Published in final edited form as:

Mol Cancer Res. 2014 July ; 12(7): 1002–1015. doi:10.1158/1541-7786.MCR-13-0623.

Differential Expression of Stress and Immune Response Pathway Transcripts and miRNAs in Normal Human Endothelial Cells Subjected to Fractionated or Single-Dose Radiation

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Abstract

While modern radiotherapy technologies can precisely deliver higher doses of radiation to tumors; thus, reducing overall radiation exposure to normal tissues, moderate dose and normal tissue toxicity still remains a significant limitation. The present study profiled the global effects on transcript and miR expression in Human Coronary Artery Endothelial Cells (HCAEC) using single-dose irradiation (SD, 10Gy) or multi-fractionated irradiation (MF, 2Gy × 5) regimens. Longitudinal timepoints were collected after a SD or final dose of MF irradiation for analysis using Agilent Human Gene Expression and miRNA microarray platforms. Compared to SD, the exposure to MF resulted in robust transcript and miR expression changes in terms of the number and magnitude. For data analysis, statistically significant mRNAs (2-fold) and miRs (1.5-fold) were processed by Ingenuity Pathway Analysis (IPA) to uncover miRs associated with target transcripts from several cellular pathways post-irradiation. Interestingly, MF radiation induced a cohort of mRNAs and miRs that coordinate the induction of immune response pathway under tight regulation. Additionally, mRNAs and miRs associated with DNA replication, recombination and repair, apoptosis, cardiovascular events and angiogenesis were revealed.

Keywords

Cell cycle checkpoints; Gene expression profiling; Immunomodulation; Molecular targets of radiation response; Normal tissue response to radiation; Cytokines in radiation response; Radiation-induced gene expression

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Disclosure of Potential Conflicts of Interest:
None

Introduction

Radiation oncology remains a mainstay of cancer therapy as both curative and palliative therapy used alone or as a component of combined modality therapy. Routinely, in clinical practice radiation therapy is administered as multiple fractions of 2–2.5 Gy per day for 5 days per week for 1–7 weeks to allow repair, repopulation and recovery of the collateral damage to the normal tissue (1–2). Individualized radiation therapy with development of modern techniques such as intensity modulated radiation therapy (IMRT) and image-guided radiation therapy (IGRT) can deliver more controlled single or fewer fractions of high dose radiation (hypofractionation) to tumor focusing on areas deemed at highest risk (3–5). The newer technology can reduce high doses to normal tissues but can increase the amount of tissue receiving daily dose (6). The incidental radiation exposure of normal tissues is a topic of concern in radiotherapy (7).

Recent work from our laboratory showed that prostate carcinoma cells that survive multi-fractionated radiation exposure have a different genomic signature compared to the cells exposed to single dose radiation (8–10). Exposure to 10 Gy radiation delivered as fractionated irradiation (1Gy $\times$ 10 or 2 Gy $\times$ 5) resulted in more robust differential gene expression changes in PC3 and DU145 cells whereas in LNCaP cells 10Gy radiation delivered as a single dose was more effective (9–10). These studies also revealed that the mRNA expression profiles following fractionated irradiation were influenced by p53 status. In LNCaP cells harboring wild-type p53 DNA replication/recombination/repair and cell cycle were the top gene ontology categories affected by radiation whereas in p53 mutated PC3 cells genes from interferon, immune and stress response categories were altered significantly. MicroRNAs (miRNAs) play an important role in regulation of gene expression at the post-transcriptional level by base-pairing with the complementary sequences within 3'-untranslated regions (3'-UTR) of target mRNAs, resulting in translational repression or mRNA degradation (11). As observed for the mRNA expression profiles, in the prostate carcinoma cells, treatment with fractionated irradiation significantly altered more miRNAs as compared to the cells exposed to single dose radiation (12).

Although normal tissue exposure remains a major concern in radiation therapy, few studies have investigated the molecular effects of various radiation treatment regimens in normal cells. The purpose of the present study was to investigate global gene and miRNA alterations in normal cells exposed to radiation protocols simulating hypofractionated and conventionally fractionated radiation regimens typically used for radiotherapy in clinic. For this study we treated normal human coronary artery endothelial cells (HCAEC) with 10 Gy radiation delivered as a single dose radiation (SD) or as 5 fractions of 2 Gy radiation (MF). The differentially expressed mRNAs and miRNAs were identified by microarray analysis at 6h and 24h after a SD and 6h and 24h after the final dose of fractionated irradiation. The data showed that in HCAEC more mRNAs and miRNAs were differentially expressed by exposure to MF compared to SD and the magnitude of changes was higher in MF irradiated cells. Gene ontology classification showed that in addition to cell cycle, genes regulating DNA replication, DNA damage stimulus and DNA repair, and genes related to immune response were significantly altered following exposure to MF. Using Ingenuity target filter program we identified miRNAs associated with the target genes from different cellular

pathways that were differentially expressed in response to SD and MF. The present study suggests that endothelial cells may play an important role in the outcome of radiotherapy in the clinical settings.

Materials and Methods

Cells

Cryopreserved human coronary artery endothelial cells (HCAEC) and the media were purchased from Lonza Walkersville Inc (Walkersville, MD). Cells were thawed and maintained in EBM-2 basal medium supplemented with fetal bovine serum and growth factors (EGM-2 MV BulletKit CC-3202) according to the supplier's instructions. Cells from passages P1 to P3 were used.

Radiation

Cells were irradiated in a PANTAK high frequency X-ray generator (Precision X-ray Inc., N. Bedford, CT), operated at 300kV and 10MA. The dose rate was 1.6 Gy per min. Cells were plated into T75cm² flasks ($1-1.5 \times 10^6$ for single dose radiation and $0.6-0.8 \times 10^6$ for fractionated radiation). After 24h, cells were exposed to a total of 10 Gy radiation administered either as a single dose radiation (SD), or as multi-fractionated radiation of 2 Gy $\times$ 5 (MF). These non-isoeffective doses were selected to simulate clinical hypofractionated and conventionally fractionated radiotherapy regimens. For the MF protocol cells were exposed to 2 Gy radiation twice a day, at 6h interval. The cells were approximately 90% confluent at the time of harvesting. For both protocols radiation-induced changes were analyzed at 6h and 24h after a SD and 6h and 24h after the final dose of fractionated irradiation. Separate controls were maintained for SD and MF radiation protocols.

RNA isolation

Cells were pelleted at 6h and 24h after a SD and 6h and 24h after the final dose of MF irradiation and stored in liquid nitrogen. Total RNA including small RNAs was isolated using phenol/chloroform extraction followed by purification over spin columns (Ambion Cat. No. AM9738). The concentration and purity of total RNA was measured by spectrophotometry at OD260/280 and the quality of the total RNA sample was assessed using an Agilent Bioanalyzer with the RNA6000 Nano Lab Chip (Agilent Technologies).

mRNA microarray analysis

The mRNA microarray analysis was performed using Agilent Technologies Human Gene Expression 4 $\times$ 44K V2 microarrays (product number G4845A, design ID 026652) designed to target 27,958 Entrez Gene RNAs.

miRNA microarray analysis

The miRNA microarray analysis was performed using Agilent Technologies Human miRNA 8 $\times$ 15K V2 microarrays (product number G4470B, design ID 019118) with probes for 723 human and 76 human viral miRNAs sourced from Sanger miRBase (release 10.1).

The mRNA and miRNA microarray data were analyzed using Gene Spring Software (Agilent Technologies) as described previously (12). To ensure that mRNAs and miRNAs were reliably measured, ANOVA was used to compare the means of each condition ($n=3$). For mRNA analysis, cutoff ratios of gene expression greater than 2.0 and less than 0.5 and a P value < 0.05 relative to the respective control group were selected. For miRNA analysis, cutoff ratios greater than 1.5 with a P value < 0.05 relative to the respective control were selected.

The mRNA and miRNA microarray data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series Accession No. GSE57059 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE57059>), and accession No. GSE56824 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE56824>), respectively.

Real-time Reverse-transcription Polymerase Chain Reaction

Separate experiments were set up to extract RNA at 6h and 24h after a SD and 6h and 24h after the final dose of fractionated irradiation for real-time RT-PCR analysis. RNA was isolated using RNeasy mini kit (Cat. No 74104, Qiagen, US) as described previously (12). Purified RNA was reverse transcribed to cDNA and RT-PCR was carried out as described previously (13). Alterations in selected differentially expressed genes were confirmed using Taq-Man Custom Express Plate (Part # 4391524, Applied Biosystem, Foster City, CA) and ABI PRISM 7500 Sequence Detection System instrument equipped with the SDS version 1.4 software. Each plate was designed to contain 18S and PES1 endogenous controls and 22 individual Taqman Gene Expression Assays in quadruplets in specified well locations (see Supplemental data for assay IDs and expanded method).

Ingenuity Pathway Analysis (IPA)

The functional significance of differentially expressed mRNAs (2-fold change and $P < 0.05$) following SD and MF irradiation was evaluated using Ingenuity Pathway analysis (IPA) software (Ingenuity Systems Version 8.7–3203, Redwood City, CA) as described previously (12–13). Data sets were uploaded into the IPA, which were next mapped to the functional networks available in the Ingenuity Pathway Knowledge Base and ranked by score as described previously (12).

miRNA -Target Filter Analysis

To identify the target mRNAs associated with differentially expressed miRNAs, data set of differentially expressed miRNAs (1.5-fold change and $P < 0.05$) and differentially expressed mRNAs (2-fold change and $P < 0.05$) were uploaded into IPA “MicroRNA Target Filter” program. For the data analysis, only the experimentally verified and highly predicted targets from IPA database were selected.

Cell cycle Analysis

Cells were fixed in 70% ethanol 6h and 24h after a SD and 6h and 24h after the final dose of MF. Data were collected and analyzed as described previously (13).

Western Blotting

Cell extracts were prepared 6h and 24h after a SD and 6h and 24h after the final dose of MF. Proteins were separated and protein bands were captured by digital CCD camera (Fuji, LAS 3000) as described previously (13). The membranes were stripped and reprobed for actin. Signal intensities were quantified using Image J 1.44p software (National Institutes of Health, Bethesda, MD), normalized to the loading control actin, and expressed as fold change compared to the unirradiated control.

Antibodies

p53 (Sc-6243), p21 (Sc-756), MDM2 (Sc-5304), RAD51 (Sc-8349) and STAT-1 (Sc-346) (Santa Cruz biotechnology, Inc), cyclin D2 (3741) and caspase 1 (3866) (Cell Signaling technologies), and actin (MAB1501R) (Millipore, USA).

Data analysis

Each data point represents average $\pm$ SEM of 3 experiments. Differences between the groups were statistically evaluated by two-tailed paired *t* test. A *P* value of <0.05 was considered statistically significant.

Results

Surviving Fractions of HCAEC following SD and MF Irradiation

The surviving fraction (SF) of HCAEC exposed to 2 Gy $\times$ 5 fractionated irradiation (MF) was 0.003 ± 0.0002 and for those exposed to 10 Gy single dose irradiation (SD) was 0.00008 ± 0.00002 . This is in keeping with the selected SD regimen having a higher biologically effective dose than the MF regimen.

Gene Expression Analyses in HCAEC following SD and MF Irradiation

Global Gene Expression Changes—Of the total 27,958 genes represented in the Agilent microarrays, treatment with 10 Gy SD and 2Gy $\times$ 5 MF resulted in differential expression (>2 fold, $P < 0.05$) of combined 2,255 genes in HCAEC cells. The Venn diagrams and the heat map in figure 1 show that the MF exposure resulted in more robust gene expression changes compared to the SD radiation (Figure 1). Of the total 2,255 genes altered, 89 genes were differentially expressed in response to SD, 1873 genes were differentially expressed in response to MF; and 293 genes were commonly differentially expressed following SD and MF treatment (Figure 1A & B). In cells irradiated with SD more genes were differentially expressed at 24h compared to 6h time point (Figure 1 C). Significant gene expression changes were evident at 6h in cells irradiated with MF, and the changes persisted up to 24h (Figure 1C).

Enrichment of Genes by Gene Ontology Classification—The 2,255 differentially expressed genes were classified in to functional categories by gene ontology classification (Supplementary Table 1). The enrichment factor of the cell cycle-regulatory genes was the highest. The other significantly altered categories were stress response, DNA replication, response to DNA damage, DNA repair, immune response, apoptosis, p53 and inflammatory

response. The number of genes altered and the magnitude of change in these categories was much higher after exposure to MF than SD. Genes from cytokines, inflammatory response and growth factor activity categories that could influence other cells and tissues were significantly altered only by MF.

Ingenuity Pathway Analysis (IPA)—Functions associated with top 10 networks (score>10) of genes differentially expressed by SD and MF are shown in Table 1. At 6h following SD only 4 networks with score more than 10 were generated, with cell cycle and cell death as the top functions. At 24h time point after SD irradiation there were 14 networks with scores more than 10. In addition to cell cycle the other top functions included DNA replication, recombination and repair. At 6h and 24h time point after the final dose of MF irradiation there were >20 networks with score >10. The main functional category in cells treated with MF at 6h and 24h time point included DNA replication, recombination and repair. RNA post-transcriptional modification was another significant category observed at both time points following MF (Table 1).

Cell Cycle Analysis—Since cell cycle was the top most category affected by radiation, cell cycle distribution in the HCAEC treated with single and fractionated radiation was examined. Figure 2 shows the distribution of cells in G1, S and G2 compartments after treatment with 10 Gy SD (A) and 2 Gy $\times$ 5 MF (B). Exposure to SD resulted in reduction in cells in G1 at 6h compared to the untreated cells, which persisted at 24h. There was an increase in the percentage of cells in G2 at 24h. Exposure to MF resulted in reduction in the percentage of cells in S phase at 24h.

Heat maps—Radiation-induced changes in individual genes from selected functional categories were color coded to demonstrate the expression patterns of individual genes within a category for each radiation treatment regimen. Figure 3A shows heat map of genes from stress response category, which includes DNA damage stimulus and DNA repair gene subsets and other stress response genes. The majority of genes from DNA damage stimulus and DNA repair subsets were downregulated in response to SD and MF. However, the magnitude of downregulation was much higher with MF. Many of the downregulated genes following SD did not pass the cutoff (<2 fold, $P > 0.05$) because the ratio of fold change although statistically significant, was less than 2 fold. A complete list of genes from stress response category with fold changes is given in the supplementary data (Supplementary Table 2). Some of the downregulated genes involved in homologous recombination from DNA repair category included *H2AFX* (*H2AX*), *BRCA1*, *BRCA2*, *BARD1*, *RPA1*, *RAD51*, *RAD51AP*, *RAD54B*, *RAD54L*, and *BLM*. Other downregulated genes from DNA repair category were DNA polymerases *POLA1*, *POLD2*, *POLD3*, *POLE2*, and *POLQ*, DNA primase *PRIM1*, replication factor *RFC5*, ribonucleotide reductases *RRM1*, *RRM2* and *DCK*. The downregulated genes related to p53 included *RFWD3*, *GTSE1*, *BLM*, *BRCA1*, *BRCA2*, *HSPD1* and *MTBP*; the upregulated genes related to p53 were *PML*, *MDM2*, *C16orf5*, *CDKN1A* (p21), *ATM*, *TP53INP1* and *TP53INP2*.

Figure 3B shows heat map of genes from immune response category, which includes inflammatory genes subset. In the immune response category the majority of genes were upregulated following fractionated irradiation. These included adhesion molecules

ICAM1 and *VCAM1*, chemokines *CXCL10*, *CXCL11*, *CXCL12*, *CXCL16*, *CCL2*, *CCL5*, *CCL20* and *CCL23*, cytokines *IFNE*, *IFNA4*, *IL1A*, *IL1B*, *IL15*, *TGFB1* and *TGFB2*, receptors for chemokines *CXCR4* and *CXCR7* and cytokines *FAS*, interferon induced proteins and transcription factors, and molecules in integrin signaling pathways *ITGA4*, *ITGB3* and *ITGAV*. Genes regulating HLA-A, B, C, F, G, and J major histocompatibility complex class I molecules were upregulated following fractionated irradiation. From HLA class II, *HLA DPA1* and *DPB1* were downregulated and *HLA DQB1* was upregulated. A complete list of genes from immune response category with fold changes is given in the supplementary data (Supplementary Table 3).

miRNA analyses in HCAEC following SD and MF Irradiation

Global miRNA changes in HCAEC following SD and MF—The miRNA microarray analysis revealed that 123 miRNAs were differentially expressed with high confidence (> 1.5 fold, $P < 0.05$) in the irradiated cells and the majority of them were upregulated (Figure 4, A, B and C). Exposure to SD resulted in the differential expression of 17 miRNAs whereas exposure to MF altered 101 miRNAs. Five miRNAs were commonly expressed after SD and MF irradiation. At 6h time point more miRNAs were differentially expressed in cells exposed to MF compared to the 24h time point, and the majority of them were upregulated (Figure 4C). These included the members of tumor suppressor let-7 family (let-7a, let-7e and let-7f). Tumor suppressor miR-34a was common for SD and MF and was upregulated at 24h after SD, and 6h and 24h after MF. The members of the oncomir miR-17-92 cluster (miR-17, miR-18a, miR-18b, miR-19a, miR-19b, miR-20a and miR-92a) were all downregulated after MF at 24h. The miRNAs associated with cardiovascular functions (miR-195, miR-21, miR-221, miR-222, miR-27b, miR-29b, all upregulated), hypoxia response (miR-210, miR-424, upregulated) and senescence (downregulated: miR-15a, miR-20a, upregulated: miR-410 and miR-431) were also differentially expressed in cells exposed to MF.

mRNA Targets of the miRNAs differentially expressed in the irradiated HCAEC

—IPA miRNA-mRNA target filter program was used to identify the target mRNAs associated with the differentially expressed miRNAs in HCAEC treated with SD and MF radiation. Only the highly predicted or experimentally verified targets were included for the target analysis. The differentially expressed miRNAs and their target mRNAs showed inverse correlation (altered in opposite direction) as well as direct correlation (both altered in same direction).

Table 2A shows the differentially expressed miRNAs after SD at 6h and 24h and the number of mRNA targets of these miRNAs observed in the present data. At 6h time point 3 miRNAs were upregulated and 5 mRNAs were downregulated showing inverse correlation between the miRNAs and mRNAs. At the same time point 5 upregulated miRNAs showed direct correlation with 8 mRNAs that were also upregulated. At 24h time point 5 differentially expressed miRNAs showed inverse correlation with 13 differentially expressed mRNAs. At the same time point a total of 8 differentially expressed miRNAs showed direct correlation with 16 differentially expressed mRNAs. Exposure to MF altered more number of miRNAs and mRNAs compared to SD, especially at 6h time point (Table 2B). At 6h after the final

dose of MF a total of 48 miRNAs were differentially expressed (47 up and 1 down) and showed inverse correlation with 617 target mRNAs (596 down and 21 up). Upregulated 44 miRNAs also showed direct correlation with 997 upregulated mRNAs at this time point. At 24h after the final dose of MF treatment 10 miRNAs were upregulated and 10 were downregulated. They showed inverse and direct correlation with 264 and 270 mRNAs respectively.

Pathway analyses—Table 3A shows the differentially expressed mRNAs from ATM, p53 signaling and cell cycle checkpoint pathways, and the inversely regulatory miRNAs associated with these mRNA targets, identified by IPA target filter analysis. Most of the genes from ATM signaling pathway were downregulated in cells treated with MF (Table 3A). Activation of H2AFX (H2AX) immediately after DNA double strand break results in recruitment of specific DNA repair proteins in ATM signaling pathway. *H2AX* was downregulated at 6h after the final dose of MF (MF 6h) and its regulatory miRNA miR-24 was upregulated. Several other genes including *CDC25A*, *FANCD2*, *SMC1A*, *SMC2*, *BRCA1*, *CHEK1*, *CDK1* and *CCNB1* were downregulated after MF exposure. The miRNAs showing inverse correlation with these target genes are shown in the table (Table 3A). *CDC25A* was downregulated after both SD and MF and its regulatory miRNA miR-34a was upregulated in SD 24h, MF 6h and MF 24h. However, at MF 6h in addition to miR-34a several other miRNAs regulating *CDC25A* were also upregulated. At 6h after MF *BRCA1* showed inverse correlation with miR-146a as well as miR-24; but only with mir-146a at 24h time point. In addition to *BRCA1*, miR-24 inversely correlated also with *E2F2* and *CDK1*. MiR-17–92 cluster was downregulated at 24h after MF irradiation. At this time point p53 regulated targets *CCND2*, *CDKN1A* (p21) and *SERPINE2* were upregulated showing inverse correlation with members of the miR-17–92 cluster and miR-16. Other upregulated gene in p53 pathway was *FAS* and it showed inverse correlation with miR-1275. MiR-23a/b was upregulated and showed inverse correlation with *TOPBP1*.

The mRNA targets associated with cell cycle checkpoints and miRNAs inversely correlated with these targets are shown in Table 3A. *E2F2* was commonly downregulated in all radiation treatment groups and showed inverse correlation with miR-326 after SD 6h, SD 24h and MF 6h. In addition to miR-326 several other miRNAs (let-7a/f, miR-24, miR-222/221, miR-365, and miR-495) also showed inverse correlation with *E2F2* in MF at 6h. However, 24h after fractionated irradiation these miRNAs were no longer differentially expressed and *E2F2* showed inverse correlation with miR-31. *CCND2* was upregulated at 6h and 24h in cells treated with fractionated radiation. However, at 6h time point all the miRNAs associated with *CCND2* were also upregulated (not shown). At 24h point several miRNAs from miR-17–92 cluster and miR-16 showed inverse correlation with *CCND2*. *CCNE2* was downregulated and showed inverse correlation with miR-34. Several other miRNAs (miR-30b, miR-374a/b and miR-495) also showed inverse correlation with *CCNE2* at 6h after MF. MiR-195 inversely correlated with targets *CDC25B*, *CHEK1* and *PLK1*. Other miRNAs and their inverse targets associated with cell cycle checkpoints were *PKMYT1*/miR-27b/a, *TOP2A*/miR-410, *CKS1B*/miR-361-5p and *CCNB1*/miR-379.

The table 3A also demonstrates that at some time points, although target genes were differentially expressed, no regulatory miRNA were identified. For example *PLK1* was

downregulated in SD 6, SD 24, MF 6 and MF 24. However, miR-195 which showed inverse correlation with *PLK1* was differentially expressed only in MF 6.

Immune response pathway—Table 3B shows the differentially expressed genes from immune response category that showed inverse correlations with the differentially expressed miRNAs in the present microarray data, by target filter analysis. At 24h following SD miR-7 showed inverse correlation with *RELB*. Some of the immune response genes and the inversely correlated miRNAs in cells exposed to fractionated radiation were chemokines *CXCL10* / miR-16, *CXCL12* / miR-19b/a; cytokine *TNFSF9* / miR-16 and cytokine receptors *TNFRSF9* / miR-1275, *TNFRSF1B* / miR-338-3p, and genes associated with integrin signaling *ITGB3* / miR-19b/a, *ITGA4* / miR-20a/miR-17-5p, and *ITGAV* / mir-92a. Expression of interferon regulatory transcription factor *IRF9* was inversely correlated with miR-20. Other prominent upregulated immune response genes and the miRNAs inversely correlating with them were *FAS* / miR-1275, *STAT2* / miR-19b/a and *PTGS2* / miR-16. *IL1RAP*, associated with synthesis of acute phase and proinflammatory proteins was down regulated and showed inverse correlation with upregulated mir-31 and miR-146a.

Cardiovascular pathway—Radiation-induced differentially expressed miRNAs associated with cardiovascular diseases and angiogenesis in HCAEC are given in the supplementary data. MiRNAs and genes associated with cardiac hypertrophy, hypoxia signaling, atherosclerosis signaling, and β adrenergic signaling in cardiovascular pathway are shown in the Supplementary Table 4. Many of the miRNAs differentially expressed in HCAEC treated with fractionated irradiation have been implicated in cardiovascular events and angiogenesis and are shown in the Supplementary Table 5.

Conformation of mRNA microarray data—Selected differentially expressed stress and immune genes from the microarray data were analyzed by real-time RT-PCR. The RT-PCR data substantially confirmed the microarray data (Supplementary Table 6).

The expression of selected differentially expressed genes, *P53*, *CDKN1A*, *MDM2*, *RAD51*, *Cyclin D2*, *CASP1* and *STAT1* at protein level was confirmed by western blot analysis. P53 and P53-regulated P21, MDM2 proteins were upregulated in response to SD and MF while RAD51 was downregulated only after MF. CYCLIN D2, CASPASE 1 and STAT-1 were upregulated after MF (Supplementary Figure 1).

Discussion

Current radiation therapy techniques expose both normal tissue and tumors to a wide range of dose size and fractionation, with a substantial amount of normal tissue potentially being irradiated (6). This study was undertaken to understand the effect of SD and MF radiation on endothelial cells using clinical relevant schedules to complement our recently reported tumor data(6, 8–10, 12). The radiation protocols for the present study were selected to simulate hypofractionated (10 Gy SD) and conventionally fractionated (2 Gy $\times$ 5 MF) regimens typically used for radiotherapy in the clinic. The microarray data showed that exposure to MF resulted in more robust changes in gene and miRNA expressions in terms of number and magnitude, compared to the SD. The MF radiation induced a cohort of mRNAs

and miRNAs associated with stress response, immune response, cell cycle, apoptosis, fibrosis, cardiovascular events and angiogenesis. Using the ingenuity pathway target filter program we identified the miRNAs that showed inverse and direct correlations with the differentially expressed target genes in response to single and fractionated irradiation.

Cell cycle and DNA replication/DNA damage stimulus/repair were the top gene ontology categories altered in the irradiated cells and the effect was more pronounced in cells exposed to MF. Ionizing radiation-induced DNA damage results in activation of various DNA repair pathways and cell cycle checkpoints resulting in a temporary arrest in cell cycle progression to allow cells to repair damaged DNA. If the damage is too severe cells are eliminated by apoptosis (14). BRCA1, ATM and P53 play key roles in DNA damage response (15–17). The two major pathways of DNA damage repair are homologous recombination (HR) and non-homologous end joining (NHEJ) (18). BRCA1 regulates DNA repair by promoting HR in concert with BRCA2 and RAD51, and also inhibits NHEJ to restrict the extent of deletion at the break site (17). The present gene expression analysis revealed that in addition to *BRCA1*, both *BRCA2* and *RAD51* from HR pathway were downregulated in cells exposed to MF. Treatment with single and fractionated radiation resulted in the upregulation of *ATM*, and several p53-regulated genes including *MDM2*, which in turn controls p53 activity, and *CDKN1A*, which regulates cell cycle checkpoints (19–20). Cdkn1a, cyclin-dependent kinase (cdk) inhibitor (p21), inhibits cyclin E-cdk2, cyclin D-cdk4 and cyclin A-cdk2 complexes (21). The p53-independent check points following ionizing radiation operating at the G2M transition are mediated by the ATM-Chk1-cdc25C-cyclin B/cdc2 pathway (21). In the present study many cell cycle regulatory genes including those encoding cyclins *CCNB1*, *CCNE2*; kinases *CDK1* (*CDC2*), *CDK2*; and phosphatases *CDC25A*, *CDC25B* and *CDC25C* were downregulated. While p53-regulated DNA repair genes *DDB2*, *GADD45a*, *DDIT4* and *TRIM22* were upregulated, the majority of DNA repair genes including those encoding DNA polymerases, primases, and replication factors were downregulated in the irradiated HCAEC.

MiRNAs play an important role in controlling the regulation of DNA damage response (14). The present study identified several miRNAs associated with target genes from ATM and P53 signaling pathways and cell cycle checkpoints in the irradiated HCAEC. Although a few microRNAs were differentially regulated in HCAEC by SD such as miR-34a, miR-136, miR-140-3p, miR-326, miR-338-3p and miR-874, these miRNAs have been implicated to coordinate the induction of cell death by apoptosis under various stresses (22–26). MiR-874 has been shown to induce G2/M arrest and cell apoptosis by targeting HDAC1 (25). In agreement with these findings exposure to SD resulted in a reduction in the percentage of HCAEC in G1 and an increase in cells in G2. The surviving fraction of cells exposed to the higher biologically effective dose regimen SD, was much lower than the surviving fraction of cells treated with MF. The data suggest that the majority of cells accumulated in G2 block following SD exposure did not recover and were eliminated. On the contrary, exposure to MF resulted in differential expression of pro-apoptotic as well as anti-apoptotic miRNAs indicating that the final outcome would depend on the cumulative effect of these opposing miRNAs. For instance, while miR17–92 cluster and miR-7 which are shown to be inhibitors of apoptosis (27) were reduced in HCAEC by MF, miR-15 and miR16a which induce

apoptosis by targeting BCL-2 (28) were downregulated and miR-21 which is implicated in suppression of apoptosis (29) was upregulated suggesting that miRNAs coordinate apoptotic pathway in the irradiated HCAEC. Importantly, the upregulation of FAS by SD and MF may also contribute to apoptosis in the irradiated cells.

The irradiated endothelial normal cells showed significant downregulation of DNA repair genes. Similar results were observed in the irradiated LNCaP prostate cancer cells harboring wild-type p53 (10). This is in contrast to the response of p53 mutated PC3 prostate cancer cells to radiation treatment observed in our earlier study (9). Also, no significant change in DNA repair genes was observed in the irradiated MCF-7, SF539 and DU145 tumor cells although MCF7 and SF539 cells express wild type p53 (8). These findings indicate that the expression of DNA repair genes in response to radiation exposure in tumor cells is not strictly dependent on the p53 status. In fact p53-independent pathways for repair such as P21-PCNA have been reported (30).

Radiation-induced vascular damage is considered to be related to the inflammatory changes in the microvasculature (31). Preclinical in vitro and in vivo studies have demonstrated that ionizing radiation triggers pro-immunogenic and inflammatory changes in the tumor cells/tumor microenvironment making tumors more susceptible to immunotherapy (32–36). The ability of radiation to promote the anti-tumor immunity has been a subject of great interest and preclinical studies have reported that the outcome depends on the radiation dose and fractionation protocols employed (1, 5). Moreover, in 2 mouse tumor models the combination of fractionated radiotherapy and anti-CTLA-4 antibody to one tumor site induced systemic tumor control as observed by a complete regression in a second palpable tumor outside the radiation field (abscopal effect) (37). The molecular changes in the irradiated tumor cells that contribute to immunogenic cell death include degradation of proteins, release of “danger signals” calreticulin and high mobility group protein B1 (HMGB1), and ATP which promote priming of antitumor T cells by dendritic cells (33, 36). Radiation induced upregulation of chemokines enhances immune cell trafficking to attract activated T cells to the irradiated tumor site (35). The cancer cells that survive the radiation insult display enhanced expression of adhesion molecules ICAM-1, death receptor Fas, and major histocompatibility complex class 1 (MHC-1) antigen-presenting molecules, resulting in an improved recognition and killing by anti-tumor T-cells (38). Interestingly, the microarray analysis revealed that several genes from immune response category were differentially expressed in the irradiated HCAEC. The majority of genes in this category were upregulated and the gene expression was more robust in cells exposed to MF. The immune response genes differentially expressed in the irradiated HCAEC included genes regulating adhesion molecules, chemokines and cytokines, receptors for chemokines and HLA MHC class I and II antigens. There is increasing evidence that miRNAs function as an effective system to regulate the magnitude of inflammatory responses (39). Accordingly, many miRNAs which activate and dampen the immune response are altered in HCAEC exposed to SD or MF indicating that this process is very tightly regulated. For instance, at 24h after fractionated radiation exposure the miRNAs from miR-17–92 cluster were downregulated and showed inverse correlation with several of the immune response genes upregulated at this time point. MiR-146 is considered to be a key regulator in innate as well as adaptive immune responses. Although miR-146a was upregulated by SD and MF in

HCAEC, the target filter analysis revealed inverse correlation between miR-146a and only 2 differentially expressed immune response genes. However, *CCL5*, *CXCR4*, *DDX58*, *IL1F10*, *IRAK2*, *LTB*, *MR1* and *STAT1* showed direct correlation with miR-146a. The target filter analysis identified several other miRNAs showing inverse correlations with immune response genes emphasizing the role of miRNAs in immune response and inflammation. These data suggest that the irradiated endothelial cells may contribute to radiation-induced immune response during radiation therapy.

The activation of growth factor, cytokine and chemokine cascades in response to the radiation-induced vascular injury also contributes to the radiation-induced fibrosis of normal tissue (40). As mentioned above several genes regulating cytokines and chemokines were upregulated in the irradiated endothelial cells. Among all radiation-induced cytokines, TGF- β activation is of particular relevance, as it elicits strong and long lasting microenvironmental changes (7, 41). TGF- β plays a central role in fibrosis by stimulating production of new matrix proteins such as fibronectin, collagens and proteoglycans (7, 42). The present gene expression analysis showed an increase in *TGFB* as well as *COL1A2* and *FBN1* at 6h and 24h after MF. The upregulation of *COL1A2* inversely correlated with the downregulation of miR-7 and miR-92a at 24h time point. *FBN1* showed inverse correlation with miR-1275 and miR-92a. Previous studies showed that upregulation of collagens and fibrillin 1 in the regions adjacent to infarct during remodeling after myocardial infarction is regulated by downregulation of miR-29 (43). Post-infarct cardiac fibrosis on the other hand was inhibited by forced expression of miR-101 (44). Interestingly, miR-29 blocks fibrosis by inhibiting the expression of ECM components, whereas miR-21 promotes fibrosis in SMCs after vascular injury by stimulating mitogen-activated protein kinase (MAPK) signaling (45). In the irradiated HCAEC, miR-29, miR-21 and miR-101 were upregulated, while miR-7 and 92a were downregulated. These observations indicate that induction of fibrosis is also a balance between the actions of these miRNAs, in agreement with the mechanism of action of miRNAs acting as rheostats to fine tune and modulate the outcome based on the intensity of damage (11).

As seen with the HCAEC, the miRNA-based gene-regulatory system provides a flexible and conditional option that would be particularly useful when mRNA expression must be fine-tuned to different levels in different cell types. The post-transcriptional dampening of gene expression by miRNAs not only offers both a mechanism for more uniform gene expression for cells of a particular type and a simple means to customize this expression level for each distinct cell type, but also offers a mechanism to rapidly respond to stress situations. The cohort of miRNAs influenced by different regimens of radiation indicates this. While miRNAs expressed in response to SD coordinate the induction of immune response factors and apoptosis, the miRNAs in MF fine-tune several processes such as DNA-repair, fibrosis, angiogenesis in addition to immune response and apoptosis.

Our previous studies have shown that the tumor cells that survive MF have substantially different phenotype than the untreated cells or the cells treated with SD and present a unique opportunity to exploit the radiation-induced changes to improve cancer therapy (9–10), with the underlying theme of radiation as “focused biology” (46). The gene expression profile of endothelial cells in response to fractionated radiation resembles to that seen in p53 wild-type

(cell cycle, DNA replication/repair) as well as p53 mutated (immune response) tumor cells observed in our previous studies (9–10). Several studies have demonstrated a potential role for radiation as an immunological adjuvant (1, 5, 32–33, 36, 38). The present study suggests that endothelial cells may contribute to systemic changes during radiotherapy recognizing that modern radiation therapy techniques can be used to target tumors and reduce normal tissue hot spots, but at the same time more normal tissue, and thus endothelial cells, receive some dose.

The importance of the tumor microenvironment is greatly emphasized in cancer therapy. Tumors are complex structures with the stroma and infiltrating cells impacting tumor survival and progression, and the acquired ability for epithelial-mesenchymal transition (47). Radiotherapy significantly alters tumor microenvironment. Certainly, normal tissue effects of radiation depend on changes to parenchyma cells that are organ-specific and also to endothelial cells that are ubiquitous. Recent studies indicate that tumor-derived endothelial cells differ from normal endothelial cells at both functional and molecular levels, and endothelial cells derived from different tumors are shown to be divergent dependent on the origin of the tumor(48). While these differences remain to be better understood and exploited, dissecting out the contributions of the various tissue components to radiation response is necessary to best understand the aggregate picture. The present data warrant further investigations on radiation response in both normal as well as tumor-derived endothelial cells.

Improving the therapeutic ratio is critical to effective clinical radiotherapy and treatment in general. In our current focus on understanding and targeting the cells that survive MF (6), the changes in endothelial cells would be of strategic importance. Furthermore, it may be possible to use changes induced by the endothelial cells including factors found in the blood to understand how the tumor is responding and use this information for immunotherapy or other molecularly targeted treatments. While much remains to be done, having normal tissue data facilitate developing better and improved therapeutic approaches.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the intramural research program of the NIH, National Cancer Institute, Center for Cancer Research. We wish to thank Dr. T. Adilakshmi for critical reading of the manuscript and editorial help. We are grateful to Dr. Charles B. Simone II (Department of Radiation Oncology, Hospital of the University of Pennsylvania, Philadelphia, PA) for his expert advice for application of clinical radiotherapy regimens in the laboratory settings.

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Implications

Radiation-induced alterations in stress and immune response genes in endothelial cells contribute to changes in normal tissue and tumor microenvironment and impact the outcome of radiotherapy.

Figure 1.

Venn diagrams (A, B) depict the numbers of differentially expressed genes (>2 fold change, $P < 0.05$) in HCAEC exposed to 10 Gy single dose (SD) and 2 Gy $\times$ 5 fractionated (MF) radiation. (A) Genes upregulated and (B) Genes downregulated by SD and MF irradiation, and (C) Heat map of differentially expressed genes at 6h and 24h after a SD and 6h and 24h after the final dose of MF irradiation. Yellow to red indicates upregulated and blue indicates downregulated genes.

Figure 2. Cell cycle perturbations in HCAEC exposed to (A) SD (10 Gy) and (B) MF (2 Gy $\times$ 5) radiation at 6h and 24h a SD and 6h and 24h after the final dose of MF irradiation. * $P < 0.05$.

Figure 3. Heat maps depicting differentially expressed genes from (A) stress response (includes DNA damage stimulus, DNA repair and other stress response genes), and (B) immune response (includes inflammatory subset) categories following SD and MF irradiation in HCAEC.

Figure 4.

Venn diagrams (A, B) depict the numbers of differentially expressed miRNAs (>1.5 fold change, $P < 0.05$) in HCAEC exposed to 10 Gy single dose (SD) and 2 Gy $\times$ 5 fractionated (MF) radiation. (A) miRNAs upregulated by SD and MF, (B) miRNAs downregulated by SD and MF irradiation and (C) Heat map of differentially expressed miRNAs at 6h and 24h after a SD and 6h and 24h after the final dose of MF irradiation.

Table 1

Functions Associated with Networks of Genes Differentially Expressed by SD and MF Irradiation. Ingenuity Pathway Analysis (IPA) of differentially expressed genes in HCAEC treated with 10 Gy single (SD) and 2 Gy $\times$ 5 fractionated (MF) irradiation at 6h and 24h after a SD and 6h and 24h after the final dose of MF irradiation. The network ID, score, number of focus molecules (in bracket) and the functions associated with top 10 networks with score >10 are shown.

Functions Associated with Top 10 Networks of Genes Differentially Expressed in Irradiated HCAEC

Radiation	ID	Score	Top Functions
SD-6h	1	66	Cellular Development, Hematopoiesis, Cell Death (27)
	2	31	Cell Cycle , Cancer, Cell Death (16)
	3	20	Cell Cycle , Molecular Transport, Protein Synthesis (11)
	4	20	Cellular Function and Maintenance, Cell Cycle , Connective Tissue Development and Function (11)
SD-24h	1	53	Cellular Assembly and Organization, DNA Replication, Recombination, and Repair , Cell Cycle (29)
	2	40	DNA Replication, Recombination, and Repair , Cell Cycle , Cancer (24)
	3	34	Cell Death, Free Radical Scavenging, Lipid Metabolism (21)
	4	33	Free Radical Scavenging, Drug Metabolism, Endocrine System Development and Function (21)
	5	30	Cell Morphology, Cancer, Reproductive System Disease (20)
	6	30	Cellular Movement, Cell Morphology, Cell-To-Cell Signaling and Interaction (20)
	7	28	Cellular Assembly and Organization, Cellular Compromise, Cell Morphology (21)
	8	25	Cellular Development, Cellular Growth and Proliferation, Cell Cycle (17)
	9	24	Cellular Compromise, Cancer, Hematological Disease (17)
	10	23	Cellular Development, Hematopoiesis, Cell Death (18)
MF-6h	1	45	Cell Cycle , Cellular Assembly and Organization, DNA Replication, Recombination, and Repair (34)
	2	40	Cellular Assembly and Organization, DNA Replication, Recombination, and Repair , Amino Acid Metabolism (32)
	3	38	Infectious Disease, DNA Replication, Recombination, and Repair , Gene Expression (31)
	4	35	Cell Cycle , Genetic Disorder, Ophthalmic Disease (30)
	5	35	Cell Cycle , Cellular Assembly and Organization, Cellular Function and Maintenance (30)
	6	35	Small Molecule Biochemistry, Lipid Metabolism, Molecular Transport (30)
	7	33	DNA Replication, Recombination, and Repair , Cell Cycle , Cellular Assembly and Organization (29)
	8	32	Cellular Assembly and Organization, Cellular Function and Maintenance, DNA Replication, Recombination, and Repair (30)
	9	31	Cellular Assembly and Organization, DNA Replication, Recombination, and Repair , Cell Cycle (28)
	10	31	Cell Cycle , Cell Morphology, Cellular Function and Maintenance (30)
MF-24h	1	50	Cellular Growth and Proliferation, Cancer, Gastrointestinal Disease (34)
	2	47	Cellular Assembly and Organization, DNA Replication, Recombination, and Repair , Cell Cycle (33)
	3	40	Cellular Assembly and Organization, DNA Replication, Recombination, and Repair , Cancer (30)
	4	38	Cell Cycle , Cellular Assembly and Organization, DNA Replication, Recombination, and Repair (29)
	5	36	Infectious Disease, Dermatological Diseases and Conditions, Genetic Disorder (28)

Radiation	ID	Score	Top Functions
	6	36	Cell Cycle , Cellular Assembly and Organization, DNA Replication, Recombination, and Repair (28)
	7	34	Cancer, Genetic Disorder, Ophthalmic Disease (27)
	8	34	RNA Post-Transcriptional Modification, Gene Expression , Genetic Disorder (27)
	9	30	DNA Replication, Recombination, and Repair, Cell Cycle , Cell Death (27)
	10	30	DNA Replication, Recombination, and Repair, Cell Cycle , Cellular Development (25)

Table 2

Differentially Expressed miRNAs and the Number of mRNA Targets Showing Inverse and Direct Correlations with Each miRNA. The differentially expressed mRNA targets of the differentially expressed miRNAs were identified using IPA miRNA/mRNA target filter analysis program. The table gives the number of mRNAs showing inverse and direct correlations with the miRNA differentially expressed at 6h and 24h after 10 Gy single dose (SD) (Table 2A) and 2 Gy $\times$ 5 fractionated (MF) (Table 2B) irradiation.

Number of mRNA Targets Showing Inverse and Direct Correlations with Differentially Expressed miRNAs at 6h and 24h Following

A. SD Irradiation									
SD-6h				SD-24h					
Inverse correlation		Direct correlation		Inverse correlation		Direct correlation			
miRNA	Targets	miRNA	Targets	miRNA	Targets	miRNA	Targets	miRNA	Targets
miR-326	1	miR-329	1	miR-1225-5p	1	miR-1225-5p	1		
miR-329	1	miR-484	1	miR-136	2	miR-136	1		
miR-32	3	miR-532-5p	2	miR-326	2	miR-140-3p	1		
3 up	5 down	miR-543	2	miR-34a	3	miR-326	5		
		miR-32	2	miR-7	5	miR-338-3p	3		
		5 up	8 up	4 up	8 down	miR-34a	2		
				1 down	5 up	miR-874	1		
						miR-7	2		
						7 up	14 up		
						1 down	2 down		
B. MF Irradiation									
MF-6h				MF-24h					
Inverse correlation		Direct correlation		Inverse correlation		Direct correlation			
miRNA	Targets	miRNA	Targets	miRNA	Targets	miRNA	Targets	miRNA	Targets
let-7a/f	28	let-7a/f	54	miR-137	22	miR-137	25		
miR-101	10	miR-101	41	miR-140-3p	2	miR-181a	26		
miR-103	12	miR-103	21	miR-146a	19	miR-140-3p	4		

miR-136	12	miR-136	17	miR-154	7	miR-146a	13
miR-137	20	miR-137	41	miR-181a	17	miR-154	5
miR-140-5p	6	miR-140-5p	14	miR-23b	37	miR-23a/b	28
miR-146a	15	miR-146a	26	miR-31	12	miR-31	9
miR-195	41	miR-195	41	miR-338-3p	6	miR-338-3p	14
miR-181a	21	miR-181a	51	miR-34a	11	miR-34a	13
miR-185	7	miR-185	25	miR-1275	12	miR-1275	4
miR-193a-5p	4	miR-193a-5p	3	miR-16	28	miR-16	31
miR-21	12	miR-21	22	miR-18a/b	11	miR-18a/b	9
miR-22	10	miR-210	1	miR-19a/b	27	miR-19a/b	30
miR-222/221	8	miR-22	14	miR-17/20a	28	miR-17/20a	33
miR-23a/b	40	miR-222/221	17	miR-7	9	miR-7	12
miR-24	14	miR-23a/b	51	miR-92a	16	miR-92a	14
miR-26a/b	20	miR-24	19				
miR-27b/a	30	miR-26a/b	38	10 up	133 down	10 up	137 up
miR-299-5p	3	miR-27b/a	54	10 down	131 up	10 down	133 down
miR-29b	18	miR-299-5p	3	Upregulated miRNAs and mRNAs are shown in Bold			
miR-30b	27	miR-29b	49				
miR-326	11	miR-30b	58				
miR-329	10	miR-326	23				
miR-338-3p	8	miR-329	21				
miR-342-3p	8	miR-338-3p	18				
miR-410	32	miR-342-3p	9				
miR-361-5p	8	miR-410	41				
miR-365	8	miR-361-5p	14				
miR-374a/b	31	miR-365	14				
miR-376c	5	miR-374a/b	46				
miR-377	18	miR-376c	26				
miR-379	1	miR-377	27				
miR-409-3p	7	miR-379	6				
miR-409-5p	2	miR-409-3p	12				

miR-431	2	miR-431	10				
miR-34a	11	miR-34a	30				
miR-487b	2	miR-487b	2				
miR-495	42	miR-495	38				
miR-543	25	44 up	997 up				
miR-654-3p	4						
miR-28-5p	3						
miR-7	21						
47 up 1 down	596 down 21 up						

Table 3

A Target Filter Analysis of Target Genes and miRNAs from ATM and P53 Signalling Pathways and Cell Cycle Check Points Showing Inverse Correlation after Single and Fractionated Irradiation

Pathways and check points	miRNA Target	SD 6h miRNA	SD 24h miRNA	MF 6h miRNA	MF 24h miRNA
ATM	<i>H2AFX</i> ↓	-	NC	miR-24↑	-
ATM, G1/S	<i>CDC25A</i> ↓	-	miR-34a↑	miR-365↑, miR-34a↑, let-7a/f↑, miR-195↑	miR-34a↑
ATM	<i>FANCD2</i> ↓	-	-	miR-21↑, let-7a/f↑, miR-23a/b↑	miR-23b↑
ATM	<i>SMC1A</i> ↓	-	-	let-7a/f↑, miR-137↑, miR-342-3p↑	miR-137↑
ATM	<i>SMC2</i> ↓	-	-	miR-410↑	NC
ATM, P53, G2M	<i>BRCA1</i> ↓	-	-	miR-146a↑, miR-24↑	miR-146a↑
ATM, P53, G2M	<i>CHEK1</i> ↓	-	-	miR-195↑	NC
ATM, G2M	<i>CCNB1</i> ↓	NC	-	miR-379↑	NC
ATM, P53, G1S	<i>CDKN1A</i> ↑	NC	-	NC	miR-20↓, miR-17↓
P53, G1/S	<i>E2F1</i> ↓	-	-	miR-136↑, miR-21↑	NC
P53	<i>TOPBP1</i> ↓	NC	NC	miR-23a/b↑	miR-23b↑
P53, G1/S	<i>CCND2</i> ↑	NC	NC	NC	miR-16 ↓, miR-17 ↓, miR-18a/b ↓, miR-19b/a ↓, miR-20a/b ↓
P53	<i>SERPINE2</i> ↑	-	-	NC	miR-16 ↓
P53	<i>FAS</i> ↑	NC	NC	NC	miR-1275 ↓
G1/S	<i>E2F2</i> ↓	miR-326↑	miR-326↑	let-7a/f↑, miR-24↑, miR-326↑	miR-31↑
G1/S	<i>CCNE2</i> ↓	-	miR-34a↑	miR-222/221↑, miR-365↑, miR-495↑	miR-34a↑
G2M	<i>CDC25B</i> ↓	-	-	miR-34a↑, miR-30b↑, miR-374a/b↑, miR-495↑	NC
G2M	<i>PLK1</i> ↓	NC	NC	miR-195↑	NC
ATM, G2M	<i>CDK1</i> ↓	-	-	miR-195↑	NC
G2M	<i>PKMYT1</i> ↓	-	-	miR-24↑, miR-410↑	NC
G2M	<i>TOP2A</i> ↓	-	-	miR-27a/b↑	NC
G2M	<i>CKS1B</i> ↓	-	-	miR-410↑	NC
		-	-	miR-361↑	NC

Target Filter Analysis of Target Genes and miRNAs from Immune response pathway Showing Inverse Correlation after Single and Fractionated Irradiation

SD-24h		MF-6h		MF-24h	
Targets	miRNA	Targets	miRNA	Targets	miRNA
<i>CCNE2</i> ↓	miR-34a↑	<i>CCNA2</i> ↓	mir-146a↑, miR-24↑, miR-410 ↑	<i>COL1A2</i> ↑	miR-7↓, miR-92a↓
<i>RELB</i> ↑	miR-7↓	<i>CCNE2</i> ↓	miR-30b↑, miR-374a↑, miR-34a↑, miR-495↑	<i>CCNA2</i> ↓	miR-146a↑
		<i>COL1A2</i> ↑	miR-7↓	<i>CCND2</i> ↑	miR-20a↓, miR-18a/b↓, miR19b/a↓, miR-16↓
		<i>HMGB1</i> ↓	miR-410 ↑, mir-495 ↑	<i>CCNE2</i> ↓	miR-34a↑
		<i>HMGB2</i> ↓	miR-23a/b ↑	<i>CXCL10</i> ↑	miR-16↓
		<i>LMNB1</i> ↓	miR-23a/b ↑	<i>CXCL12</i> ↑	miR-19b/a↓
		<i>LMNB2</i> ↓	miR-24↑, miR-30b ↑	<i>DUSP10</i> ↑	miR-20a↓, miR-92a↓
		<i>PAK1</i> ↓	let7a/f ↑, miR-221↑	<i>FAS</i> ↑	miR-1275↓
		<i>PPP1CC</i> ↓	miR-140-5p↑, miR-27b↑	<i>HMGB2</i> ↓	miR-23b↑
		<i>RELB</i> ↑	miR-7↓	<i>IFIT2</i> ↑	miR-92a↓
		<i>TNFRSF1B</i> ↓	let7a/f ↑, miR-22↑, miR-338-3p↑, miR-495↑	<i>IGF1</i> ↑	miR-18a/b↓, miR-1275↓, miR19b/a↓, miR-16↓
		<i>UNG</i> ↓	miR-195↑, miR-495↑	<i>IL1RAP</i> ↓	miR-31↑, miR-146a↑
				<i>IRF9</i> ↑↑	miR-20a↓
				<i>ITGA4</i> ↑	miR-20a↓
				<i>ITGAV</i> ↑	miR-92a↓
				<i>ITGB3</i> ↑	miR19-b/a↓
				<i>LMNB1</i> ↓	miR-23b↑
				<i>MMP2</i> ↑	miR-20a↓
				<i>PARP1</i> ↓	miR-31↑
				<i>PTGS2</i> ↑	miR-16↓
				<i>RAG1</i> ↑	miR-92a↓
				<i>RUNX1</i> ↑	miR-20a↓, miR-18a/b↓
				<i>STAT2</i> ↑	miR19-b/a↓
				<i>TGFα</i> ↑	miR-7↓
				<i>TIFA</i> ↓	miR-181a↑
				<i>TNFRSF1B</i> ↓	miR-338-3p↑
				<i>TNFRSF9</i> ↑	miR-1275↓
				<i>TNFSF9</i> ↑	miR-16↓

NC- Only the target mRNA was differentially expressed, no corresponding differentially expressed miRNA identified

Neither the target mRNA nor any corresponding miRNA were differentially expressed in that protocol

Upregulated target genes and miRNAs are shown in bold