amplified with the QuantStudio system. For six samples, technical duplicates were performed.

OpenArray data processing and analysis

Threshold cycles were assigned to each amplification reaction using ExpressionSuite software (Life Technologies). This software uses a proprietary algorithm to calculate a "relative threshold cycle" or Crt, similar to the Cycle of quantitation (Cq) or threshold cycle (Ct) assigned by other platforms but taking amplification efficiency into account for each individual reaction.. Additionally, the software assigns an "amplification score" to each reaction for rapid assessment of data reliability. Data were filtered to exclude reactions with a minimum amplification score and maximum Crt of 1.24 and 32, respectively (almost all features had Crt < 28). Several features with abnormally early Crt values were discarded after amplification curve inspection; these included mmu-miRs-1894-3p, -1896, and -1942, and hsa-miRs-188-3p and -5p. Data were exported to Microsoft Excel. Features detected in fewer than 14 of 18 runs (12 samples, 6 replicates) were excluded from further consideration, with the exception of miR-122, which was detected in all flaxseed-fed samples but in only two others. Following application of this detection filter, replicate values were averaged.

Data normalization by quantiles, as well as analysis, was performed with R/Bioconductor tools including affy/preprocesscore; the MultiExperiment Viewer (MeV; http://www.tm4.org/mev.html); and Microsoft Excel as described previously.[58,59] Mean-centered normalized data were used to cluster samples hierarchically (Pearson distance, average linkage). Differences between treatment groups were assessed by two-way ANOVA, and features displaying differential regulation ($P < 0.005$) as assessed by diet or radiation exposure comparisons were used for clustering analysis. Interaction of diet and radiation was also assessed.

Validation by individual stem-loop qPCR assays

Individual stem-loop primer reverse transcription/hydrolysis probe qPCR assays (Life Technologies) were used to quantitate 12 mature miRNAs (miRs-10a, -16, -21, -25, -26a, -34a, 142-3p, -143, -150, -181c, -340, and -375) and two structural small RNAs, snoRNA-135 and snRNA U6, following manufacturer's recommendations and with minor modifications as described previously.[60,61] Data were normalized to the geometric mean of all measured RNAs using the delta-delta Cq method. Differences between groups were assessed by the Kruskal-Wallis test followed by Dunn post-test for multiple comparisons.

miRNA target prediction

TargetScan[19] was used to predict targets of the significantly upregulated miR-34a.

Data availability

Raw and normalized data have been deposited with the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE57123.

Disclosure of Potential Conflicts of Interest

The authors declare that they have no competing interests.

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Author Contributions

M.C.S. designed the study and individual experiments, analyzed data, wrote the manuscript, and supervised project and lab personnel; R.P. performed animal experiments, biochemical assays and conducted data analysis; E.A. conducted animal experiments and tissue analyses; M.A.M. performed RNA purification and qPCR assays and analysis; K.W.W. performed miRNA analysis, qPCR confirmation, and data analyses, and contributed in writing of the manuscript.

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