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Radiation Survivors: Understanding and exploiting the phenotype following fractionated radiation therapy

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Abstract

Radiation oncology modalities such as intensity-modulated and image-guided radiation therapy can reduce the high dose to normal tissue and deliver a heterogeneous dose to tumors focusing on areas deemed at highest risk for tumor persistence. Clinical radiation oncology produces daily doses ranging from 1 to 20 Gy, with tissues being exposed to 30 or more daily fractions. Hypothesizing that cells that survive fractionated radiation therapy have a substantially different phenotype than the untreated cells, which might be exploitable for targeting with molecular therapeutics or immunotherapy, three prostate cancer cell lines (PC3, DU145 and LNCaP) and normal endothelial cells were studied to understand the biology of differential effects of multi-fraction (MF) radiation of 0.5, 1 and/or 2 Gy fraction to 10 Gy total dose, and a single dose (SD) of 5 and 10 Gy. The resulting changes in mRNA, miRNA and phosphoproteome were analyzed. Significant differences were observed in the MF radiation exposures including those from the 0.5 Gy MF that produces little cell killing. As expected, p53 function played a major role in response. Pathways modified by MF include immune response, DNA damage, cell cycle arrest, TGF- β , survival and apoptotic signal transduction. The radiation-induced stress response will set-forth a unique platform for exploiting the effects of radiation therapy as “focused biology” for cancer treatment in conjunction with molecular targeted or immunologically directed therapy. Given that more normal tissue is treated, albeit to lower doses with these newer techniques, the response of the normal tissue may also influence long-term treatment outcome.

Introduction

The biological consequences of radiation exposure are of interest for cancer etiology and treatment. The potential negative consequences of ionizing radiation have been recently highlighted through reports of treatment errors in radiation therapy (RT) (1), risk from CT scans (2) and radionuclide release from the Fukushima Nuclear Power Plant disaster (3–4). On the positive side of the ledger, RT is a mainstay of both curative and palliative cancer treatment used either alone or as a component in adjuvant therapy. Benefits include improved local tumor control (such as in head & neck, breast and lung cancers) survival (such as in breast and brain tumors) and organ preservation (such as in breast, head & neck, gastrointestinal and bladder cancers).

Radiation is administered in multiple fractions usually five days per week for a total of one to seven weeks. As discussed in this review, the tumor and normal tissue cells that survive multi-fraction radiation have a different genetic and proteomic profiles in comparison to the non-irradiated cells at the start of treatment, and the resulting pattern is dependent on the size of the individual radiation dose per fraction. While more studies are needed, it may be possible to take advantage of the new patterns of gene expression and proteomic profiles to select a dose and radiation schedule to increase the efficacy of molecular targeted drugs and immunotherapy, a concept of “focused biology”(5). The impact of the multi-fraction lower dose on the increased volume of irradiated normal tissue remains to be elucidated.

Based on our group's interest in understanding the cellular response to radiation-induced stress, we put forward and tested a hypothesis that the molecular response of cells to single-dose (SD) radiation would be different from the response to multi-fractionated (MF) radiation; and that the cells that survived MF would become more alike by virtue of their adaptation to radiation (6). This review comprises of analyses of the findings from our laboratory (7–9) and published reports by others groups (6, 10–14) on effects of SD and MF on cellular response. There are several translational goals that need to be considered which include:

- Understanding the molecular characteristics of tumor cells that survive fractionated radiation and the impact of genetic background impact on MF-induced changes.
- What the impact of the size of the dose per fraction, (including low doses that might occur at the tumor periphery and in the surrounding normal tissue) is.
- Since molecular-targeted drugs require the presence and persistence of a target, can MF be used to induce such targets rather than depend on its presence and, therefore, make molecular-targeted therapy more effective and broadly applicable?
- Is there a preferred “omics” approach or combination of methods (mRNA, miRNA or phosphoproteomics) with which to assess these changes?

Dose-heterogeneity in clinical radiation therapy

In the clinic, MF radiation is used due to its normal tissue sparing effects (15) with the maximum radiation dose delivered based on normal tissue tolerance from clinical experience (16). The less complicated RT modalities including opposed pairs, 4-field configuration and, to some extent, 3-D conformal RT use a limited number of fields with a relatively uniform energy deposition across the beam and a homogeneous dose within the tumor. In contrast, new technological approaches such as Intensity-Modulated Radiation Therapy (IMRT) (17), Image-Guided Radiation Therapy (IGRT) (17) and heavy ion therapy (protons and carbon) (18) are designed to reduce the high doses delivered to normal tissues. IMRT and IGRT utilize dose painting and sculpting to deliver radiation within the target so that dose is often not homogenous within a tumor. A specified target may receive a higher dose based on local tumor burden, the presence of a physiological area at risk for local failure (such as hypoxia) or persistence of an imaging abnormality (such as PET) (19). IMRT and IGRT use multiple and often non-coplanar intensity-modulated beams such that there is more normal tissue treated to some moderate dose. Thus, there is more tissue treated with increased dose heterogeneity. Recently, the advances in diagnostic imaging, the ability to stay on target during radiation treatment by accounting for patient and tumor intra-fraction motion, and an interest in shortening treatment time for biological (prostate cancer) and/or patient convenience (breast cancer) have also led to the use of larger doses-per-fraction (i.e. hypofractionation). Thus, in current clinical radiation therapy, tissues are exposed to MF radiation in a range of doses for one to seven weeks.

Figure 1 provides an example of dose heterogeneity in clinical radiation oncology. The IMRT plan is for a pharyngeal tumor, which by design covers the tumor well, but also has significant dose distribution to the nearby normal tissue with the majority of the tissue behind the vertebral region receiving >100 cGy/day as a result of multiple field configurations. The daily treatment dose is heterogeneous including a maximum dose of almost 230 cGy/day with much of the tissue receiving as much as 150 cGy/day, and other regions receiving between 50–150 cGy/day.

Molecular phenotype depends on fractionation, fraction size and underlying genetic profile

In RT, the multiplicity of doses to which patients are exposed may engage different biological processes and molecular pathways. The daily dose ranges include high doses from ablative hypo-fractionation that are >10 Gy (often close to 15–20 Gy) (20–21), moderate dose of 1–10 Gy (22), low doses including treatments designed to take advantage of low-dose hypersensitivity ~0.5 Gy (23). [Note: There are lower doses (~10 cGy) that elicit low dose “adaptive” responses such as the non-targeted/bystander effects in unirradiated cells (24) discussed below]. Differences in the effects of fractionated and single-dose radiation including those from our laboratory have been previously documented, (6–7, 10–12) and include the induction of stress-response (7, 12) and immune-related (6–7, 10–11) genes by fractionated radiation.

In studies comparing MF and SD from our laboratory, Tsai et al. demonstrated significant differences in the gene expression profiles following MF and SD, with MF uniquely up-regulating interferon-related genes and TGF- β associated genes in a prostate cancer xenograft model and *in vitro* in prostate, breast and gliosarcoma cells lines (6). Our analyses of the global gene profiles following radiation (MF and SD) show a more robust MF-induced gene expression in HCAEC (in preparation), LNCaP (9) and PC3 (7) in comparison to SD. The observed gene expression patterns have shown MF radiation to be more immunomodulatory in comparison to SD in LNCaP (9) PC3 (7) and normal HCAEC (Human Coronary Artery Endothelial Cells) (in preparation) but not in DU145 cells (7). Figure 2 serves as a representative illustration, highlighting the differences between single and fractionated radiation by depicting differences in expression of immune response gene using SD 10 Gy and MF 1Gy \times 10. It also illustrates that there is an inflection point after 6–7 fractions in MF. In comparison to SD, the majority of gene and miRNA changes were also more robust and stable up to 72h after the end of MF (7), suggesting a sufficiently stable phenotype that might affect tissue/tumor response and also potentially be targetable with drug therapy (7).

Dewan et al. demonstrated an MF-induced immune-mediated abscopal response with anti-CTLA-4 antibody, in breast and colon cancer xenograft experimental models. The same abscopal response was not observed with SD; in comparison to SD, MF dramatically improved the local and secondary tumor control (10). More recently, following MF, Postow et al. observed immune activation and elevated antibody levels against a NY-ESO-1 (a cancer antigen expressed in 30–40% of melanomas) in a melanoma patient treated with anti-CTLA-4 antibody, ipilimumab (14). Clinical trials combining fractionated radiation and ipilimumab are currently underway in stage IV melanomas (ClinicalTrials.gov number, NCT01449279) (25) and advanced prostate cancer (ClinicalTrials.gov number, NCT00861614).

As much as radiation dose, quality, dose rate and fractionation (10) all play an important role in cellular response to radiation, observed differences in response to SD and MF radiation regimens are also dependent on the underlying genetic profiles. Following

radiation (MF and SD), our studies have shown 2255 differential expressed genes in HCAEC (in preparation), 978 in LNCaP (9), 343 in PC3 and 116 in DU145 (7). The genes most altered by both radiation protocols was also different between the three prostate cancer cell lines (7, 9). A list of top 10 upregulated and 10 downregulated genes in the three prostate cancer cell lines are provided as supplementary information. As previously stated, MF altered more genes than SD in HCAEC (normal endothelial cell), LNCaP (*p53*-wildtype) and PC3 (*p53*-null). In contrast, SD altered more genes in DU145 (*p53*-mutant). *p53*, a vital tumor suppressor, is associated with activation of DNA repair, apoptosis and radiosensitivity. The diverse *p53* status of these cells likely plays a central role in their radiation response and could provide a partial explanation as to why different pathways were top-ranked in the functional gene assessments. A more detailed study of the effect of the underlying genotype on the phenotypic change following MF and SD is planned.

In addition to cell cycle, DNA repair, and cell death pathways stress response, signal transduction (6–7, 11), metabolic, respiratory (26–27), immune response(7–8, 10–11) pathways, have been reported to be altered by radiation (SD and MF) in various *in vitro* and *in vivo* systems. A list of representative examples of pathways altered by radiation (SD and MF) are listed in table 1; many of which have also been implicated in low dose radiation-induced bystander effects (6–8, 26–27). Klammer et al recently reported a low-dose radiation-induced adaptive phenotype as a bystander effect in mouse fibroblasts (28). An adaptive response is a phenomenon observed after a low dose radiation (<10 cGy) primes cells for a response to a higher radiation dose, such that the cells are more resistant following a priming dose than without it. The genomic alterations resulting from an adaptive response, such as mutations, formation of micronuclei and chromosome aberrations, are often transmitted to the viable progeny or “Radiation Survivors” (24, 28–29). Contributing factors to the maintenance of an adaptive phenotype across generations from both irradiated and non-irradiated bystander cells), include the genetic background of the cells (28) and epigenetic drivers such as histone modifications, DNA methylation and miRNA signaling (29). In the case of fractionated radiation, it is possible that exposure to repeated doses of sublethal radiation could induce an adaptive phenotype different from the classic adaptive response of the “low priming dose +higher dose”. Our data (6) using 1 Gy fractions demonstrate that for some genes there is an inflection point after 6–7 fractions. The MF-induced signal persistence in irradiated cells (7, 30) could be as a result of an “MF-adaptive” response. It is speculative that epigenetic changes from MF could be transmitted across generations; which could also induce a bystander response in the neighboring non-irradiated cells, causing them to send out stress signals and in turn, generate an extended abscopal effect in other tissue and organs.

The role of miRNAs in (radiation and cytotoxic) stress response is becoming increasingly clear (31–32). They are key regulators of gene expression by post-transcriptional interference of mRNAs and function as oncogenes (miR-17–92 cluster, miR21, miR106b-93–25 cluster, miR-155, miR221, miR-222), tumor suppressor (let-7, miR-15–16 cluster, miR-34, miR203) and epigenetic modulators. Mounting evidence associating miRNAs and epigenetics show some miRNAs to be epigenetically regulated (let-7, miR-34 family, miR-124, miR -137, miR -148, miR-203 and miR-200 family) (33–34), while some have been reported to repress epigenetic drivers by directly targeting key genes such as DNA methyl transferases, HDACs and histones(miR-26, miR-29, miR-101, miR-148, mir-203, miR-205, miR-214and miR-449) (30, 33–34).

Their dysregulation has been implicated in multiple cancers; the radiation-induced differential expression of miRNA is also well documented (31, 35–36). Mueller and colleagues report that a family of miRNAs (miR-99 family) is upregulated in response to radiation (2Gy x2) and reduces the efficiency of DNA repair in two *p53*+ breast (MCF7) and

prostate (LNCaP) cancer cell lines. They further showed decreased DNA repair efficiency with mir-99 expression following MF (13). miR-99 expression has also been reported to be decreased in advanced and more radioresistant cancers (13, 37). This finding supports earlier reports from our group; we demonstrate increased expression of select miRNA after radiation exposure, especially MF in LNCaP and PC3(8). Further, we showed increased miR-99 expression following MF in PC3 but not in LNCaP. In contrast, let-7, which is also typically underexpressed in cancers and associated with poor prognosis in patients (38), was upregulated after MF in LNCaP and PC3 (8). let-7, a tumor suppressor miRNA, regulates cellular proliferation and expression of oncogenes such as RAS/c-MYC and HMGA-2; its over-expression has been reported to inhibit tumor development and growth (38–40).

miRNAs are crucial to the maintenance of radiation-induced response and although, miRNA profiling is a promising diagnostic tool, as expression levels of specific miR have been associated with major cancer outcomes, it should be noted that the baseline miRNA expression levels in different cancers are not always predictive of response (8, 13) so that post-exposure analysis will be informative in addition to baseline assessment. Further studies are necessary to assess the role of epigenetics and p53 function in radiation-induced miRNA response and to understand the how miRNAs contribute to the maintenance of MF-induced adaptation, as cells pass on their “survivor” phenotype to subsequent generations.

Tumor Heterogeneity

Inter- and intra-tumoral heterogeneity is a feature of carcinogenesis and tumor progression including malignant transformation, metastasis, immune evasion, and treatment resistance. Gerliger *et al.* recently mapped the heterogeneity in four renal cancer patient samples and the associated metastases using exome-sequencing. They report multiple genetic and functional mutations within a single tumor for mTOR (41) and its downstream targets. Recent reports have also highlighted the heterogeneity within different tumors (42–44). This has implications for biomarkers, diagnostics, therapeutic efficacy and relatively specific molecular-targeted therapies. (41–44). We are interested in exploring the effect of radiation on tumor heterogeneity, particularly how the adaptation to MF could be exploited to therapeutic advantage.

The new perspective for radiation oncology

The novel observation from these studies is that changes in the molecular characteristics of the surviving tumor cells and normal tissues after MF may affect treatment outcome and could potentially be exploited. IMRT and IGRT techniques create a heterogeneous dose distribution within the tumor; hypofractionation uses large doses in either a single or a limited number of fractions. While physical techniques allow for two beneficial outcomes—increasing the dose to regions of interest within the tumor volume and decreasing the maximal normal tissue dose near the tumor, more tissue is exposed to some radiation, much of it receiving approximately half the daily tumor dose and some much lower. The normal tissue and stromal cells that receive repeated lower doses do respond and the impact of this on the tumor microenvironment remains to be elucidated. Our data show that there is a different molecular profile between tumor cell type and differences are dependent on radiation dose per fraction.

Mathematical models based on clinical experience are useful for treatment selection in the clinic (15, 38, 45) but these do not describe biological processes that are changed. Our studies have shown that cells that survive and remain clonogenic after MF are phenotypically different from the starting cells. Furthermore, cells may be lethally irradiated in terms of clonogenic potential yet survive and could influence outcome, such as late effects (46–47). Proteomics data being analyzed indicate that a specific molecular target

(such as NF- κ B and PARP) may go in opposite directions following SD and MF. Ongoing and future plans include *in vivo* studies, a broader range of tissue types and genetic background, effect of hypoxia and utilization of MF as part of combined modality treatment and immunotherapy (10).

How does this fit with the newer models for cancer treatment?

New models of molecular-targeted therapy targets both oncogene addiction (OA) and non-oncogene addiction (NOA) (48); NOA could be potentially be produced using a specified course of MF radiation (7). Synthetic lethality takes advantage of the cell's susceptibility due to a defective pathway (49–50) and it might be possible to induce cell susceptibility using MF to replace the therapeutic dependence on mutated pathway, thereby broadening the potential for synthetically lethal drugs. The extensive heterogeneity within tumors (41–44) presents a challenge as to what targets can be effectively exploited, so that the cellular adaptation to radiation could generate a target and/or reduce heterogeneity. As noted above, baseline miRNA expression between various cell types is not always predictive of magnitude of response (8, 13) so that sampling a tumor after being challenged by radiation may provide a new approach to molecular targeting. We recognize that there is much to be done; however, there may be very innovative ways of utilizing the ability to target radiation to generate changes that can improve treatment efficacy. It is a new paradigm of using radiation “pharmacokinetics and pharmacodynamics”.

In summary, understanding and utilizing the radiation stress response and the adaptation to MF radiation for both tumors and normal tissues coupled with the advances in imaging and technological delivery of radiation provides potential for a unique targeting situation for combined modality therapy and immunotherapy, an approach we call “focused biology” (5).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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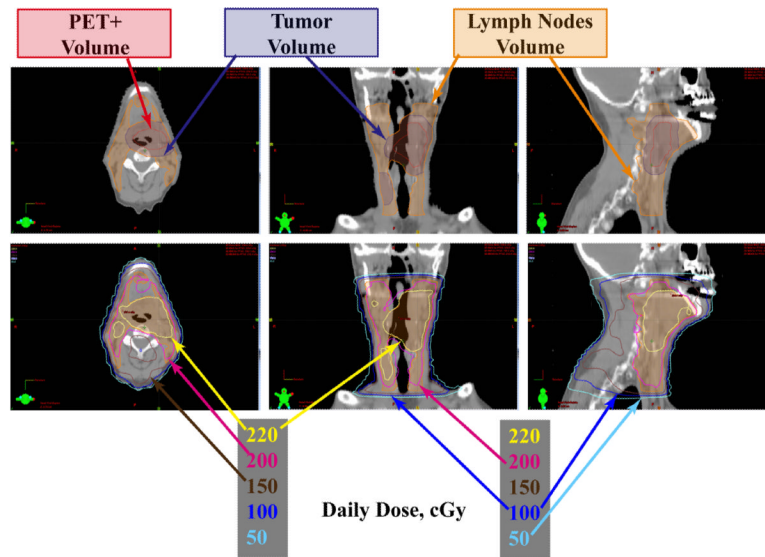


Figure 1. Dose heterogeneity during IMRT radiation therapy

Dose distribution from an IMRT treatment plan for a pharyngeal tumor. Top panel: the target includes the PET scan positive region (red outline) that is within the tumor volume (blue outline), and the lymph nodes (orange outline), which receive a lower total dose. Lower panel: the daily tumor dose is 200 cGy (pink outline) with some areas within the tumor receiving higher focal doses (220 cGy, yellow outline). The multiple field approach treats normal tissue to daily doses to 100–150 cGy per day (dark blue and brown lines respectively) and some to even lower doses per fraction of 50 cGy (light blue outline).

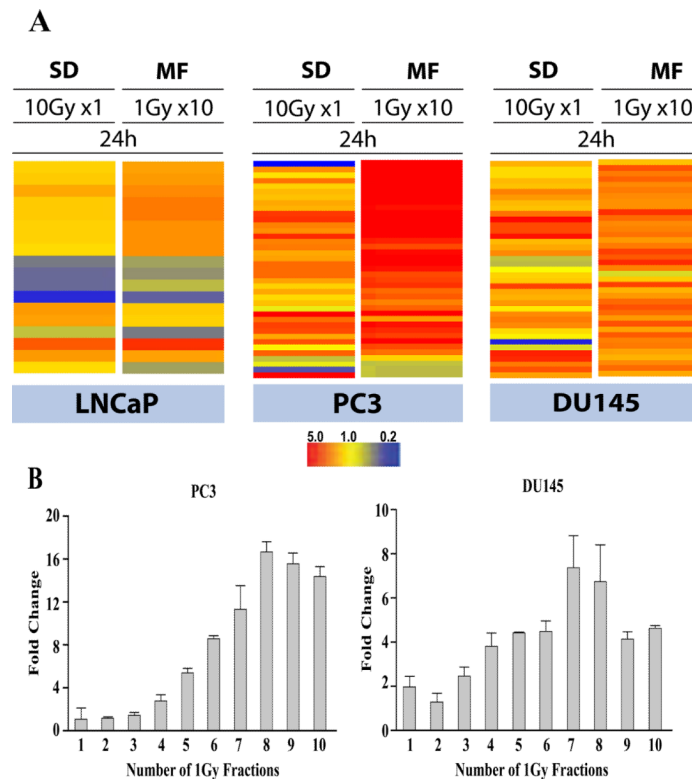


Figure 2. Adaptation takes time

A Heat maps depicting the expression patterns of immune response genes 24h following exposure to 10 Gy as single dose (SD) or 10 fractions of 1 Gy (MF) in LNCaP, PC3 and DU145. **B.** Inflection point. Samples were collected 24h after the indicated number of 1Gy radiation fractions in PC3 and DU145. For MF, there was at least a 6h window between each fraction; samples were collected 24h after final fraction. Data represents fold change in *IFI44* expression determined by RT-PCR. Data were adapted in part from (7).

Table 1

Differences in gene profiles between single dose and multi-fraction radiation. Table summarizes pathway analysis of previously reported gene, following exposure to SD and MF radiation.

Cell type	Radiation Protocol	Functional Categories	Type of Analysis	Ref
MCF-7, MCF-IR20	MF 2Gy×20	Growth regulator, Apoptosis, DNA repair, DNA replication, Cell adhesion, Angiogenesis, regulator, Cell division, Growth factor, Cytokine	<i>in vitro</i> , mRNA	Li, et al. (2001)
MCF-7	MF 2Gy ×5	Cell division, Apoptosis, GTPase regulator, Growth factor	<i>in vitro</i> , mRNA	Madhusoo dhanan, et al. (2009)
MCF-7	SD 10Gy×1	Apoptosis, Cell senescence, Cell Cycle control, DNA damage repair Angiogenesis, Signal transduction and transcription factors		
MCF-7	MF 2Gy ×5	Angiogenesis,		
SF539	MF 2Gy ×5	Apoptosis, Cell senescence, Cell Cycle control, DNA damage repair Angiogenesis, Signal transduction and transcription factors		
DUI145	MF 2Gy ×5	Cancer, Infectious Disease, Respiratory Disease		
MCF-7, SF539, DUI145	SD 10Gy×1	Cell-To-Cell Signaling and Interaction, DNA Replication, Recombination, and Repair, Nucleic Acid Metabolism	<i>in vitro</i> , mRNA	Tsai, et al. (2007)
DUI145	MF 1Gy ×5	Cancer, Infectious Disease, Respiratory Disease, Cellular Development, Cellular Growth and Proliferation, Connective Tissue Development and Function		
DUI145	SD 10Gy×1	Cancer, Infectious Disease, Respiratory Disease, Molecular Transport, Hematological Disease, Hereditary Disorder		
DUI145	MF 1Gy ×5	Cell-To-Cell Signaling and Interaction, Drug Metabolism, Lipid Metabolism	<i>in vitro</i> , mRNA	
DUI145	SD 10Gy×1	Organismal Survival, Cancer, Cell Death, Molecular Transport, Drug Metabolism, Small Molecule Biochemistry, Inflammatory Response, Cellular Development, Cellular Growth and Proliferation, Cancer, Reproductive System Disease, Endocrine System Disorders		
DUI145	SD 10Gy×1	Organismal Survival, Cancer, Cell Death, Molecular Transport, Drug Metabolism, Small Molecule Biochemistry, Inflammatory Response, Cellular Development, Cellular Growth and Proliferation, Cancer, Reproductive System Disease, Endocrine System Disorders		
DUI145	SD 10Gy×1	Cell Cycle, Gastrointestinal Disease, Hepatic System Disease, Hematological System Development and Function, Hematopoiesis, Tissue Development		
LNCaP	MF 1Gy ×10	Cell Cycle, DNA Binding, Proliferation, Response to Stress, Transcription Factor, Signal Transduction, DNA Replication, Histone, Cyclin, Immune Response	<i>in vitro</i> , mRNA	Simone, et al. (in review)
LNCaP	SD 10Gy ×1	Cell Cycle, DNA Binding, DNA Replication, Response to Stress, DNA Repair, Proliferation, Transcription Factor, Signal Transduction, Cyclin, Histone		
PC3	MF 1Gy ×10	Immune Response, DNA Binding, Response to Stress, Interferon, Signal Transduction, Proliferation, Apoptosis, Transcription Factor, Ubiquitin, Inflammatory Response	<i>in vitro</i> , mRNA	John-Aryankala yil, et al. (2010)
PC3	SD 10Gy ×1	DNA Binding, Signal Transduction, Immune Response, Cell Cycle, Response to Stress, Proliferation, Histone, Ubiquitin, Transcription Factor, Interferon		

Cell type	Radiation Protocol	Functional Categories	Type of Analysis	Ref
DUI45	MF 1Gy ×10	DNA Binding, Response to Stress, Apoptosis, Proliferation, Signal Transduction, Immune Response, Interferon, DNA Damage	<i>in vitro</i> , mRNA	
	SD 10Gy ×1	DNA Binding, Response to Stress, Immune Response, Cell Cycle, Transcription Factor, Proliferation, Protease, Histone, Interferon, Cyclin		

Table 2

Differences in miRNA Profiles between single dose and multi-fraction radiation. Table summarizes pathway analysis of previously reported microRNAs, following exposure to SD and MF radiation.

Cell type	Radiation Protocol	Functional Categories	Type of Analysis	Ref		
LNCaP	MF	0.5Gy × 10	Genetic disorder, skeletal and muscular disorders, inflammatory disease	<i>in vitro</i> , miRNA	John-Aryankala yil, et al. (2012)	
		1Gy × 10	Reproductive system disease, genetic disorder, skeletal and muscular disorder			
	SD	5Gy × 1	Cell cycle cellular growth and proliferation cellular development			<i>in vitro</i> , miRNA
		10Gy × 1	Cellular development, cellular growth and proliferation, cell cycle			
PC3	MF	0.5Gy × 10	Cell death, liver necrosis/cell death, cell cycle	<i>in vitro</i> , miRNA		
		1Gy × 10	Reproductive system disease, genetic disorder, skeletal and muscular disorders			
	SD	5Gy × 1	Cellular development, embryonic development, nervous system development and function		<i>in vitro</i> , miRNA	
		10Gy × 1	Cellular assembly and organization, cell death, skeletal and muscular system development and function			
DU145	MF	0.5Gy × 10	Cell cycle, cell death, cell morphology	<i>in vitro</i> , miRNA		
		1Gy × 10	DNA replication, recombination and repair, cell death, liver necrosis/cell death, inflammatory response, antigen presentation, cell-to-cell signaling and interaction			
	SD	5Gy × 1	None		<i>in vitro</i> , miRNA	
		10Gy × 1	Cell cycle, cell death, cell morphology			