Gene and miRNA expression profiles of mouse Lewis lung carcinoma LLC1 cells following single or fractionated dose irradiation

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Abbreviations: RT, radiotherapy; IR, ionizing radiation; SD, single dose; FD, fractionated dose

Key words: fractionated irradiation, radiation response, genome-wide expression analysis, gene and microRNA expression, Lewis lung carcinoma, syngeneic tumor model

Abstract. In clinical practice ionizing radiation (IR) is primarily applied to cancer treatment in the form of fractionated dose (FD) irradiation. Despite this fact, a substantially higher amount of current knowledge in the field of radiobiology comes from in vitro studies based on the cellular response to single dose (SD) irradiation. In addition, intrinsic and acquired resistance to IR remains an issue in clinical practice, leading to radiotherapy treatment failure. Numerous previous studies suggest that an improved understanding of the molecular processes involved in the radiation-induced DNA damage response to FD irradiation could improve the effectiveness of radiotherapy. Therefore, the present study examined the differential expression of genes and microRNA (miRNA) in murine Lewis lung cancer (LLC)1 cells exposed to SD or FD irradiation. The results of the present study indicated that the gene and miRNA expression profiles of LLC1 cells exposed to irradiation were dose delivery type-dependent. Data analysis also revealed that mRNAs may be regulated by miRNAs in a radiation-dependent manner, suggesting that these mRNAs and miRNAs are the potential targets in the cellular response to SD or FD irradiation. However, LLC1 tumors after FD irradiation exhibited no significant changes in the expression of selected genes and miRNAs observed in the irradiated cells in vitro, suggesting that experimental in vitro conditions, particularly the tumor microenvironment, should be considered in detail to promote the development of efficient radiotherapy approaches. Nevertheless, the present study highlights the primary signaling pathways involved in the response of murine cancer cells to irradiation. Data presented in the present study can be applied to improve the outcome and development of radiotherapy in preclinical animal model settings.

Introduction

Radiotherapy (RT) remains one of the most common types of therapy used alone or in combination with other therapeutics to treat cancer. In clinical practice, ~50% of all cancer patients receive radiotherapy at some point during treatment. Currently, to allow repair and recovery of radiation-induced damage to normal tissue cells, radiotherapy is administered in fractions of ~2 Gy every 24 h, 5 days/week for ≤7 weeks. However, the majority of the knowledge in the field of radiobiology comes from single dose (SD) irradiation research (1). Therefore, a detailed investigation of the molecular processes mediating the cellular response to fractionated dose (FD) irradiation is required to improve the efficiency of radiotherapy.

There is an emerging body of knowledge on the comprehensive molecular mechanisms underlying the cellular response to FD irradiation and the mechanisms associated with resistance to RT. Previous studies have shown that treatment with multiple fractions of irradiation produces a different gene expression signature in several cancer cell lines compared with SD irradiation (2,3). For instance, exposure to 10 Gy delivered as fractionated irradiation results in increased changes in differential gene expression in prostate cancer PC3 and DU145 cells (4). In addition, as demonstrated by gene expression profiles, exposure to FD irradiation can induce a significantly different microRNA (miRNA/miR) expression profile compared with SD (5,6). miRNAs perform an important role in the regulation of the expression of genes involved in the cellular response to radiation-induced DNA damage (7). Previous studies have reported that the modulation of miRNA expression levels in cancer cells can alter their sensitivity
to irradiation (8-10). Therefore, the integration of gene and miRNA signatures of radiosensitivity could lead to a reliable strategy for predicting radiation-induced cellular responses. Furthermore, the silencing of radiation-induced miRNAs could be implemented in direct antitumor therapies to improve the response of tumor cells to RT.

Several previous studies using a gene expression microarray approach indicated expression of a different set of genes in several human cancer cell xenografts following exposure to irradiation compared with cells irradiated in vitro, suggesting that the tumor microenvironment may affect the outcome of irradiation (2,11). The LLC1 cell line was established from the lung of a C57BL mouse bearing a primary Lewis lung carcinoma tumor. This cell line is highly tumorigenic and immunologically compatible with the murine immune system, unlike widely used human cancer cell xenograft models. Consequently, the LLC1 cell line is primarily used in syngeneic animal models to evaluate the efficacy of anticancer treatment in vivo (12). The present study analyzed global gene and miRNA expression changes in LLC1 cells exposed to SD of 2 or 10 Gy irradiation and FD of 5x2 Gy irradiation.

Materials and methods

Cell culture and maintenance. The LLC1 mouse Lewis lung carcinoma cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO2, with Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.,). 2 mM L-glutamine (Thermo Fisher Scientific, Inc.), 100 U/ml penicillin (Carl Roth GmbH Co., KG, Karlsruhe, Germany) and 0.1 mg/ml streptomycin (Carl Roth GmbH Co., KG).

Animals and tumor model. C57BL/6 female mice (Vilnius University, Vilnius, Lithuania) were maintained at a constant temperature (22±1°C), relative humidity (55±10%) and photoperiod (12 h light/dark cycle) in the Open Access Centre at the National Cancer Institute of Lithuania (Vilnius, Lithuania). All animal procedures were performed in accordance with the guidelines established by State Food and Veterinary Service Animal Care and Use Committee (Vilnius, Lithuania) that approved the current study (approval no. 0190). Two female mice at 10-12 weeks of age and 19-22 g body weight were injected subcutaneously with Lewis lung carcinoma LLC1 cells (1x106 cells suspended in DMEM medium) into their right groins. Animals were sedated with ketamine hydrochloride alone (0.1 mg/g body mass; ROTEXMEDICA GmbH, Trittau, Germany) by injection of 0.1-0.2 ml/animal solution in sterile normal saline (B. Braun Melsungen AG, Melsungen, Germany) into the caudal thigh muscles and sacrificed by cervical dislocation, and their tumors were excised, homogenized and their tumors were excised, homogenized and placed in a customized harness that allowed the groin to be exposed to irradiation, whereas the rest of the body was shielded by lead. In all of the experiments separate controls of non-irradiated mice tumors were used for SD or FD regimens.

Clonogenic survival assay. LLC1 cells were plated in 6-well plates 24 h prior to irradiation (500-10,000 cells/well) and treated with SD of up to 10 Gy or FD of 2 Gy of ionizing radiation (IR) daily for ≤5 days. In total, 8 days subsequent to irradiation LLC1 cell colonies (>50 cells/colony) were stained with crystal violet and counted manually. Clonogenic survival was evaluated as described previously (13). The mean cell survival fraction from 3 independent experiments was used to represent survival at each irradiation dose.

Total RNA and miRNA extraction. Total RNA enriched with small noncoding RNAs was isolated using the mirVana RNA isolation kit (Thermo Fisher Scientific, Inc.) according to manufacturer's protocol. For total RNA extraction, LLC1 cells were plated into 25 cm2 cell culture flasks for RNA isolation (0.7x106 or 0.1x106 cells/flask for the SD and FD irradiation regimens, respectively). Subsequently, ~1x106 LLC1 cells were harvested 4 h following SD (2 or 10 Gy) or FD (5x2 Gy) irradiation and were used for total RNA extraction. Following the same experimental design, untreated or irradiated animals were sacrificed, tumors excised and 100 mg of mouse tumor tissue was used for total RNA extraction. The quantity and quality of RNA were measured using a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, Inc.) and Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA).

Gene expression microarrays. RNA sample preparation, labeling and hybridization were performed using the kits described below according to the manufacturer's protocol. Briefly, 1 µg of total RNA was used for cDNA synthesis and amplification using the MessageAmp aRNA Amplification kit (Thermo Fisher Scientific, Inc.). Subsequently, 825 ng of cDNA was labeled with Cy3/Cy5 using the Arcturus® Turbo Labeling™ Cy3/Cy5 kit (Thermo Fisher Scientific, Inc.). The cDNA was then hybridized to the Mouse Whole Genome 4x44k Oligonucleotide Microarray (Agilent Technologies, Inc.) using a HS 400 Hybridization station (Tecan Group, Ltd., Männedorf, Switzerland). A total of 3 independent replicates for each sample were run. Microarray slides were scanned using the LS Reloaded laser scanner (Tecan Group Ltd.). Microarray image analysis and data generated were analyzed using ImaGene software (version 9.0; BioDiscovery, El Segundo, CA, USA) and GeneSpring GX software (version 11.5; Agilent Technologies, Inc.). Raw extracted gene expression data were normalized through Loess regression analysis to account for variation. Genes that exhibited a significant (P<0.05) fold-change in expression of >1.5 were defined as...
differentially expressed in LLC1 cells between the untreated and irradiated groups. The microarray design and data are available from the Gene Expression Omnibus (GEO) database (accession no. GSE84108; ncbi.nlm.nih.gov/geo) (14).

**miRNA expression microarrays.** miRNA labeling was performed using the miRNA Complete Labeling and Hyb kit (Agilent Technologies, Inc.) according to manufacturer's protocol. Briefly, 100 ng of total RNA was dephosphorylated and directly labeled with Cy3. Samples were dried out and resuspended in Hi-RPM Hybridization Buffer (Agilent Technologies, Inc.), containing a GE Blocking Agent (Agilent Technologies, Inc.) and denatured by heating for 5 min at 100°C. In a further step, samples were hybridized to Mouse miRNA 8x15K Microarrays (Agilent Technologies, Inc.) containing probes for 627 mouse miRNAs from the Sanger Institute. Three replicate hybridizations were performed for each sample. Slides were washed 3 times in PBS and scanned with the SureScan Microarray Scanner (Agilent Technologies, Inc.). Microarray images were extracted using Feature Extraction software (version 10.7.3.1; Agilent Technologies, Inc.). Enrichment analysis was performed using the WEB-based GEnie SeT AnaLysis Toolkit, as described previously (16). P-values were calculated using the hypergeometric test and adjusted using the Benjamini and Hochberg procedure. Functional KEGG pathway categories associated with ≥ 5 genes were considered as significantly enriched (P<0.05) in differentially expressed genes. Enrichment analysis is available at the GEO database (accession no. GSE84109).

**Enrichment analysis.** Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of gene expression data was performed using the WEB-based GEnie SeT AnaLysis Toolkit, as described previously (16). P-values were calculated using the hypergeometric test and adjusted using the Benjamini and Hochberg procedure. Functional KEGG pathway categories associated with ≥ 5 genes were considered as significantly enriched (P<0.05) in differentially expressed genes. Enrichment analysis is available at the GEO database (accession no. GSE84109).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) for the evaluation of the miRNA expression.** To validate differential gene expression changes, the RevertAid RT kit (Thermo Fisher Scientific, Inc.) was used for cDNA synthesis as described previously (20). Briefly, 0.2 µg of total RNA was added to a 20 µl RT reaction containing 1 µM specific RT primer, 1 µM dNTP mix, 20 units RNase inhibitor and 20 units reverse transcriptase. Thermocycling conditions were as follows: 25°C for 20 min; 37°C for 60 min; and 70°C for 10 min. The sequences of the specific primers were as follows: SnoRNA-135, forward (F), 5' - CTT AAT CTT CCC ACC GCC CAG-3' and reverse (R), 5'-GGA CGC ACT GAC CGA TCA TTA-3' and miR-145a-5p, forward (F), 5'-CGG GAG GCA GTG TAG TTA GCT-3' and reverse (R), 5'-GAT GGG GTA TGA GAT CA-3'; BTG anti-proliferation factor 2 (Btg2), forward (F), 5'-GGC ATTAGGGCCAGAG-3' and reverse (R), 5'-GCTTGAATACCC GTTGGAGATCA-3'; and miR-145a-5p, forward (F), 5'-GTC GCA TAT CTT GAT GG-3' and reverse (R), 5'-CGA ATC ACT AAC TCC ACT-3'. The sequences of the primers were as follows: Hprt1 forward (F), 5'-CTT AGA TTT CCC AGG A-3'; and reverse (R), 5'-GGA CGC ACT GAC CGA TCA TTA-3' and miR-145a-5p, forward (F), 5'-CGG GAG GCA GTG TAG TTA GCT-3' and reverse (R), 5'-GAT GGG GTA TGA GAT CA-3'; BTG anti-proliferation factor 2 (Btg2), forward (F), 5'-GGC ATTAGGGCCAGAG-3' and reverse (R), 5'-GCTTGAATACCC GTTGGAGATCA-3'; and miR-145a-5p, forward (F), 5'-GTC GCA TAT CTT GAT GG-3' and reverse (R), 5'-CGA ATC ACT AAC TCC ACT-3'. The sequences of the specific primers were as follows: SnoRNA-135, forward (F), 5'-CTT AAT CTT CCC ACC GCC CAG-3' and reverse (R), 5'-GGA CGC ACT GAC CGA TCA TTA-3' and miR-145a-5p, forward (F), 5'-CGG GAG GCA GTG TAG TTA GCT-3' and reverse (R), 5'-GAT GGG GTA TGA GAT CA-3'; BTG anti-proliferation factor 2 (Btg2), forward (F), 5'-GGC ATTAGGGCCAGAG-3' and reverse (R), 5'-GCTTGAATACCC GTTGGAGATCA-3'; and miR-145a-5p, forward (F), 5'-GTC GCA TAT CTT GAT GG-3' and reverse (R), 5'-CGA ATC ACT AAC TCC ACT-3'.

**RT-qPCR for the evaluation of the miRNA expression.** To validate differential changes in miRNA expression, the RevertAid RT kit (Thermo Fisher Scientific, Inc.) was used for cDNA synthesis as described previously (20). Briefly, 0.2 µg of total RNA was added to a 20 µl RT reaction containing 1 µM specific RT primer, 1 µM dNTP mix, 20 units RNase inhibitor and 20 units reverse transcriptase. Thermocycling conditions were as follows: 25°C for 20 min; 37°C for 60 min; and 70°C for 10 min. The sequences of the specific primers were as follows: SnoRNA-135, forward (F), 5'-CTT AAT CTT CCC ACC GCC CAG-3' and reverse (R), 5'-GGA CGC ACT GAC CGA TCA TTA-3' and miR-145a-5p, forward (F), 5'-CGG GAG GCA GTG TAG TTA GCT-3' and reverse (R), 5'-GAT GGG GTA TGA GAT CA-3'; BTG anti-proliferation factor 2 (Btg2), forward (F), 5'-GGC ATTAGGGCCAGAG-3' and reverse (R), 5'-GCTTGAATACCC GTTGGAGATCA-3'; and miR-145a-5p, forward (F), 5'-GTC GCA TAT CTT GAT GG-3' and reverse (R), 5'-CGA ATC ACT AAC TCC ACT-3'.

**Statistical analysis.** Data were analyzed using GraphPad Prism software (version 6.0; GraphPad Software, La Jolla, CA, USA).
A Student's t-test was performed to statistically compare differences between the untreated and irradiated groups. Pc<0.05 was considered to indicate a statistically significant difference. All experiments were independently repeated ≥3 times.

**Results**

**Clonogenic cell survival.** Clonogenic survival analysis revealed that LLC-1 cells were more sensitive to SD irradiation compared with FD irradiation (data not shown). The surviving fractions of LLC1 cells following 2-10 Gy SD irradiation were 62.2±4.1-1.1a±0.51% compared with non-irradiated cells. LLC1 cell survival decreased to 19.81±4.65% following 5x2 Gy FD irradiation compared with non-irradiated cells.

**Global mRNA expression changes.** Genome wide gene expression microarray data analysis revealed that a total of 2,294 genes were differentially expressed (fold-change >1.5; P<0.05) in LLC1 cells 4 h following treatment with 2 Gy SD (SD2), 10 Gy (SD10) or 5x2 Gy FD irradiation compared with the untreated controls (Fig. 1). The amount of differentially expressed genes following irradiation was dose delivery-dependent. The exposure of LLC1 cells to SD2 resulted in the differential expression of 422 genes. By contrast, the expression of 1,258 and 1,465 genes was significantly altered following exposure to SD10 and FD irradiation, respectively. The ratio of upregulated and downregulated genes was similar following all irradiation regimens. Microarray data analysis also revealed that 145 differently expressed genes were common between all irradiation regimens.

**KEGG pathway enrichment analysis.** In order to elucidate which pathways were significantly affected by irradiation treatment, genes identified to be differentially expressed (fold-change >1.5; P<0.05) following SD or FD were grouped into functional KEGG pathway categories (Table I). KEGG pathway analysis revealed that the ‘cell cycle’ and ‘p53 signaling pathway’ categories were the most significantly altered following all irradiation regimens, and the ‘DNA replication’ and ‘apoptosis’ categories were also significantly altered subsequent to SD10 and FD irradiation regimens. Genes associated with ‘Pathways in cancer’ were the most significantly enriched among all KEGG categories following SD10 and FD irradiation. Furthermore, subsequent to exposure to SD10 or FD the second most significantly altered functional categories were related to DNA repair (‘mismatch repair’, ‘nucleotide excision repair’ and ‘base excision repair’) and the immune response (‘cytokine-cytokine receptor interaction’, ‘hepatitis C’, ‘chemokine signaling pathway’, ‘B cell receptor signaling pathway’, ‘Janus kinase-signal transducer and activator of transcription signaling pathway’ and ‘Toll-like receptor signaling pathway’). The pathway enrichment data also revealed that SD10 and FD irradiation significantly altered the expression of genes involved in the ‘mitogen activated protein kinase (MAPK), ‘tumor growth factor-β’, ‘vascular endothelial growth factor’, ‘wingless-type MMTV integration site family’ and ‘insulin’ signaling pathways.

**Heat map analysis.** Radiation-induced changes in the expression of individual genes from the p53, cell cycle, apoptosis and immune response-associated KEGG pathway categories, which were the most significantly altered in LLC1 cells following SD and FD irradiation, were color coded to demonstrate the expression patterns of genes within each category following exposure to SD and FD irradiation protocols (Fig. 2). Typically, the heat maps demonstrated that the differential expression of genes peaked in cells exposed to SD10 or FD irradiation. In addition, the extent of certain differentially expressed genes was different in cells irradiated with FD compared with SD.

The microarray data indicated that a total of 27 genes involved in the p53 signaling pathway were significantly altered in LLC1 cells exposed to all irradiation regimens (Fig. 2A). Fig. 2B depicts a total of 77 genes associated with immune response regulation that were differentially expressed in LLC1 cells following irradiation treatment. This subset of the heat map reveals that the expression of 51 genes was upregulated and 26 genes were downregulated. The expression of chemokines ccl7 and ccl9 peaked in response to 10 Gy, whereas FD induced the expression of ccl5. The irradiation regimens also induced the expression of tumor necrosis factor-associated cytokines tnfrsf10b and tnfrsf19, which peaked following 10 Gy irradiation. FD also significantly altered the expression of tnfrsf9, tnfrsf18 and tnfrsf25. The expression of cytokines, including figf, vegfa pdgfc, ctf1 and il11, was significantly altered in LLC1 cells in response to FD irradiation. The irradiation regimens induced the expression of transcription factors Nfatc1 and Stat1, which peaked following SD10. In addition, FD significantly induced the expression of Stat5a. Exposure to radiation also altered the expression of a total of 34 genes involved in cell cycle regulation (Fig. 2C). The majority of the differentially expressed genes were downregulated, whereas the expression of only 9 genes was upregulated in this category. Heat map analysis also demonstrated that 19 apoptosis related genes were differentially expressed in cells following irradiation (Fig. 2D). The expression of a total of 14 genes was upregulated in this category, including proapoptotic Fas, Bad, Bid, Casp7, Trp53, Thfrsf10b, and anti-apoptotic Bcl2I and Cflar genes. In addition, the expression of 5 apoptosis-associated genes was downregulated in cells following exposure to irradiation in this group, including Bcl2 and Xiap peaked following SD10.

**Global miRNA expression changes.** The miRNA microarray data revealed that a total of 18 miRNAs were differentially expressed (>2-fold; P<0.05) in LLC1 cells exposed to all irradiation protocols (Table II). The expression of 2 miRNAs, miR-34c-5p and miR-145a-3p, was significantly altered by all irradiation protocols, whereas miR-34c-3p and miR-34b-3p were upregulated following exposure to SD10 and FD. Data in Table II also revealed that the highest number of miRNAs was differentially altered in LLC1 cells following exposure to FD, resulting in deregulated expression of 7 unique miRNAs. The expression of miR-186-5p, miR-145a-5p, miR-129-5p, miR-192-5p, miR-129-2-3p and miR-30c-5p was upregulated, and miR-105 was downregulated, in LLC1 cells following the FD regimen.

**miRNA target filter analysis.** In order to determine functions of the 18 miRNA significantly altered following exposure to SD and FD irradiation in the post-transcriptional regulation of gene expression, the present study identified 6,343 individual
target genes potentially regulated by these miRNA using in silico miRNA target analysis. Subsequently, negative associations between all differently expressed genes and miRNAs associated with cell cycle regulation, the p53 signaling pathway, apoptosis and the immune response were identified, indicating a potential miRNA-mRNA connection in these processes.

The miRNAs showing inverse associations with differently expressed target genes involved in selected pathways are shown in Table III. A negative association was identified between the differential expression of 6 miRNAs and 11 mRNAs in the cell cycle, p53 signaling pathway and apoptosis KEGG categories. miRNA target analysis also revealed that 20 differentially expressed genes from the immune response category were inversely associated with the differential expression of 12 miRNAs.

Microarray data validation. To validate the microarray data, the present study selected 4 upregulated genes and miRNAs for RT-qPCR analysis (Table IV). The results indicated that the expression of genes involved in the p53 signaling pathway, including Btg2, cyclin Ccng1, p21 and Thbs2, were significantly upregulated in LLC1 cells following irradiation.
The present study investigated the changes in gene and miRNA expression signatures following SD2, SD10 and 5x2 Gy FD irradiation in mouse lung carcinoma LLC1 cells and syngeneic LLC1 tumors. The obtained data revealed that the gene expression profiles of LLC1 cells were irradiation dose delivery-dependent. In addition, the present study demonstrated through KEGG pathway enrichment analysis that the p53 signaling, cell cycle, apoptosis and immune response pathways were the most significantly altered functional categories in LLC1 cells following irradiation. The extent of differential expression was also irradiation dose delivery-dependent. The results of the miRNA microarray indicated that FD irradiation induced a significantly different miRNA expression pattern compared with SD irradiation. Furthermore, miRNA target filter analysis revealed a significant association between miRNA and miRNA expression signatures in LLC1 cells following exposure to radiation. However, RT-qPCR analysis demonstrated that LLC1 tumors exhibited no significant change in the expression of selected genes and miRNAs following a FD irradiation regimen.

The microarray data revealed that FD irradiation induced differential expression in the highest number of genes. In addition, a total of 145 genes were commonly expressed between all irradiation regimens, demonstrating a significantly different gene expression pattern in LLC1 cells following exposure to SD or FD radiation. These results are supported by previous studies (2,4,5) that also indicated that different gene expression profiles in human breast and prostate carcinoma and normal endothelium cell lines were irradiation dose delivery-dependent. In addition, Palayoor et al (5) have previously identified potential therapeutic targets from investigated the response of human prostate carcinoma cells exposed to FD radiation (3). The results of previous studies and the present study suggest that FD irradiation could be a relevant approach to identifying genes and molecular pathways that are clinically important for the improving the efficacy of radiotherapy.

The present study demonstrated that the most significantly altered functional categories in LLC1 cells following all irradiation regimens were cell cycle regulation and the p53 signaling pathways. In addition, the most significant pathway enrichment was identified in cells exposed to FD. The transcription factor p53 serves an essential role in the cellular response to IR-induced DNA damage (21). The activation of p53 results in temporary cell cycle arrest to facilitate the repaired of damaged DNA, in addition to apoptosis if the damage cannot be repaired (22,23). The present study identified that the majority of cell cycle-associated genes were downregulated in LLC1 cells following SD and FD irradiation regimens, including genes promoting G1/S and G2/M transition. Notably, the expression of the kinases Atr and Chek1, which are implicated in S-phase DNA damage checkpoint arrest (24), were also downregulated in LLC1 cells following irradiation. In addition, irradiation resulted in a significant upregulation of p21, which is a master regulator of cell cycle checkpoint progression or arrest (25). However, the deregulation of gene expression was more robust in cells exposed to FD. These included differentially expressed genes associated with the progression of DNA replication and mitosis. For example, the

Figure 2. Heat maps of differentially expressed genes according to their Kyoto Encyclopedia of Genes and Genomes pathway category in LLC1 cells following single dose (2 or 10 Gy) or fractionated dose (5x2 Gy) irradiation. (A) p53 signaling pathway. (B) Immune response. (C) Cell cycle regulation. (D) Apoptosis.

compared with the untreated cells. RT-qPCR analysis also revealed that miR-34b-3p and miR-34c-5p were significantly upregulated in LLC1 cells following exposure of SD10 and FD, whereas miR-186-5p and miR-145a-5p were significantly upregulated following FD, compared with untreated cells. RT-qPCR analysis validated the gene and miRNA microarray data.

The present study compared the difference in expression of selected genes and miRNAs in LLC1 cells grown in vitro and LLC1 tumors in vivo following SD10 and FD irradiation (Fig. 3A and B). RT-qPCR analysis revealed that the expression of btg2, ccng1 and p21 was upregulated in tumors and LLC1 cells following SD treatment with 10 Gy. Thbs2 was also upregulated in vivo following irradiation of 10 Gy, whereas the expression of thbs2 was only significantly altered in vitro following FD. Notably, the expression of the selected genes was not significantly altered in LLC tumors following the FD irradiation regimen, which is in contrast to the results identified in LLC1 cells. Additionally, RT-qPCR analysis indicated no significant changes in the expression of the selected miRNAs in LLC1 tumors following irradiation (Fig. 3B).
Table II. Relative expression of differentially expressed miRNAs in LLC1 cells following single dose (2 or 10 Gy) or fractionated dose (5x2 Gy) irradiation.

| miRNA        | miRbase ID no. | 2 Gy     | 10 Gy     | 5x2 Gy    |
|--------------|----------------|---------|----------|----------|
| miR-34c-5p   | MIMAT0000381   | 2.19a   | 2.79a    | 5.30a    |
| miR-145a-3p  | MIMAT0004534   | -2.46a  | -2.08a   | -2.94a   |
| miR-878-5p   | MIMAT0004932   | -2.20a  | -2.74a   | -2.43    |
| miR-126a-5p  | MIMAT0001137   | -2.16a  | -1.6     | -2.15    |
| miR-338-5p   | MIMAT0004647   | -2.03a  | 1.10     | -1.89    |
| miR-26b-3p   | MIMAT0004630   | -1.31   | -2.32a   | -1.33    |
| miR-136-5p   | MIMAT0000148   | -1.28   | 2.17a    | -1.43    |
| miR-466a-5p  | MIMAT0004759   | -1.73   | -2.58a   | -3.28    |
| miR-710      | MIMAT0003500   | -1.71   | -2.45a   | -3.11    |
| miR-34c-3p   | MIMAT0004580   | 2.32    | 4.77a    | 24.93a   |
| miR-30c-5p   | MIMAT0000514   | -1.10   | 1.11     | 5.50a    |
| miR-126a-3p  | MIMAT0000157   | 1.12    | 1.12     | 6.99a    |
| miR-186-5p   | MIMAT0000215   | 1.46    | 1.55     | 3.55a    |
| miR-192-5p   | MIMAT0000517   | 1.55    | 1.36     | 2.62a    |

*Relative miRNA expression >2-fold and P<0.05 compared with the expression levels in untreated cells.

Table III. miRNA target filter analysis of differentially expressed target genes and miRNAs from the cell cycle, p53, apoptosis and immune response categories that demonstrated an inverse association in LLC1 cells exposed to single dose (10 Gy) or fractionated dose (5x2 Gy) irradiation.

| Category            | miRNA      | Target gene          | miRNA   | Target gene          |
|---------------------|------------|----------------------|---------|----------------------|
| Cell cycle          | miR-34c-5p | E2f3↓;E2f5↓;Ccne2↓   | miR-30c-5p | Ccne2↓;Stag1↓;Orc4↓;Skp2↓ |
|                     |            |                      | miR-34c-5p | Ccne2↓;E2f3↓          |
|                     |            |                      | miR-129-5p | Stag1↓;Orc4↓          |
|                     |            |                      | miR-145a-5p | Orc4↓                |
|                     |            |                      | miR-186-5p | Cdc27↓;Stag1↓         |
|                     |            |                      | miR-30c-5p | Ccne2↓                |
|                     |            |                      | miR-34c-5p | Ccne2↓                |
|                     |            |                      | miR-129-5p | Pten↓                |
|                     |            |                      | miR-145a-3p | Pmaip1↑;Sesn2↑     |
|                     |            |                      | miR-30c-5p | Ppp3cb↓               |
| p53 signaling       | miR-34c-5p | Ccne2↓               | miR-30c-5p | Lepr↓;Kras↓;Ppp3cb↓  |
| pathway             |            |                      | miR-34c-3p | Gng5↓                |
|                     |            |                      | miR-34c-5p | Pdk1↓                |
|                     |            |                      | miR-145a-3p | Il6ra↓;Rock1↓     |
|                     |            |                      | miR-186-5p | Vegfa↓;Pias2↓        |
|                     |            |                      | miR-192-5p | Crk↓;Pias2↓          |
|                     |            |                      | miR-105   | Tgfrb2↑;Stat1↑       |
| Apoptosis           | miR-34b-3p | Spred1↓              | miR-145a-3p | Tgfrb2↑;Cr2↑;Inpp5d↑;Ticam1↑ |
|                     | miR-34c-3p | Spred1↓              |         |                      |
|                     | miR-34c-5p | Pdk1↓                |         |                      |
|                     | miR-136-5p | Edua2r↑              |         |                      |
|                     | miR-145a-3p | Cr2↑;Inpp5d↑      |         |                      |
|                     | miR-466a-5p | Edua2r↑;Egfr↑;Inhhb↑ |         |                      |
|                     | miR-710    | Stat1↑;Pik3r3↑       |         |                      |

An upwards and downwards pointing arrow indicates increased and decreased expression, respectively. miRNA/miR, microRNA.
Table IV. Validation of gene and miRNA microarray data by RT-qPCR.

A, Gene

| Gene | Irradiation dose | Data   | 10 Gy | 5x2 Gy |
|------|-----------------|--------|-------|--------|
| Btg2 | RT-qPCR         | 5.09±0.34 | 4.51±0.73 |
|      | Microarrays     | 5.38±1.55 | 2.74±0.09 |
| Ccng1| RT-qPCR         | 4.02±0.57 | 3.2±0.47 |
|      | Microarrays     | 2.06±0.47 | 2.30±0.45 |
| P21  | RT-qPCR         | 2.64±0.06 | 2.6±0.49 |
|      | Microarrays     | 5.96±1.28 | 2.43±0.16 |
| Thbs2| RT-qPCR         | 1.30±0.10 | 2.76±0.67 |
|      | Microarrays     | 1.29±0.18 | 2.26±0.11 |

B, miRNA

| miRNA/miR, microRNA | Irradiation dose | Data   | 10 Gy | 5x2 Gy |
|---------------------|-----------------|--------|-------|--------|
| miR-34b-3p          | RT-qPCR         | 2.76±0.48 | 3.6±0.58 |
|                     | Microarrays     | 4.07±1.53 | 14.78±4.75 |
| miR-34c-5p          | RT-qPCR         | 2.67±0.66 | 2.32±0.34 |
|                     | Microarrays     | 2.79±0.33 | 5.3±0.90 |
| miR-186-5p          | RT-qPCR         | 1.62±0.48 | 2.28±0.06 |
|                     | Microarrays     | 1.55±0.59 | 3.55±0.81 |
| miR-145a-5p         | RT-qPCR         | 1.41±0.46 | 3.44±0.49 |
|                     | Microarrays     | 1.12±0.88 | 6.99±1.41 |

miRNA/miR, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

expression of anaphase-promoting complex (APC/C) encoding genes, including Cdc26, Cdc27, Anapc2 and Anapc11, was altered in cells exposed to FD radiation. In addition, FD irradiation deregulated the expression of Bub1 and Bub3, which are involved in the spindle assembly checkpoint and the regulation of APC/C catalytic activity (26). Furthermore, the irradiated LLC1 cells demonstrated significantly upregulated expression of genes involved in the regulation of apoptosis. However, microarray data indicated that the deregulation of gene expression associated with pro-apoptotic processes peaked in cells exposed to SD10. The expression levels of anti-apoptotic genes, including Bcl2l1 and Akt2, were increased in cells following the exposure to FD compared with SD. These data indicate that the survival of LLC1 cells is significantly higher following exposure to FD compared with SD10. The pro-survival effect of the radiation-induced DNA damage response of LLC1 cells treated with FD is likely dependent on the cumulative effect of the differential expression of genes.

The present study revealed that the expression of genes involved in the immune response was significantly altered in LLC1 cells following all irradiation regimens. Despite this, the number of differentially expressed genes was similar in cells exposed to SD10 and FD. However, the set of specific differentially expressed inflammatory genes was significantly different following the different irradiation protocols. This is in accordance with the results of previous studies, which identified a distinct expression profile of immune response genes between cells exposed to SD or FD (3,5). Microarray analysis performed in the current study also demonstrated that genes from the immune response category, including genes encoding chemokines, cytokines, cytokine receptors and tumor necrosis factors, were differently expressed in the irradiated LLC1 cells compared with non-irradiated cells. The results of the present study are consistent with previous studies demonstrating that RT can promote the immune recognition of tumor cells by increasing the expression of antigen-presenting molecules, pro-inflammatory cytokines and the release of ‘Damage-associated molecular signals’, leading to the attraction of immune cells to the irradiated tumor site (27-29). Additionally, the expression of transcription factors, including Nfatc1 and Stat1, were upregulated in cells exposed to SD and FD radiation. Members of the Stat family have been demonstrated to activate the transcription of genes involved in cancer cell survival, proliferation and angiogenesis (30). Furthermore, Stat1 is considered to serve an important role in regulating the expression of interferon-stimulated genes (ISGs) (31). The transactivation of ISGs by Stat1 can be induced as a part of the cellular response to IR and can lead to increased radiosensitivity (32). In the present study, treatment with FD radiation elevated the expression of Stat5a, suggesting that other Stat family members may be involved in resistance to radiotherapy. This is further supported by a previous study that demonstrated an association between the expression of Stat5a and radiosensitivity in head and neck squamous cell carcinoma cells (33). These findings suggest that the radiation-induced immune response in irradiated LLC1 cells may contribute to tumor development in a dose delivery-dependent manner. Radiation-induced alterations in the expression of inflammatory genes are considered to be pro-immunogenic, highlighting the potential of combining RT with immunotherapy for the treatment of cancer (34,35). Preclinical data also indicates that the promotion of antitumor immune response is irradiation delivery type-dependent, since RT delivered as a SD is not sufficient to induce antitumor immunity (36-38). Together these findings indicate possible directions for the development of more efficient anticancer irradiation treatment strategies, based on exploiting the pro-survival and immunogenic tumor signaling pathway alterations during FD irradiation.

It has previously been demonstrated that exposure to SD radiation results in the differential expression of miRNAs in various cancer and normal cells (39). In the present study, the expression of a total of 18 miRNAs was significantly altered in LLC1 cells exposed to IR. Microarray analysis also identified that the expression of miR-34 cluster miRNAs, including miR-34b and miR34c, was significantly
upregulated in cells exposed to SD or FD radiation. Previous studies have demonstrated that members of the miR-34 cluster are regulated by p53 and involved in the regulation of cell cycle arrest, proliferation inhibition and apoptosis (40). miR-34 cluster miRNAs have also been identified to be upregulated in different human cancer cells exposed to IR (41,42). These observations suggest that miR-34 cluster miRNAs serve an important role in the response of LLC1 cells to IR. The microarray data analysis demonstrated that FD irradiation induced the most robust deregulation of miRNA expression, indicating that the expression of miRNA is also altered in an irradiation delivery-dependent manner. Similar results were identified in previous studies, which demonstrated a high degree of alteration in the expression of miRNAs in prostate cancer and endothelium cells following FD (5,6). In addition, Leung et al (43) reported that only a small number of miRNAs differentially expressed in breast cancer cells exposed to SD or FD were the same, suggesting that FD induces a distinct miRNA signature compared with SD radiation.

To further extend the understanding of the roles miRNAs serve in the cellular response to IR, the present study performed miRNA target filter analysis to identify potential functional associations between differentially expressed miRNAs and mRNAs in LLC1 cells exposed to radiation. However, the identification of regulatory miRNAs and their target mRNAs remains a major challenge since a single miRNA may regulate multiple mRNAs and vice versa. In addition, statistical methods which are able to identify these miRNA-controlled regulations may result in thousands of putative miRNA-mRNA pairs, leading to an inability to extract a biologically relevant understanding of the collective function of differentially expressed miRNAs (44). Therefore, the present study investigated the negative association between the expression of miRNAs and genes associated with p53, cell cycle regulation, apoptosis and immune response pathways, which were shown to be the most prominently altered in LLC1 cells following SD and FD irradiation.

It has been shown that the transcription factor p53 performs an important role in the regulation of the transcription of several miRNAs, which in turn control the expression of p53-regulated genes that mediate cell cycle arrest and apoptosis (40,45). The present miRNA target analysis revealed an inverse association between the expression of miR-34c and E2f3, E2f5 and Ccne2, suggesting that the upregulation of miR-34c could be associated with G1 phase regulation in LLC1 cells exposed to radiation. This is also supported by previous studies that have highlighted the role of miR-34c in the induction of G1 and G2/M cell cycle arrest (46,47). In addition, Li et al (48) have demonstrated that the expression of E2F3 is reduced following the upregulation of miR-34c in endometrial carcinoma cells, indicating that E2F3 could be a target of miR-34c. The present study identified a negative association between several miRNAs and genes that were differentially expressed in cells exposed to FD radiation. For example, the upregulation of miR-30c was associated with the downregulation of Ccne2, Stag1, Orc4 and Skp2. In addition, the expression of Stag1 and Orc4 was inversely associated with the expression of miR-129, miR-145a and miR-186. These observations suggest that these miRNAs perform important roles in the cell cycle arrest response to radiation in LLC1 cells in a dose delivery-dependent manner. However, no significant association was observed between differentially expressed miRNAs and genes involved in apoptosis, with the exception of a negative association between the expression of miR-30c and Ppp3cb. Nevertheless, several miRNAs that...
were differentially expressed subsequent to irradiation have previously been demonstrated to be associated with the regulation of apoptosis, indicating that they may serve a similar role in irradiated LLC1 cells. For example, the overexpression of miR-129 was identified to promote the death of irradiated breast cancer cells by targeting high mobility group box 1 for degradation (49). In addition, miR-30c was demonstrated to serve an important role in the radiation-induced hematopoietic cell damage response (50).

There is emerging evidence that miRNAs are involved in the radiation-induced regulation of inflammatory responses (5-7). miRNA target filter analysis revealed that the majority of miRNAs differentially expressed in LLC1 cells exposed to SD and FD radiation were inversely associated with several genes associated with the immune response, highlighting the role of miRNA in the inflammatory response to irradiation. Target filter analysis also indicated that the regulation of miRNAs and inflammatory response-associated genes in LLC1 cells treated with fractionated irradiation was significantly different. In addition, the upregulation of Stat1 was associated with the downregulation of miR-710 in cells exposed to SD and with the downregulation of miR-105 in cells exposed to FD. The present study demonstrated that treatment with FD also upregulated the expression of miR-145-5p, which has previously been identified to target Stat1 (51), indicating that certain differentially expressed genes could be regulated by distinct miRNAs in cells exposed to SD or FD radiation. These data suggest that the regulation of the immune response by miRNAs in irradiated LLC1 cells may involve radiation dose delivery-dependent.

The results of the present study demonstrate that the expression of genes and miRNAs is different in LLC1 tumors and LLC1 cells in vitro following exposure to SD10 or FD irradiation. Despite the fact that treatment with SD10 increased the expression of genes involved in the p53 signaling pathway in LLC1 tumors, no significant change in the expression of selected genes and miRNAs were observed in vitro following the exposure to FD radiation, suggesting that the radiation-induced changes in gene and miRNA expression may be modulated by the tumor microenvironment. Similar results of different cellular response to irradiation under the different microenvironment conditions were obtained by previous studies, which have applied different strategies to validate in vitro data. Camphausen et al (11) reported that glioblastoma U87 and U251 cells exposed to 6 Gy SD radiation in vivo exhibited a different set of differentially expressed genes compared with cells grown in vitro. In addition, Tsai et al (2) demonstrated that prostate cancer DU145 cell xenografts exhibited a different profile of genes induced by SD and FD compared with the same cells exposed to irradiation in vitro, indicating that a 10 Gy exposure in vivo could only reach an effect of up to 3 Gy exposure under in vitro growth conditions. These data suggest that investigations into the effects of gene and miRNA expression will require more biologically relevant experimental conditions.

In conclusion, the present study indicates that the gene and miRNA expression profiles in LLC1 cells exposed to radiation are dose delivery type-dependent. In addition, data analysis revealed that the altered expression of miRNAs and targeted miRNAs may affect radiation-induced DNA damage response pathways differently in LLC1 cells exposed to SD and FD irradiation. The results of the present study may be applied to improve the outcome of radiotherapy. However, experimental in vitro conditions, including the tumor microenvironment, should be considered in more detail in further investigations.

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