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Fractionated Radiation Alters Oncomir and Tumor Suppressor miRNAs in Human Prostate Cancer Cells

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Abstract

We have previously demonstrated that prostate carcinoma cells exposed to fractionated radiation differentially expressed more genes compared to single-dose radiation. To understand the role of miRNA in regulation of radiation-induced gene expression, we analyzed miRNA expression in LNCaP, PC3 and DU145 prostate cancer cells treated with single-dose radiation and fractionated radiation by micro-array. Selected miRNAs were studied in RWPE-1 normal prostate epithelial cells by RT-PCR. Fractionated radiation significantly altered more miRNAs as compared to single-dose radiation. Downregulation of oncomir-17-92 cluster was observed only in the p53 positive LNCaP and RWPE-1 cells treated with single-dose radiation and fractionated radiation. Comparison of miRNA and mRNA data by IPA target filter analysis revealed an inverse correlation between miR-17-92 cluster and several targets including *TP53INP1* in p53 signaling pathway. The base level expressions of these miRNAs were significantly different among the cell lines and did not predict the radiation outcome. Tumor suppressor miR-34a and let-7 miRNAs were upregulated by fractionated radiation in radiosensitive LNCaP (p53 positive) and PC3 (p53-null) cells indicating that radiation-induced miRNA expression may not be regulated by p53 alone. Our data support the potential for using fractionated radiation to induce molecular targets and radiation-induced miRNAs may have a significant role in predicting radiosensitivity

INTRODUCTION

MicroRNAs (miRNA) are short non-coding single-stranded RNAs of approximately 22 nucleotides in length that have emerged as predominantly negative modifiers of gene expression (1). It is well known that hundreds of miRNAs are found in the human genome and that a single microRNA can potentially regulate a wide range of target genes resulting in a global effect on gene expression (2). miRNAs play a significant role in regulating cellular processes, such as proliferation, apoptosis, differentiation, signal transduction, senescence, invasion and angiogenesis, many of which are aberrant in cancer (3–8).

The importance of miRNAs in cancer was highlighted by the observation that more than 50% of human miRNA genes are frequently located in fragile sites and in genomic regions involved in cancers (9). Some of the miRNAs have been identified as tumor promoters or tumor suppressors by the modulation of gene expression in the oncogenic or tumor suppressor networks (6, 10–12). The miR-17–92 cluster, one of the first identified oncogenic miRNAs, was shown to regulate cell survival, proliferation, differentiation, and angiogenesis (11, 13–15). A well known tumor suppressor miRNA miR-34a was found to be downregulated in drug-resistant prostate cancer cells, and ectopic expression of miR-34a resulted in increased sensitivity to camptothecin (16). Reduced expression of tumor suppressor let-7 miRNAs was shown to be associated with a poor prognosis and shortened postoperative survival in lung cancers (17).

Many human epithelial cancers, including prostate cancer, contain miRNA signatures that differ from their normal counterparts (14, 18–20). Salter *et al.* suggested that an optimal strategy for predicting chemotherapeutic response would be to integrate the miRNA and mRNA signatures of chemosensitivity (21). Since miRNAs can act as oncogenes or tumor suppressors, the interference of cancer-specific miRNAs could be exploited to produce a direct anticancer effect, and improve the response of tumor cells to conventional anticancer therapies (22, 23).

Exposure to ionizing radiation significantly alters miRNA expression patterns in normal cells as well as in cancer cells (24–28). Furthermore, some studies have reported modulation of radiosensitivity by altering miRNA levels (27–29). In most of these studies, the changes in miRNA expression in the irradiated cells were evaluated after treating cells either with different doses of radiation or at different times after administering single-dose radiation. In the clinic, radiation is usually administered in daily treatments of 1.8–2.0 Gy, 5 days per week for 7–9 weeks. Recent studies from our laboratory demonstrated that compared to single-dose radiation, fractionated radiation treatment resulted in robust differential gene expression in prostate carcinoma cells (30). To understand the role of miRNAs in the regulation of gene expression, we employed a combined approach of comparing the gene expression profiles of mRNAs and miRNAs in irradiated prostate carcinoma cells after single and fractionated radiation protocols. Since ionizing radiation-induced DNA double-strand breaks result in the phosphorylation of the p53 pathway and regulation of cellular processes, we studied the miRNA expression in p53-positive LNCaP cells, and p53-mutant PC3 and DU145 cells. We also studied the expression pattern of selected miRNAs in normal human prostate epithelial cells (RWPE-1) after exposure to radiation.

Differential expression patterns of miRNAs were evaluated after two regimens of single-dose radiation (5 Gy and 10 Gy) and fractionated radiation (0.5 Gy $\times$ 10, 1 Gy $\times$ 10). Data revealed that miRNAs showed differential expression patterns in response to different radiation protocols. Compared to fractionated radiation, cells treated with single-dose radiation differentially expressed fewer miRNAs. For miR-17-92 cluster miRNAs, the response to fractionated radiation differed in p53 positive LNCaP cells, and p53 mutant PC3 and DU145 cells. These miRNAs were downregulated only in p53-positive LNCaP cells after single and fractionated dose of radiation, but not in p53 mutant PC3 or DU145 cells. Interestingly, despite the different p53 status, LNCaP and PC3 cells exhibited similar

miRNA expression patterns for let-7 and miR-34a in response to fractionated radiation. miR-146a, implicated in innate immune response, was differentially expressed after fractionated radiation only in DU145 cells.

MATERIALS AND METHODS

Cells

LNCaP (p53-wt), PC3 (p53-null), and DU145 (p53-mutated) human prostate carcinoma cells and RWPE-1 (p53-wt) normal human prostate epithelial cells were obtained from American Type Culture Collection (ATCC) (Manassas, VA). LNCaP cells were maintained on RPMI 1640 and 10% FBS from ATCC and supplemented with antibiotics. PC3 and DU145 cells were grown in RPMI 1640 supplemented with 10% FBS, glutamine and antibiotics (Life Technologies, Inc., Grand Island, NY). RWPE-1 cells were grown in serum-free keratinocyte medium with growth factors, 25 µg/mL Bovine Pituitary Extract (BPE) (catalog no. 3028), and 0.1 ng/mL Recombinant Epidermal Growth Factor (rEGF) (catalog no. 10450), purchased from Life Technologies.

Radiation

Cells were plated into T75 culture flasks (1×10^6 for single-dose radiation and 0.5×10^6 for fractionated radiation). After 24 h, cells were exposed to a total of 5 Gy and 10 Gy radiation administered either as single-dose radiation, or as multi-fractionated radiation of $0.5 \text{ Gy} \times 10$ and $1 \text{ Gy} \times 10$ (fractionated). For the $0.5 \text{ Gy} \times 10$ protocol, cells were exposed to 0.5 Gy radiation twice a day for 5 days and for the $1 \text{ Gy} \times 10$ protocol, cells were exposed to 1 Gy radiation twice a day, at 6 h intervals for 5 days. RNA was collected at 24 h after the final dose of radiation. Separate controls were maintained for single dose and fractionated radiation protocols.

Clonogenic Assay

Cells were treated with 2, 4, 6 and 8 Gy radiation delivered as single doses. Cells were trypsinized, counted, and plated for clonogenic assay 20 h after the final dose of radiation. Colonies were stained with crystal violet after 12 days (DU145 and PC3) or 21 days (LNCaP) and colonies of 50 cells were counted. Survival curve parameters were determined using a computer program that represents cell survival after radiation by a linear-quadratic equation (31).

RNA Isolation

Total RNA and miRNA were isolated from the harvested cells at 24 h after single-dose and fractionated-dose radiation from three separate biological replicates. Small RNA (less than 200 nucleotides) were isolated using the RNAeasy kit (Qiagen, U.S.) followed by an enrichment procedure for small RNA recovery (Applied Biosystems, Foster City, CA). Total RNA was isolated with RNAeasy kit (Qiagen). The quantity and quality of the total RNA and miRNA was determined by Nanodrop and Bioanalyser.

miRNA Microarray Analysis

Microarray analysis was performed on LNCaP, PC3 and DU145 cells using RNA isolated from 3 separate experiments. The miRNA microarray analysis was performed using Agilent human miRNA Microarray Kit (V2). The chip contains probes for 723 human and 76 human viral microRNAs from the Sanger database v. 10.1. Data were analyzed using Gene Spring Software (Agilent Technologies). Data for all arrays were filtered for intensity values that were above background in at least two of any set of three replicates for any condition within each radiation protocol. To ensure that miRNAs were reliably measured, ANOVA was used to compare the means of each condition ($n = 3$). For miRNA analysis, a cut-off ratio >1.5 with a $P < 0.05$ relative to the control was selected for this study.

Real-Time RT-PCR for miRNA Expression with Taqman MicroRNA Assay

A total of 10 ng of RNA was used to reverse transcribe specific miRNAs of interest into cDNA using the Taqman miRNA reverse transcription kit (Applied Biosystems, no. 4367038). This was followed by real-time PCR using miRNAs specific Taqman probe assays for miR-34a, miR-19a and miR-146a in a 7500 Real-time PCR machine (Applied Biosystems). Standard curves were examined in triplicate for both the miRNA of interest and the internal control gene U6 or RNU 48, and miRNA expression levels were normalized to respective controls and calculated using the delta CT method (32).

miR-146a Transfection

Our previous study showed that PC3 cells differentially expressed a large number of immune response genes after exposure to fractionated radiation, whereas only a few genes were altered in DU145 cells. Since miR-146 has been implicated in innate immune response, the miR-146 levels were modulated by transfection of pre- and anti-146a in PC3 and DU145 cells. For transfection cells were seeded at 25,000 cells/wells in a 6-well plate. The cells were incubated overnight at 37°C and 5% CO₂ and then transfected with precursor, pre-miR-146a (PM10722), and inhibitor, anti-miR-146a (AM10722), at a final concentration of (50 nM) combined with siPORT amine transfection reagent in accordance with the manufacturer's instructions (AM4502, Applied Biosystems, Carlsbad, CA). For each experiment, vehicle controls were kept for each condition and used as normalization controls.

Real-Time RT-PCR for mRNA Expression of Immune Response Genes in Cells Transfected with Pre-miR and Anti-miR-146a

The expression of *IRAK1* and selected immune response genes *IFI27*, *IFI44*, *OAS1* and *OASL* in PC3 and DU145 cells transfected with pre- and anti-miR-146a was examined by real-time RT-PCR using Taqman gene expression assays and the ABI PRISM 7500 Sequence Detection System equipped with the SDS version 1.4.0 software (Applied Biosystems, Foster City, CA). Forward and reverse primers and probes were designed and produced by Applied Biosystems. cDNA was prepared from total RNA using cDNA Reverse Transcription Kit (part no. 4368814) and PCR was carried out using TaqMan Universal PCR Master Mix (part no. 4324018). Each sample was analyzed in duplicate, and *GAPDH* was used as an endogenous control (Hs.99999905.s1). Negative controls were

processed under the same conditions without RNA template. Data are presented as the average fold change in the target genes in irradiated/transfected cells normalized to the internal control gene (*GAPDH*) and relative to unirradiated control/vehicle control cells from 3 separate experiments. The following probes were used in this study: *IRAK1* (Hs01021686_m1), *IFI27* (Hs00271467_m1), *IFI44* (Hs00197427_m1), *OAS1* (Hs00242943_m1), and *OASL* (Hs00984390_m1).

Ingenuity Pathway Analysis (IPA)

The functional significance of differentially expressed miRNAs altered by radiation was evaluated using Ingenuity Pathway analysis (IPA) software (version 8.8, Redwood City, CA). Differentially expressed miRNAs (>1.5-fold change and $P < 0.05$) were selected for network generation and pathway analyses implemented in IPA tools. Agilent probe IDs and miR-based IDs were uploaded into the IPA, which were then mapped to the functional networks available in the Ingenuity Pathway Knowledge Base (32). Each network was given a score reflecting the negative logarithm of the P value, based on the chance of the significant molecules falling in to the network at random. A score of 2 implies that there is a 1 in 100 chance that the focus genes are together in a network because of random chance. Therefore, scores of 2 or higher have at least a 99% confidence of not being generated by random chance.

IPA Network Generation

Data set containing miRNAs with corresponding expression values with 1.5-fold cutoff ($P < 0.05$) was uploaded into the IPA application (version 9.0–3206). Each identifier was mapped to its corresponding object in the Ingenuity® Knowledge Base. These molecules, called Network Eligible molecules, were overlaid onto a global Ingenuity Systems, 2011 (www.ingenuity.com) and molecular network was developed from information contained in the Ingenuity Knowledge Base. Networks of Network Eligible Molecules were then algorithmically generated based on their connectivity.

miRNA-Target Gene Analysis

An miRNA-target gene analysis data set of differentially expressed miRNAs (1.5-fold cut off, $P < 0.05$) and differentially expressed mRNAs (2-fold cut off, $P < 0.05$) were uploaded into IPA “Micro RNA Target Filter” program. For data analysis, only the experimentally observed and highly predicted targets were selected.

Data Analysis

Each data point represents $AV \pm SEM$ of 3 experiments. Differences between the groups were statistically evaluated by two-tailed paired t test. A $P < 0.05$ was considered statistically significant. Statistical significance of the data shown in Fig. 5 was analyzed using DataAssist Software v3.0 (Applied Biosystems). This software was used to calculate P values by t test using Benjamini-Hochberg False Discovery Rate.

RESULTS

Radiosensitivity of DU145, PC3 and LNCaP Cells

Figure 1 shows the radiation survival curves of DU145, PC3 and LNCaP cells. The surviving fractions (SF) for DU145, PC3, and LNCaP cells at 2 Gy were 0.66 ± 0.03 , 0.53 ± 0.007 and 0.44 ± 0.12 , respectively, and for 8 Gy were 0.06 ± 0.007 , 0.003 ± 0.002 , and 0.007 ± 0.004 , respectively.²

Global miRNA Expression Pattern

Microarray analysis of miRNA revealed 116 miRNAs differentially expressed with high confidence (>1.5 - fold change, $P < 0.05$) in LNCaP, PC3, and DU145 cells exposed to 5 and 10 Gy single-dose (SD) and 0.5 Gy $\times$ 10 and 1 Gy $\times$ 10 fractionated (MF) radiation (Fig. 2A and B). In LNCaP, PC3 and DU145 cells exposure to single-dose radiation resulted in differential expression of 37, 7 and 2 miRNAs, respectively. Treatment with fractionated radiation protocols resulted in differential expression of 48, 47 and 6 miRNAs in LNCaP, PC3 and DU145 cells. PC3 and DU145 cells each had only 1 miRNA in common with LNCaP cells after treatment with single-dose radiation (Fig. 2A). Fractionated radiation exposure resulted in 8 common miRNAs between LNCaP and PC3 cells (Fig. 2 B). A significant increase in the number of downregulated miRNAs was seen in LNCaP cells after treatment with single and fractionated radiation (Fig. 2C). In PC3 cells, treatment with fractionated radiation resulted in a greater number of upregulated miRNAs, but in LNCaP cells equal numbers of miRNAs were up- and downregulated after fractionated irradiation (Fig. 2C).

Heat Maps

Fold changes in the individual miRNAs differentially expressed at 24 h after single-dose and fractionated radiation were color-coded to demonstrate the pattern of miRNAs in the three cell lines. More miRNAs were differentially expressed in LNCaP and PC3 cells compared to DU145 cells (Fig. 3). Between the two fractionation protocols, 1 Gy $\times$ 10 regimen resulted in differential expression of more miRNAs compared to the 0.5 Gy $\times$ 10 regimen (Fig. 3B). In LNCaP cells, the majority of the miRNAs differentially expressed after single-dose irradiation were downregulated (Fig. 3A), whereas more than 50% of miRNAs were upregulated after the 2 fractionated radiation protocols (Fig. 3B). Compared to LNCaP and PC3 cells, in DU145 cells very few miRNAs were altered after single-dose and fractionated radiation exposure.

Differential Expression of miRNAs in LNCaP, PC3 and DU145 Cells Treated with Single and Fractionated Radiation

The miRNA microarray analysis revealed differential expression of several miRNAs including miR-17-92 cluster, miR-34a, let-7 family miRNAs and has-miR-146a in prostate cancer cell lines after single and fractionated irradiation (Table 1 and Fig. 4). In LNCaP cells, miR-17-92 cluster was significantly downregulated after single and fractionated

²The α/β ratios for the DU145, PC3 and LNCaP cells were 7.593, 3.325, and 5.481, respectively.

radiation exposure, whereas upregulation of miR-34a was seen only after fractionated irradiation in both LNCaP and PC3 cells (Table 1). Figure 4A and B show the mini-heat maps of miR-17-92 cluster and the let-7 family of miRNAs differentially expressed after exposure to single-dose and fractionated radiation protocols. In general, more miRNAs were differentially expressed after fractionated irradiation compared to single-dose irradiation. Figure 4C shows the basal expression levels of the let-7 family miRNAs in unirradiated LNCaP, PC3 and DU145 control cells. The base levels of the individual let-7 family members (three cell lines) were different (Fig. 4C). Let-7d, let-7e, let-7f and let-7i were significantly lower in LNCaP cells compared to DU145 cells.

Base Levels of miR-34a, miR-19a, and miR-146a in LNCaP, PC3 and DU145 Cells in Comparison with Normal Prostate Epithelial Cell Line RWPE-1

Figure 5 shows the fold changes in basal expression levels of miR-19a, miR-34a and miR-146a in LNCaP, PC3 and DU145 cells in comparison with normal prostate epithelial RWPE-1 cells. RT-PCR analysis of unirradiated prostate cancer cell lines showed that there were substantial differences in the base levels of individual miRNAs in each cell line and across the 3 cell lines. Among the three cell lines, the highest basal expression of miR-19a was seen in PC3 cells. The basal expression of miR-34a was larger in LNCaP cells compared to RWPE-1 cells, whereas it was significantly lower in PC3 and DU145 cells (Fig. 5). There was a statistically significant difference in the basal expression of miR-146 in the 3 cell lines, miR-146a expression highest in PC3 cells and lowest in LNCaP cells (Fig. 5).

Validation of Differentially Expressed miRNAs by RT-PCR

Fold changes in miRNAs after exposure to single (SD10) and fractionated (MF 1 Gy $\times$ 10) irradiation shown in Table 1 were confirmed by RT-PCR analysis. Figure 6 shows fold changes in selected miRNAs in irradiated RWPE-1, LNCaP, PC3, and DU145 cells in comparison with the unirradiated control cells. miR-19a was downregulated by single-dose and fractionated irradiation in RWPE-1, LNCaP and PC3 cells but was upregulated in DU145 cells after fractionated irradiation (Fig. 6A). miR-34a was upregulated by fractionated irradiation in LNCaP and PC3 cells, but not in RWPE-1 and DU145 cells (Fig. 6B). There was a threefold increase in miR-146a in DU145 cells after fractionated radiation exposure (Fig. 6C). However, there were no significant changes in miR-146a in the irradiated RWPE-1, LNCaP and PC3 cells. The RT-PCR data (Fig. 6) showed substantial correlation with the miRNA microarray data (Table 1). A comparison of single-dose and fractionated radiation regimens showed a significant difference for miR-19a expression only in DU145 cells. In LNCaP cells, there was a significant difference in miR-34a increase between the single-dose and fractionated radiation protocols. An increase in miR-146a after single-dose and fractionated regimens was significantly different in both RWPE-1 and DU145 cells. Significant differences in expression of miRNAs were observed between the cell lines and are indicated in Fig. 6.

Modulation of miR-146a and Target Evaluation

Our previous mRNA microarray data showed that in PC3 cells, immune response was the top most gene ontology category altered by fractionated radiation. In DU145 cells, very few

immune response genes were differentially expressed by fractionated irradiation (30). Since miR-146a is implicated in the regulation of innate immune response, we examined the effect of anti-miR- and pre-miR-146 transfection on the expression of the selected immune response genes identified from our previous mRNA micro-array study in PC3 and DU145 cells.

IRAK1, a predicted target of miR-146a, was used as an internal control to evaluate the effect of miR-146a modulation in prostate cancer cell lines. Real-time RT-PCR analysis confirmed that, compared to the vehicle control, *IRAK1* expression was upregulated in PC3 and DU145 cells transfected with anti-miR-146a and downregulated in the cells transfected with pre-miR-146a (Table 2). Four selected immune response genes identified from our previously reported data showed similar up- or downregulation in pre- and anti-miR146a transfected PC3 cells. A similar trend was also observed in DU145 cells (Table 2). Consistent with our earlier observation, exposure to fractionated radiation significantly upregulated immune response gene *IFI27* in PC3 cells (Table 2). In PC3 cells transfected with anti-miR-146a, fractionated irradiation further upregulated the *IFI27* expression. Transfection with pre-miR-146a significantly reduced the expression of *IFI27* compared to the increase observed after irradiation alone. A similar, but less significant trend was observed also for immune response genes *IFI44*, *OASL* and *OAS1* upon irradiation of pre-miR-146a transfected PC3 cells. The expression level of the predicted target *IRAK1* was not significantly altered after radiation exposure in PC3 and DU145 cells. However, *IRAK1* was significantly downregulated after fractionated irradiation in DU145 cells transfected with pre-miR-146a, compared to fractionated irradiation alone.

The magnitude of radiation-induced upregulation of *IFI27* was much lower in DU145 cells compared to the increase observed in PC3 cells. In contrast to PC3, in DU145 cells the inverse correlation between miR-146a levels and *IFI27* was observed only with pre-miR transfection. Upregulation of *IFI27* was not observed with anti-miR-146a transfection. Compared to the vehicle treated XRT controls, in pre-miR-146a transfected DU145 cells the expression of four immune response genes was significantly downregulated by fractionated irradiation (Table 2).

IPA Analysis

To identify the functions of miRNAs in the post transcriptional regulation of gene expression patterns, miRNAs significantly altered after radiation exposure (> 1.5 -fold change, $P < 0.05$) were mapped to their functional networks in the IPA database and ranked by score (Table 3). Table 3 shows the scores, the names of molecules (miRNAs) and the top functional categories for all radiation protocols in each line. In LNCaP cells, cell cycle and cellular development, growth and proliferation were the top categories altered after single-dose irradiation. The categories detected following 1 Gy $\times$ 10 fractionated radiation regimen common for LNCaP and PC3 cells included skeletal and muscular disorder, and reproductive and genetic disorder. There are limited data from DU145, with fractionated radiation exposure showing inflammatory response and antigen presentation categories that were not seen after single-dose exposure. The common theme in all cell lines is the difference between single-dose and fractionated regimens. Top net works of miRNAs and

connecting molecules are shown in Supplementary Fig. 1 (<http://dx.doi.org/10.1667/RR2703.1.S1>).

A long-term goal of this project is to identify radiation-inducible targets and to see how best to select the target from mRNA, miRNA or proteomic analysis. Table 4 shows the Micro RNA Target Filter IPA analysis of the major differentially expressed miRNAs from the present study paired with the differentially expressed mRNA targets identified from our mRNA microarray data published previously [(30); Simone *et al.*, unpublished data]. Table 4 shows names, fold changes and functions of some of the predicted target genes of miR-19a, miR-34a and let-7 miRNAs differentially expressed in LNCaP and PC3 cells treated with single-dose and fractionated radiation protocols. These targets showed inverse correlations with the miRNAs. A complete list of the miRNA/target gene analysis is given in Supplementary Tables 1–6 (<http://dx.doi.org/10.1667/RR2703.1.S2–S7>). The targets of miR-19a, prostate transmembrane protein, androgen-induced 1 (*PMEPA1*), and tumor protein p53 inducible nuclear protein 1 (*TP53INP1*), along with 9 other targets showed inverse correlations in LNCaP cells (Table 4). Alpha-kinase 2 (*ALPK2*) and angiopoietin 2 (*ANGPT2*) showed more specific upregulation after fractionated radiation exposure. Even though let-7 miRNAs were upregulated in LNCaP and PC3 cells, more targets were downregulated in LNCaP cells. In PC3 cells, *E2F2* was the only target of let-7 that showed inverse correlation. miR-34a was also upregulated in both LNCaP and PC3 cells, and cyclin E2 (*CCNE2*) was the only target gene that was downregulated in both cell lines.

DISCUSSION

We recently demonstrated significant differences in the gene expression profiles of p53 mutant PC3 and DU145 prostate carcinoma cells irradiated with single-dose compared to fractionated radiation (30). The present study investigated the changes in miRNA expression pattern after exposure to single-dose and fractionated radiation in PC3 and DU145 cells, and also included LNCaP cells expressing wild-type p53. The data revealed that the miRNA expression profile of the cells that received and survived repeated exposure of radiation was significantly different from the starting cell population as well as from the cells exposed to single higher dose of radiation. Of the two radiation protocols, fractionated irradiation altered more miRNAs compared to single-dose regimen in p53-wt LNCaP and in p53-mutant PC3 and DU145 cells. The current study also investigated the impact of fractionation of a dose of radiation, 0.5 Gy, that would induce little cell killing, so that we can better understand the changes in cells that survive repeated radiation exposure. The 0.5 Gy dose per fraction is also a dose that out-of-field micrometastases and normal tissue may encounter during a course of radiation.

Several studies have shown that exposure to radiation results in differential expression of miRNAs in a variety of cancer and normal cells (25–28, 33–40). In the present study, a total of 116 miRNAs were differentially expressed in prostate cancer cells exposed to radiation, of these miR-34a, let-7 family miRNAs and miR-146a were upregulated specifically after

¹*Editor's Note.* The online version of this article (DOI: 10.1667/RR2803.1) contains supplementary information that is available to all authorized users.

fractionated irradiation. Tumor suppressor p53 plays an important role in the expression of several miRNAs. It has been shown that p53 binds to and transcriptionally activates miR-34a, which in turn regulates the expression of p53-regulated genes controlling cellular processes such as cell cycle arrest and apoptosis (34, 41–43). The normal prostate epithelial RWPE-1 cells, as well as the LNCaP cells are p53 positive. However, the base level of miR-34a in LNCaP cells was significantly higher compared to the RWPE-1 cells. The responses of these two cell types to single or fractionated irradiation were also significantly different. In RWPE-1, cells exposed to fractionated radiation downregulated miR-34a, whereas, in LNCaP cells, it was significantly upregulated. These data suggest that the basal miR-34a miRNA levels, as well as radiation-induced miRNA expression, may not be regulated by p53 alone.

Radiation-induced upregulation of miR-34a has been previously reported in p53-wt A549 human non-small cell lung cancer cells and p53-wt HCT116 colon carcinoma cells, but not in their p53-null derivative (28, 33, 38). However, in the present study a significant upregulation of miR-34a after fractionated radiation exposure was observed in both LNCaP (wt-p53) and PC3 (null-p53) cells. A similar induction of miR-34a after exposure to X rays was reported in human lymphoblast cell lines with different p53 status (44). A p53-independent expression of miR-34a was also reported in p53-null K562 cells after treatment with phorbol ester (TPA) by activating an alternative TPA-responsive promoter (45). The mechanism of upregulation of miR-34a in irradiated p53-null PC3 cells is not clear. Nevertheless, the present data show that pathways other than p53 can regulate miR-34a.

miR-34a acts as a tumor suppressor in some p53-mutant cells. In p53-mutant U251 glioma cells, miR-34a expression was low in comparison with glioma cells expressing wild-type p53. Overexpression of miR-34a in U251 resulted in less malignant phenotype including reduced *in vitro* migration and invasive capabilities (46). In a panel of lung cancer cell lines devoid of functional p53, exogenous miR-34a provided strong evidence of the tumor suppressive ability of miR-34a *in vitro* and *in vivo*, suggesting that pathways down-stream of miR-34a are sufficient to block cancer cell growth (47). It appears that chemotherapy resistance may be related to miR-34 expression level. Earlier reports have shown that miR-34a was downregulated in drug-resistant prostate cancer cells and the ectopic expression of miR-34a decreased chemoresistance to camptothecin through inducing apoptosis (16). In the present study, exposure to fractionated irradiation resulted in upregulation of miR-34a in radiosensitive LNCaP and PC3 cells, but not in radioresistant DU145 cells. Radiation-induced miR-34a increase may be used as a predictor of radiation sensitivity. Currently, we are evaluating the effect of modulating miR-34a on radiosensitivity in a variety of cell lines with different p53 status.

In this study, we have shown for the first time that the miR-17-92 cluster of miRNAs is markedly downregulated by both single and fractionated irradiation in p53 positive LNCaP and RWPE-1 cells. The miRNA-17-92 cluster, also known as oncomiR-1, promotes cell cycle progression (48) and proliferation (13) and inhibits apoptosis (11, 49, 50). In lymphomas and other solid tumors, overexpression of miR-17-92 is associated with radioresistance (29, 50). Overexpression of miR-17-92 in tumor cells significantly enhanced the resistance to radiation-induced cell damage and G₂/M arrest (29). Among the three

prostate carcinoma cell lines, miR-19a was downregulated in p53 positive radiosensitive LNCaP cells exposed to single dose, as well as fractionated radiation regimens suggests that inhibition of the miRNA-19a may provide a new therapeutic strategy for radioresistant prostate cancers with mutated p53.

Human let-7 family members are downregulated in several cancers and restoration of let-7a, 7b and 7c can inhibit the growth of cancer cells (5, 51). However, the role of different let-7 family members in radiation response is not clear. It has been shown that exposure to radiation downregulated the expression of several let-7 miRNAs in A549 lung cancer cells (28). Furthermore, *in vitro* overexpression of let-7a and let-7b radiosensitized the cells, and downregulation of let-7a and let-7b resulted in an increased radioresistance (28). On the contrary, other studies observed no change in let-7 miRNAs after irradiation (35), or upregulation of let-7 miRNAs after irradiation (25). In the present study, fractionated irradiation significantly upregulated let-7b, c and e in radiosensitive LNCaP and PC3 cells, whereas these miRNAs were downregulated in radioresistant DU145 cells. Further studies are required to assess if radiation-induced let-7 expression could be a predictive factor for radiosensitivity.

miR-146a was upregulated in DU145 cells after fractionated irradiation. miR-146 is implicated in tumor cell invasion (52, 53) as well as innate immune response (54, 55). Previous studies from our laboratory demonstrated that the immune response gene expression pattern of PC3 and DU145 cells treated with fractionated irradiation was significantly different (30). The present study showed that the miR-146 miRNA response to radiation in these two cell lines was also different. As expected, modulation of miR-146a with anti- and pre-miR-146a significantly altered *IFI27* gene expression in PC3 cells. The effect was less pronounced for other genes examined in PC3 cells. Transfection with anti-miR had no significant effect on the immune response genes examined in DU145 cells, most likely because miR-146a base level expression was already significantly low in DU145 compared to PC3 cells. However, upregulation of miR-146a by pre-miR transfection significantly downregulated all the genes examined in DU145 cells. Further studies are required to determine the role of miR-146a in irradiated cells.

It is well-known that a single miRNA can regulate several target genes (56) and that miRNAs are believed to regulate their targets in inverse fashion (1). A combined analysis of the present miRNA data and our previously published mRNA data using IPA micro-RNA target filter program identified targets for the miRNAs differentially expressed following single-dose and fractionated irradiation. Although the miRNA/target gene pairing did show inverse correlation, in many instances several targets exhibited changes in the same direction. In the irradiated LNCaP cells mir-17-92 cluster, miRNAs were downregulated after single-dose and fractionated irradiation and tumor protein p53 inducible protein 1 (*TP53INP1*), which is a highly predictable target of mir-19a was upregulated showing an inverse correlation. However, *TP53INP1* is also a highly predictable target of miR-22 that was upregulated in the LNCaP cells after radiation (see Supplementary Table 2; <http://dx.doi.org/10.1667/RR2703.1.S3>). In this case, a comparison of miR-22 with target *TP53INP1* does not show an inverse correlation.

Our data show that there is no correlation between basal level of miRNA and response to radiation. Thus, should miRNA be used to select the radiation-inducible target, profiling the untreated tumor cell would not be adequate. In summary, as part of our investigation as to the potential to use fractionated irradiation as a means of inducing susceptibility to a molecular therapeutic, this study identified miRNAs differentially expressed by single-dose and/or fractionated radiation regimens. This study included normal prostate epithelial cells and LNCaP cells with wild-type p53, and PC3 and DU145 cells with mutated p53. In prostate cancer cell lines the miRNA expression pattern after radiation exposure was not dependent on the p53 status alone. The IPA demonstrated differences in pathway induction between single-dose and fractionated irradiation in all 3 cancer cell lines with more changes after fractionated radiation exposure compared to single-dose radiation exposure. In terms of radiation-inducible targets, 1 Gy $\times$ 10 irradiation produced the most changes and the IPA pathways were similar between the LNCaP and PC3, primarily due to upregulation in let-7 and miR-34a. Those pathways are inducible at the 0.5 Gy fraction size, and are different for the p53 proficient LNCaP compared to mutated PC3 and DU145. This may have implications for normal tissue effects of radiation as well as the potential to select a radiation fractionation scheme to induce a desired effect.

Taken together, the data in this paper support the potential for using fractionated irradiation to induce molecular targets. In addition to mRNA and miRNA analyses, our ongoing work includes phosphoprotein analyses to facilitate identification of potential druggable targets in cancer cells exposed to fractionated radiation. The parental genotype will likely impact the inducible target as demonstrated by the differences seen between LNCaP and PC3, some of which is likely due to p53. Given the multiple targets of miRNAs, we do not yet know if they will be useful for ultimate drug target selection, although miRNA analysis after a few radiation fractions will be more informative than baseline measure. We also included a low dose 0.5 Gy per fraction to see the effect of fractionated radiation where there would be little cell killing per fraction and showed that cells that survive this radiation regimen exhibit a robust miRNA expression profile. This applies to normal tissues and possibly microscopic tumor cells that may be beyond the tumor margin, which is consistent with the idea that one might be able to select a fractionation dose and schedule that could optimize induced drug susceptibility. The therapeutic usage of tumor suppressor miRNAs or inhibitors of oncogenic miRNAs and synthetic miRNA mimics have been previously reported (57, 58). Targeting the radiation -inducible miRNAs involved in regulating radiation responses would be a useful approach to enhance radiation sensitivity of prostate cancer cells. Currently, we are in the process of modulating miRNAs in order to identify important pathways for use as a molecular targeted therapy.

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FIG. 1. Clonogenic survival of LNCaP, PC3 and DU145 cells exposed to 0, 2, 4, 6 and 8 Gy of radiation. Each data point represents $AV \pm SEM$ of 3 separate experiments.

FIG. 2.

Venn diagrams depicting the number of differentially expressed (> 1.5 -fold and $P < 0.05$) miRNAs in LNCaP, PC3 and DU145 cells 24 h after exposure to (panel A) 5 Gy and 10 Gy as single dose (SD) and (panel B) 0.5 Gy $\times$ 10 and 1 Gy $\times$ 10 as multi fractionated (MF) radiation. There was only 1 commonly altered miRNA between LNCaP and PC3 cells and between LNCaP and DU145 cells after single-dose radiation. For fractionated radiation, 8 miRNAs were common between LNCaP and PC3 cells. Panel C: Data indicate up- or down-regulated miRNAs (> 1.5 -fold and $P < 0.05$) in LNCaP, PC3 and DU145 cells after 5 Gy and 10 Gy single-dose radiation and 0.5 Gy and 1 Gy $\times$ 10 fractionated radiation.

FIG. 3.

Heat map showing miRNA profiles in LNCaP, PC3 and DU145 cells at 24 h after exposure to (panel A) 5 Gy and 10 Gy as single-dose and (panel B) 0.5 Gy $\times$ 10 and 1 Gy $\times$ 10 as fractionated radiation. Yellow to red indicates up regulated miRNAs. Blue indicates downregulated miRNAs. Arrows indicate miR-34a and miR-146a that are differentially expressed by fractionated radiation.

FIG. 4. Mini heat map showing differential expression of (panel A) miR-17-92 cluster of miRNAs in LNCaP, PC3 and DU145 cells after fractionated and single-dose irradiation and (panel B) let-7 family of miRNAs after fractionated and single-dose irradiation. Panel C: Base levels of let-7 family of miRNAs in unirradiated controls for LNCaP, PC3 and DU145 cells. Data shown are AV ± SEM of 3 separate experiments. **P* < 0.05, statistical difference between LNCaP and DU145 cells.

FIG. 5.

Real-time RT-PCR analysis of base level expression values of miR-19a, miR-34a and miR-146a in LNCaP, PC3 and DU145 cells normalized to RWPE-1 normal prostate epithelial cells. RNA was prepared from cells grown under identical conditions for 24 h in 6-well plates. *Statistical difference between RWPE-1 and prostate cancer cell lines.

FIG. 6.

Real-time RT-PCR validation of differentially expressed miRNAs (panel A) miR-19a, (panel B) miR-34a, and (panel C) miR-146a in RWPE-1, LNCaP, PC3 and DU145 cells. Cells were irradiated with 10 Gy single-dose or 1 Gy X 10 fractionated radiation and RNA was collected 24 h after the final dose. Data shown are AV $\pm$ SEM of 3 separate experiments. Significant statistical differences between the cell lines are indicated by different symbols. *RWPE-1 compared to prostate cancer cells (*P < 0.05, **P < 0.001, ***P < 0.0001), (PC3) compared to DU145, †LNCaP compared to DU145, ▲LNCaP

compared to PC3. Significant statistical differences between single-dose and fractionated radiation regimens are indicated by *P* values.

Microarray Analysis of Fold Changes in miRNAs Differentially Expressed (>1.5 -fold $P < 0.05$) in LNCaP, PC3, and DU145 Cells Treated with Single-Dose Radiation (10 Gy and 5 Gy) and fractionated Radiation (1 Gy $\times$ 10 and 0.5 Gy $\times$ 10)

TABLE 1

Accession no.	miRNA ID	LNCaP				PC3				DU145			
		10 Gy		5 Gy		1 Gy $\times$ 10		0.5 Gy $\times$ 10		10 Gy		5 Gy $\times$ 10	
		1 Gy		0.5 Gy		1 Gy		0.5 Gy		1 Gy		0.5 Gy	
	<i>miR-17-92</i>												
MI0000072	hsa-miR-18a	1.82*	-1.64*	-4.55*	-2.33*	1.03	1.15	1.04	-1.20	-1.14	-1.22	1.23	1.15
MI0001518	hsa-miR-18b	-1.64	-1.19	-2.94*	-2.86*	1.19	1.19	1.09	-1.27	1.15	-1.11	-1.27	-1.22
MI0000073	hsa-miR-19a	-1.61*	-1.69*	-9.09*	-6.67	-1.15	1.28	1.37	-1.43	-1.14	1.36	1.31	1.19
MI0000074	hsa-miR-19b	-2.63	-2.70	-4.76*	-3.85*	-1.12	1.20	-1.45	-1.39	-1.85	1.05	1.13	-1.05
MI0000268	hsa-miR-34a	1.34	1.34	3.38*	2.72*	1.19	-1.54	4.05*	2.48	-1.61	-2.17	1.10	-1.18
	<i>let-7 family</i>												
MI0000063	let7b	-1.05	-1.02	1.87*	1.41	1.73	1.62	2.91*	1.68	-1.10	-1.2	-1.75	-1.37
MI0000064	let7c	1.04	1.18	1.51*	1.17	1.09	1.14	3.35*	2.98*	-1.72	-1.35	-1.82	-1.47
MI0000066	let7e	1.08	1.16	2.46*	1.72	1.43	1.40	3.17*	1.33	-1.09	-1.22	-1.47	-1.32
MI0000433	let7g	1.00	1.07	2.36	2.20	1.48	1.56	3.09*	1.43	-1.18	-1.23	-1.08	-1.08
MI0000434	let7i	-1.05	1.06	2.11	2.02	1.37	1.42	2.31*	1.35	-1.19	-1.27	-1.23	-1.20
MI0000477	hsa-miR-146a	-1.14	-1.33	1.25	1.20	1.10	1.29	1.59	1.19	1.00	1.00	2.27*	1.68

Note. Each data point is an average from three separate experiments.

* $P < 0.05$.

Relative Expression of *IRAK1*, and Selected Immune Response Genes by Real-Time RT-PCR Analysis in PC3 and DU145 Cells after Transfection with Anti and Pre-miR-146a

TABLE 2

Gene ID	Gene symbol	antimiR	premiR	XRT	antimiR + XRT	premiR + XRT
<i>PC3</i>						
NM_001569	IRAK1	1.23 ± 0.03	0.70 ± 0.06	0.82 ± 0.03	0.72 ± 0.03	0.44 ± 0.03
NM_005532	IFI27	1.42 ± 0.02**	0.30 ± 0.04**	29.39 ± 2.02*	36.45 ± 1.41**	15.52 ± 1.17**
NM_006417	IFI44	1.21 ± 0.09	1.13 ± 0.02*	4.70 ± 0.37	5.01 ± 0.35	3.53 ± 0.20
NM_003733	OASL	1.74 ± 0.05*	0.72 ± 0.07*	6.82 ± 1.30*	6.80 ± 0.27	3.71 ± 0.30
NM_002534	OAS1	1.40 ± 0.08*	0.83 ± 0.03*	6.24 ± 0.41	5.10 ± 0.25	2.73 ± 0.06*
<i>DU145</i>						
NM_001569	IRAK1	1.25 ± 0.08	0.63 ± 0.02*	1.15 ± 0.01	1.28 ± 0.04	0.42 ± 0.04*
NM_005532	IFI27	0.92 ± 0.11	0.71 ± 0.00*	4.44 ± 0.50	2.83 ± 0.21	1.72 ± 0.11*
NM_006417	IFI44	1.22 ± 0.12	0.72 ± 0.13	4.02 ± 0.51	3.03 ± 0.52*	1.92 ± 0.41*
NM_003733	OASL	1.31 ± 0.02*	0.73 ± 0.12*	2.10 ± 0.12*	1.82 ± 0.23	1.30 ± 0.12*
NM_002534	OAS1	1.50 ± 0.08*	0.92 ± 0.05	4.81 ± 0.40	2.91 ± 0.14*	1.80 ± 0.13*

Notes. Cells were transfected anti-miR and pre-miR and irradiated (1 Gy × 8) starting at 24 h after transfection (as described in Materials and Methods). RNA was collected 24 h after the final dose of fractionated radiation. Each value is an AV ± SEM of 3 separate experiments.

* $P < 0.05$ and
** $P < 0.005$.

TABLE 3

IPA Analysis Showing Networks and Associated Functional Categories of miRNAs Differentially Expressed at 24 h in LNCaP, PC3, and DU145 Cells Treated with Single-Dose and Fractionated Radiation

Radiation protocol	Score	Focus molecules	Molecules	Top functions
LNCaP (SD-5 Gy)	19	8	miR-8, miR-15, miR-17, miR-19, miR-27, miR-28, miR-130, miR-193	Cell cycle, cellular growth and proliferation, cellular development
LNCaP (SD-10 Gy)	7	2	miR-15, miR-19	Cellular development, cellular growth and proliferation, cell cycle
LNCaP (MF-0.5 Gy × 10)	4	1	miR-154	Genetic disorder, skeletal and muscular disorders, inflammatory disease
LNCaP (MF-1 Gy × 10)	40	14	let-7 , miR-101, miR-8, miR-17, miR-19, miR-22 , miR-28 , miR-29, miR-30, miR-34 , miR-130, miR-193 , miR-221 , miR-19B	Reproductive system disease, genetic disorder, skeletal and muscular disorder
PC3 (SD-5 Gy)	4	1	miR-7	Cellular development, embryonic development, nervous system development and function
PC3 (SD-10 Gy)	4	1	miR-10	Cellular assembly and organization, cell death, skeletal and muscular system development and function
PC3 (MF-0.5 Gy × 10)	6	2	let-7 , miR-361	Cell death, liver necrosis/cell death, cell cycle
PC3 (MF-1 Gy × 10)	53	19	let-7 , miR-101 , miR-23 , miR-28 , miR-29 , miR-30 , miR-34 , miR-99 , miR-125 , miR-182 , miR-183 , miR-192 , miR-193 , miR-331 , miR-368 , miR-378 , miR-181B , miR-376A , miR-7	Reproductive system disease, genetic disorder, skeletal and muscular disorders
DU145 (SD-5 Gy)	NA	NA	NA	NA
DU145 (SD-10 Gy)	4	1	miR-342	Cell cycle, cell death, cell morphology
DU145 (MF-0.5 Gy × 10)	4	1	miR-342	Cell cycle, cell death, cell morphology
DU145 (MF-1 Gy × 10)	6	2	miR-146 , miR-193	DNA replication, recombination and repair, cell death, liver necrosis/cell death, inflammatory response, antigen presentation, cell-to-cell signaling and

Notes. Score refers to the statistical significance and focus molecules indicate the number of miRNAs that could be mapped to molecules out of a possible 35 molecules in each network. Names of the miRNAs in each functional category are given. Upregulated miRNAs are indicated as bold.

TABLE 4

Radiation-Induced Target Genes of Selected miRNAs in LNCaP and PC3 Cells

GenBank	Gene symbol	Fold change			Function	
		5 Gy	0.5 Gy × 10	1 Gy × 10		
miR-19a targets in LNCaP (mir-19a is down-regulated by single-dose and fractionated radiation)						
NM_020182	PMEPA1	1.47	2.00	1.60	2.10	androgen receptor signaling and apoptosis
H95228	AAK1	1.50	2.17	1.65	2.72	protein serine/threonine kinase activity
NM_052947	ALPK2	1.33	2.77	1.48	3.66	colony formation, transcription factor activity
NM_001147	ANGPT2	2.01	5.65	2.73	7.12	angiopoietin signaling, IL-8 signaling
BF510526	DHRS3	1.10	1.92	1.34	2.51	G ₁ /S-phase transition, double-stranded DNA break repair
NM_003633	ENC1	1.62	2.09	1.76	3.03	NRF2-mediated oxidative stress response
CF139514	FAM102A	1.26	1.93	1.58	2.24	actin cytoskeleton regulation
NM_022740	HIPK2	1.67	1.93	1.34	2.09	molecular mechanisms of cancer, p53 signaling
BC016384	MPRIIP	1.12	1.79	1.24	2.25	actin cytoskeleton signaling, Cdc42 signaling
AA664076	MXD1	1.25	1.76	1.37	2.50	polyamine regulation in colon cancer VDR/RXR activator
NM_033285	TP53INP1	1.88	1.88	1.92	2.37	p53 signaling
let-7 targets in LNCaP (let-7 is up-regulated by fractionated radiation)						
NM_004217	AURKB	0.09	0.70	0.08	0.29	proliferation, apoptosis, colony formation, adhesion
NM_207406	CCDC4	0.82	0.54	0.78	0.44	actin binding
NM_000393	COL5A2	1.42	0.59	1.30	0.49	protein binding
NM_004091	E2F2	0.10	0.91	0.08	0.42	G ₁ phase of mitotic cell cycle regulation and apoptotic process
NM_005497	GJA7	0.47	0.59	0.36	0.36	cell adhesion; cell-cell signaling
BQ020156	C6orf167	0.79	0.49	0.27	0.35	protein binding
NM_003038	SLC1A4	0.39	0.41	0.34	0.31	sugar binding; transferase activity, transferring glycosyl groups
let-7 targets in PC3 (let-7 is up-regulated by fractionated radiation)						
NM_004091	E2F2	NA	NA	0.49	0.43	G ₁ phase of mitotic cell cycle regulation and apoptotic process
miR-34a targets in LNCaP (miR-34a is up-regulated by fractionated radiation)						
NM_004702	CCNE2	0.21	0.20	0.80	0.48	regulation of cyclin-dependent protein kinase activity
AL_602715	PDCD4	1.27	0.56	0.89	0.49	negative regulation of cell cycle and apoptotic process

GenBank	Gene symbol	Fold change			Function
		5 Gy	0.5 Gy × 10	10 Gy	
miR-34a targets in PC3 (miR-34a is up-regulated by fractionated radiation)					
NM_004702	CCNE2	NA	NA	0.55	0.62
					regulation of cyclin-dependent protein kinase activity

Notes. Differentially expressed (> 1.5 -fold, $P < 0.05$) miRNAs from the present study and differentially expressed genes (> 2 -fold, $P < 0.05$) from our previously published study (John-Aryankalayil *et al.*, 2011) were analyzed using the Micro RNA Target Filter program as described in Materials and Methods. The table shows fold changes in the target genes that showed inverse correlation with miRNAs. A complete list of the target genes of all differentially expressed miRNAs is given in the Supplementary Tables 1-6 (<http://dx.doi.org/10.1667/RR2703.LS2-S7>). NA: mRNA microarray analysis was not carried out for PC3 cells for 5 Gy (single-dose) and 0.5 Gy × 10 (fractionated) radiation protocols.