Gene Expression and Early Radiation Response of Two Distinct Neuroblastoma Cell Lines

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Abstract

Simple Summary: Neuroblastoma is a common pediatric cancer that spreads systemically, making therapy difficult. Tumors are frequently resistant to radiation therapy, a last line of defense in patients with aggressive disease. In this study, we found that there are distinct gene expression patterns dependent on neuroblastoma subtype; however, both neuroblastoma types examined showed only subtle gene expression differences early following radiation exposure. This indicates that while neuroblastoma tumors are diverse in phenotype and gene expression, they have similarly stable gene expression and resistance during early response to stressors such as radiation.

Abstract: External beam radiation is infrequently used for treatment of pediatric cancer such as neuroblastoma, typically reserved for palliative care in patients with aggressive metastatic disease who fail to respond to alternative treatments. Understanding effects of radiation on neuroblastoma cells could improve efficacy of this final means of therapy to decrease tumor burden and stabilize the disease. In this study, clonogenic assays were used to establish radiation responses for SK-N-AS and SK-N-DZ cell lines; cells were then irradiated at doses that cause 90% cell killing based on clonogenic assay and their RNA isolated and subjected to microarray analysis. In addition, cells were transfected with pre-miRNA constructs that led to overexpression of microRNAs miR-34a and miR-1228. Statistically significant differences were detected for expression of several thousands of genes when the two cell lines were compared with each other. In comparison, radiation led only to minor gene expression differences of less than two-fold at the 1h post-irradiation timepoint in both cell lines. Overexpression of miR-34a and miR-1228 in either cell line did not change this outcome. Therefore, neuroblastoma cell lines SK-N-AS and SK-N-DZ are phenotypically diverse and gene expression differences between them are extensive. At the same time, the regulation of gene expression in both of these cell lines is in a stable equilibrium at early timepoints after exposure to ionizing radiation.

1. Introduction

Neuroblastomas are pediatric solid tumors that develop from the peripheral sympathetic nervous system, originating from multipotent neural crest cells [1], and show significant diversity in disease severity and prognosis. Heterogeneity of neuroblastoma as a disease is echoed by heterogeneities between cell lines derived from individual neuroblastoma cancers [2,3]. This study is focused on cell lines SK-N-AS and SK-N-DZ, both isolated from bone marrow metastatic sites but showing diverse genetic backgrounds and many disparate responses to potential cancer treatments, including ionizing radiation (Supplemental Table S1). Over the years, these two cell lines were sometimes used as a source for investigation of commonalities between different neuroblastomas, e.g. with regard to their proteomic profiles and secretion [4-6], cellular signaling [7] and response to therapy [8-12]; and sometimes as models for differences between different neuroblastoma types. Implanted in mice, SK-N-AS cells generate larger tumors with larger diameter tumor vessels compared to SK-N-DZ cells, possibly attributed to the increased VEGF protein expression in the SK-N-AS cell line [13]. In addition, only SK-N-AS cells cause osteolytic bone metastases in nude mice [14], which is ascribed to high *COX-2* and prostaglandin E2

(*PGE2*) expression in this cell line. Different *MYCN* amplification status in these cells [15,16], with SK-N-AS having only a single copy while SK-N-DZ carries amplified *MYCN*, led to their use in studies focused on *MYC* related treatments such as combination of uridine and dihydroorotate dehydrogenase [17]. In a study focused on cell-line dependent response to the inhibitor of ganglioside sialidase, different responses to chemical agents were noted where de-differentiation and increased proliferation occurred in SK-N-DZ as a type of cholinergic/adrenergic cells, while no response was found in adrenergic type cell line SK-N-AS [18]. Equal doses of deferoxamine had a cytolytic effect on SK-N-DZ cells but lead only to a growth reduction of SK-N-AS cells [19]. On the other hand, IFN-gamma induced caspase-8 expression resulted in apoptosis of SK-N-AS cells, while in their counterpart SK-N-DZ cells activation of the same signaling cascade did not lead to *TRAIL* mediated apoptosis [20]. Likewise, Toll-like receptor-3 (*TLR3*) expression is lower in SK-N-DZ cells and as a consequence, they survive treatment with poly(I:C) better than their *TLR3*-high counterpart SK-N-AS cells [21].

In this work, our aim was to reveal the first, early timepoint gene expression changes in SK-N-AS and SK-N-DZ cells in response to radiation, as cell-specific “strategies” to recovery from insult. Because ionizing radiation exposure has different effects on cells in different stages of cell cycle, it should be noted that these cell lines demonstrate different cell cycle arrest behaviors in response to, for example, doxorubicin treatment [22]. Viability of these cells after exposure to ionizing radiation differs significantly, as previously demonstrated in preliminary work by MTS assay using a cesium-137 gamma irradiator on SK-N-AS and SK-N-DZ cells [23]. In this study, we used a variety of assays (Supplemental Figure S1) and demonstrated that exposure to X-rays results in significantly greater survival of SK-N-AS cells compared to SK-N-DZ cell line. However, changes in overall gene expression in either cell line one hour after exposure to doses that cause 90% cell killing is surprisingly modest. In addition, we explored possible modulation of radiation response in the two cell lines associated with overexpression of hsa-miR-1228-3p (miR-1228) and hsa-miR-34a-5p (miR-34a) by transfection of synthetic pre-miRNAs. These two microRNAs have opposite patterns of baseline expression in untreated SK-N-AS and SK-N-DZ cells (Supplemental Figure S2), implying possible involvement in cell-specific repair following radiation. However, transfection of either of these two pre-miRNAs to SK-N-AS cells 24h before irradiation did not lead to significant modulation of survival in a clonogenic assay. Gene expression patterns in either cell line were not markedly different in response to microRNA overexpression with or without radiation exposure. Similarly, qPCR analysis of select genes or ELISA assays for nuclear expression of NF-kB proteins p65 and c-Rel and c-Myc protein at 24h after irradiation, showed that only cell type differences lead to much more prominent gene expression differences regardless of exposures to radiation, alone or in combination with transfection of microRNAs miR-34a or miR-1228.

2. Materials And Methods

2.1 Cell Culture, Transfections and Irradiation Treatments

Human neuroblastoma cell lines SK-N-AS and SK-S-DZ (catalog numbers CRL-2137 and CRL-2149, respectively, ATCC, Manassas, VA) were maintained in high-glucose Dulbecco’s modified Eagle medium

(Corning, Glendale, AZ) supplemented with 10% fetal bovine serum (Gibco^{TM}, Waltham, MA). All cells were cultivated in presence of 1x antibiotic antimycotic solution (Corning, Glendale, AZ) and 1x Essential amino acids solution (Corning, Glendale, AZ), at 5% CO_2 at 37°C. For all transfection experiments, 6 x 10^5 cells per well were seeded in 6 well plate one day prior transfection. Plates intended for SK-N-DZ cells wells coated with Geltrex (Invitrogen, Waltham, MA) before use. Schematic of experimental treatments, cell collection, and assays are given in Supplemental Figure S1.

Transfections were done with Ambion Pre-miR miRNA precursors (#AM17100; miR-34a assay #PM11030, miR-1228 assay #PM13532; ThermoFisher Scientific, Waltham, MA), and respective negative controls (#AM17110, ThermoFisher) using Lipofectamine RNAiMax (Invitrogen, Waltham, MA) at 24h prior to intended assays. Total quantity of 700pmol of pre-miRs was resuspended into 2ml of medium with Lipofectamine mix following standard protocol. Transfection mixtures also contained a fluorescent dye that enabled additional confirmation that transfection was successful. Native expression and overexpression of miR-34a and miR-1228 was confirmed by corresponding TaqMan Assay 2x mix (catalog #4427975, assay IDs #000425 for miR-34a and #002919 for miR-1228, Applied Biosystems, Waltham, MA)(Supplemental Figures S2 and S3).

For clonogenic assays (Fig S1A), cells in T25 flasks were irradiated with 160 kVp X-rays at dose rate of 3.12Gy/min (RS200 Irradiator, RadSource, Buford, GA) with doses of 1, 2, 4, 6 or 7Gy. Cells were provided with fresh media immediately prior to irradiation and were trypsinized at 15 minutes post-irradiation to be seeded at different cell densities in 12, 24 and 48 well plates. Three to four weeks after plating, media was removed and cell colonies were fixed and stained over night with 0.001% crystal violet dissolved in 10% Neutral Buffered Formalin. The following day, plates were washed and dried prior to colony counting. This approach avoided extensive washes prior to fixation that could remove loosely attached SK-N-DZ cell colonies. Colonies with more than 50 cells were counted. Colonies that grew in six wells of a multi well plate (technical replicates) were counted for each separate experiment (biological replicate). Proportion of untreated and non-irradiated cells that successfully grew into colonies varied between experiments. This cell number was considered as 100% growth, and colony growth numbers for all treated cell samples were presented as percentages of this value.

For clonogenic assays with transfected cells, cells in six well plates were transfected with pre-miRNA molecules for 20 to 24h or non-transfected cells were irradiated with the same X-ray source with doses of 4 or 6 Gy. Again, cells were harvested by trypsinization and seeded at different cell densities; after several weeks of growth in the incubator, colonies were stained, washed, dried and counted as described above.

For RNA isolation and protein isolation, cells were irradiated twenty hours after transfection (Supplemental Figure S1B). For each experiment, transfections were done simultaneously with the same cell density and incubation times. Doses used for RNA and protein isolation from SK-N-AS cells were 4Gy and 6Gy, and for SK-N-DZ cells 2Gy and 4Gy. Based on clonogenic assay data, the radiation doses chosen gave roughly equivalent cell survival rates between the two cell lines. Cells were incubated 1h (Supplemental Figure S1B.a-c) or 24h (d-e) after irradiation prior to RNA isolation; cells used for

preparation of nuclear extracts were harvested 24h (Supplemental Figure S1B.f) after irradiation. In each case, experiments were done as biological triplicates for each transfection, irradiation and post-irradiation timepoint.

2.2 RNA Isolation for Quantitative Real-Time Polymerase Chain Reaction

Gene and microRNA expression levels were analyzed in SK-N-AS and SK-N-DZ cell lines, with and without radiation exposure. Total RNA and miRNA fractions were isolated with the mirVana^{TM} miRNA Isolation kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocol. miRNA overexpression was induced by transfecting SK-N-AS and SK-N-DZ cells with control miR, miR-34a, or miR-1228 constructs 24h prior to radiation treatment. Non-irradiated cultures were used as baseline control; SK-N-AS cells were irradiated at 4Gy and 6Gy while SK-N-DZ irradiated with 2Gy and 4Gy, consistent with prior sections. To confirm miRNA overexpression, cDNA was generated using specific primers for miR-34a, miR-1228, and endogenous control U6 snRNA (Thermo Fisher Scientific, Waltham, MA) and TaqMan MicroRNA Reverse Transcription kit (#4366496, Applied Biosystems, Waltham, MA). Overexpression of selected miRNA was confirmed using TaqMan assay for corresponding miRNA (Applied Biosystems, Waltham, MA) at 24h after transfection. Relative miRNA expression level was determined using control, non-irradiated sample (Supplemental Figure S3). Although pre-miR concentration used was the same for each cell line, a range of overexpression was detected, which may be correlated with factors that regulate different endogenous expression of these miRNAs in the two cell lines.

Quantitative real-time polymerase chain reaction (qPCR) for target genes of interest was performed with the Fast SYBR Green Master Mix (Applied Biosystems, Waltham, MA) by using the Applied Biosystems thermal cycler Model 7300. Expression was normalized against *GAPDH* expression as before [32], and relative quantification was calculated using the ΔΔCt method.

Results are represented as fold increase in the test samples compared with the sample transfected with control pre-miRNA. For qPCR, all samples were analyzed in three technical and biological replicates. The sequences of oligonucleotides used as qPCR primers are listed in Supplemental Table S1.

2.3 Gene Expression Array Analysis

cDNA for gene expression analysis was generated from 1 μg of DNase-treated total RNA using SuperScript® III First-Strand Synthesis SuperMix (Thermo Fisher Scientific, Waltham, MA). Next, RNA samples were diluted with DEPC treated water to final concentration of 50ng/ul and submitted to Northwestern University's NUSeq Core Facility for gene expression analysis.

The Human Clariom™ D GeneChip (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA), which allows to probe the expression of over 540,000 transcripts was used for the microarray study. The GeneChip WT PLUS Reagent Kit was used for RNA sample preparation for array hybridization. For each sample, 50 ng total RNA was used for cRNA target preparation, array hybridization, washing, staining and image scanning. The washing and staining steps were performed on a GeneChip Fluidics Station 450

and the scanning of hybridized arrays was conducted on a GeneChip Scanner 3000 7G. After generation, the array data was first examined for quality using QC criteria set by Affymetrix. All hybridizations were done in triplicate for biological replicates for each transfection, irradiation and post-irradiation timepoint. Sample and hybridization quality controls met the criteria for this array type.

Probeset data from the raw CEL files were summarized using the “affy” package (v. 1.68) [24] of Bioconductor (v. 3.12) [25] in R (v. 4.0.4) [26]. Genes were annotated with a custom CDF (v. 25) from the Molecular and Behavioral Neuroscience Institute (Brainarray) at the University of Michigan [27].

Signals were then standardized across arrays using the Supervised Normalization of Microarrays (SNM) method (v. 1.38) [28] in R/Bioconductor. The SNM model was fit with three biological variables: cell line, radiation dose level, and miRNA treatment. Variance due to batch processing was removed by modeling the microarray scan date as the adjustment variable and setting the “Rm = True” option.

Differential expression analysis was performed using the R package Limma (v. 3.46) [29]. Normalized intensities were fit to a linear model with coefficients for each of the 16 factor combinations of cell line (SK-N-AS or SK-N-DZ), radiation dose (0Gy or high dose) and miRNA treatment (miR-34a, miR-1228, control miRNA, or no miRNA). All pairwise comparisons of interest were extracted from this model as contrasts using empirical Bayes smoothing [30]. The false discovery rate (FDR) was controlled using the Benjamini and Hochberg correction. Probes with adjusted p-value < 0.05 and fold-change (FC) greater than 1.5 (that is, |log2FC| > 0.58) were judged to be differentially expressed.

For cellular pathways analyses, the gene expression lists were ordered based on differential gene expression in SK-N-AS vs. SK-N-DZ cells or vice versa under different conditions, and ENSEMBL codes for genes were submitted to g-profiler (https://biit.cs.ut.ee/gprofiler_beta/gost) [31]. Query conditions were: ordered gene list: TRUE; sources of pathways GO:MF, GO:CC, GO:BP, KEGG, REAC, TF, MIRNA, HPA, CORUM, HP, WP; significance threshold method g_SCS, threshold 0.05. Versions of the g-profiler used work with Ensembl 106 and Ensembl Genomes 53 as sources of ENSEMBL codes.

2.4 Isolation of nuclear extracts and ELISA assays for c-Myc and NF-kB

Content of c-Myc and NF-kB in nuclear extracts was detected using TransAM™ (Active Motif, Carlsbad, CA) ELISA based kits. Nuclear extracts were isolated from non-transfected and transfected, irradiated and non-irradiated SK-N-AS and SK-N-DZ cells using Nuclear Extract Kit (Active motif, Carlsbad, CA). Transfections were performed as described previously, cells were irradiated 24h after transfection and extracts isolated after a 24h post-irradiation incubation. Ten to seventeen micrograms per well were used for nuclear protein extract ELISAs for detection of NF-kB proteins p65, c-Rel and c-Myc. Detection was performed according manufacturer instructions using ClarioStar plate reader (BMG Labtech, Cary, NC 27513). The experiments were done in triplicates, with cell nuclear extracts isolated from three independently performed transfection-irradiation experiments.

3. Results

3.1 Clonogenic assays show marked differences in radiation response between neuroblastoma cell lines SK-N-AS and SK-N-DZ

Neuroblastic SK-N-DZ cells [3,33] have low adhesion, which hampers manipulation required for irradiation of already plated cell culture dishes with single cells. In previous ionizing radiation experiments using Cs-137 gamma rays, we have used MTS assays in order to circumvent this problem [23]. In this work, we utilized a different approach by irradiating relatively confluent cell culture dishes, followed by trypsinization and seeding clonogenic assays after a 15 minute post-irradiation incubation (Supplemental Figure S1A). The same approach was used for SK-N-AS cell line, which is more substrate-adherent. Single cells generated by trypsinization of irradiated or sham irradiated 70% confluent cells were permitted to attach to the plate and grow into colonies over a period of 3-6 weeks depending on cell line. Figure 1 shows the survival curves for SK-N-AS and SK-N-DZ cell lines, demonstrating that a dose of 6Gy causes 90% cell killing of SK-N-AS cells while equivalent cell killing of SK-N-DZ requires only 4Gy.

3.2 Neuroblastoma cell lines SK-N-AS and SK-N-DZ show opposite pattern of expression for microRNAs miR-1228 and miR-34a

A preliminary gene expression evaluation of cell lines SK-N-AS and SK-N-DZ using Cs-137 gamma rays (not published) showed numerous messenger RNA expression differences as well as opposite expression patterns for microRNAs miR-1228 and miR-34a. We repeated evaluation of expression for the two miRs in the two cell lines by qPCR (Supplemental Figure S2) and found a robust and reproducible expression of miR-1228 in SK-N-AS cells while expression of miR-34a was several-fold lower in the same cell line. MicroRNA miR-1228 is one of the handful of miRs known to be increased in response to ionizing radiation in human embryonic stem cell line H1 [34]. MiR-1228 is also a so-called mirtron – a microRNA released from an intron of low density lipoprotein receptor related protein 1 precursor (LDP1) [35].

Conversely, in SK-N-DZ cells (Supplemental Figure S2) relative quantification obtained by qPCR showed a several fold higher expression of miR-34a compared to miR-1228. In previous neuroblastoma studies, expression of miR-34a was found to be associated with a good prognosis [36,37]. In neuroblastoma cell lines *in vitro*, overexpression of miR-34a was found to be cytotoxic [38,39].

To evaluate whether presence of these two miRNAs at the time of irradiation would change cell survival or response to radiation, we transfected both cell lines with miR-34a and miR-1228 (Supplemental Figure S1) prior to irradiation and additional studies. Overexpression of the two miRNAs was robust in both cell lines in all subsequent experiments (Supplemental Figure S3).

3.3 Clonogenic assays show no cell survival differences between native and microRNA transfected neuroblastoma cell line SK-N-AS

SK-N-AS cells were transfected with pre-miRNAs for mature miR-1228 and miR-34a as described in the methods section. Successful transfection was monitored by fluorescence readings of the cells used for clonogenic assay, and by qPCR of the cells transfected by pre-miRs in parallel, simultaneously and

with the same transfection mixtures as cells used for clonogenic assay (Supplemental Figure S3). Twenty-four hours after transfection, cells were irradiated and 15 minutes later trypsinized and seeded as single cells to grow into colonies. Four biological replicates and three technical replicates of each experiment were done; no effects of miRs of interest were noted. Across different experimental conditions, percentage of cells growing into colonies after 4Gy exposure varied between 0.05 and 4.03% for non-transfected cells, 0.02 and 3.8% for control miR transfected cells; 0.15 to 3.56% for cells transfected with miR-1228, and 0.14 to 4.28% for cells transfected with miR-34a. Similarly, percentage of cells growing into colonies after 6Gy exposure was 0.11 to 1.81 % for non-transfected cells, 0.16 to 1.63 % for control miR transfected cells; 0.13 to 1.73 % for cells transfected with miR-1228; and 0.18 to 1.94 % for cells transfected with miR-34a. It should be noted that these numbers are lower than what was shown for SK-N-AS cell line in previous clonogenic experiments series. This may be the result of the fact that the cells in this experiment have undergone two trypsinization steps within a period of 48h and their capacity to re-attach to the plate may have been diminished. Regardless, the data are fairly consistent for each radiation dose irrespective of the pre-miR treatment, suggesting that the overexpression of miR-1228 or miR-34a has no significant effect on viability of SK-N-AS cells in combination with 4 or 6 Gy radiation exposure.

3.4 Clariom™ D Assay Gene Expression Study of Neuroblastoma Cell Lines SK-N-AS and SK-N-DZ

Gene expression differences between different neuroblastoma cell lines are well documented [2,40]; however, gene expression comparisons between cell lines SK-N-AS and SK-N-DZ were not done until this work. In this study, cells were irradiated with X-ray doses that cause comparatively similar degree of cell death: 6 Gy for cell line SK-N-AS and 4 Gy for SK-N-DZ. For each cell line, we have also transfected the cells to overexpress microRNAs miR-1228 and miR-34a for 24h prior to X-ray radiation exposure (Supplemental Figure S1). Three biological replicates for each treatment were done and the cells were harvested for RNA isolation for microarray work 1h after radiation exposure. Gene expression was evaluated with Clariom D gene expression array; the data have been deposited into GEO database (GSE197124). Gene expression normalization and differential expression were evaluated as described in the Methods section. Interestingly, despite the fact that the dose of radiation used with each cell line was cytotoxic and resulting in no more than 10% of the cells growing into colonies, at the one hour post-irradiation timepoint gene expression differences between irradiated and non-irradiated cells were almost non-existent. For example, no differentially expressed genes were found when comparing the 0Gy and 6Gy conditions in either non-transfected or control miR transfected SK-N-AS cells. In SK-N-AS cells transfected to overexpress miR-1228, only two differentially expressed genes with FC above 1.5-fold threshold were found between the irradiated and non-irradiated conditions: the C-C motif chemokine ligand 2 (*CCL2*, ENSEMBL ID ENSG00000108691) and the U5B small nuclear RNA 1 (*RNU5B-1*, ENSEMBL ID ENSG00000200156). In SK-N-AS cells transfected to overexpress miR-34a, a single gene, NF-kB inhibitor alpha (*NFKBIA*, ENSEMBL ID ENSG00000100906), was found to be overexpressed between irradiated and non-irradiated conditions. In SK-N-DZ cells, irradiation with 4Gy dose of X-rays did not cause any differential gene expression compared with the non-irradiated cells; this was true in all miRNA treated and untreated cells.

Conversely, statistically significant ($p<0.01$) gene expression differences between the two cell lines were marked at the 1.5 FC threshold (Figure 2), with more than 10% or 4000 differentially expressed genes out of the total 38833 genes. Figure 2A depicts an Euler plot of the number of differentially expressed genes between the SK-N-AS and SK-N-DZ cell lines in each of the miRNA conditions, without radiation. Figure 2B shows that similar ratios of differentially expressed genes between the cell lines are evident one hour after irradiation as well.

The complete gene expression datasets are available in GEO (GSE197124); however, four tables with genes affected by radiation are provided in supplemental data (Supplemental Tables S3-S6). Each table shows the genes that are either repressed or induced by radiation and differently expressed in the two cell lines, for each of the four experimental conditions: no-transfection, control miR transfection or transfection with pre-miRs for 34a and 1228. Next, ordered lists of ENSEMBL gene IDs were submitted to g-profiler and used to generate lists of GO Molecular Function (GO:MF), GO Biological Processes (GO:BP) and the lists of microRNAs likely to be involved in gene expression regulation of these pathways (Supplemental Figures S4-S7).

More specifically, comparison of gene expression in non-transfected SK-N-AS cells vs. SK-N-DZ cells generated a list of genes that are not modulated by radiation (not shown) and four lists of genes either suppressed or induced by radiation in one of the cell lines compared with the other (Supplemental Table S3). In this table “AS vs DZ w/o IR” corresponds to genes that are suppressed by radiation and that are upregulated in SK-N-AS cells compared to SK-N-DZ cells in the absence of radiation. “DZ vs AS w/o IR” is a list of genes that are suppressed by radiation and that are upregulated in SK-N-DZ cells compared to SK-N-AS cells in the absence of radiation. “AS vs DZ IR” corresponds to genes that are induced by radiation and upregulated in SK-N-AS cells compared to SK-N-DZ cells at 1h after radiation. “DZ vs AS IR” corresponds to genes that are induced by radiation and upregulated in SK-N-DZ cells compared to SK-N-AS cells at 1h after radiation. ENSEMBL designations for the differentially expressed genes in Supplemental Table S3 were used in g-profiler to generate Supplemental Figure S4.

Comparison of gene expression in control-transfected SK-N-AS cells vs. SK-N-DZ cells generated a list of genes that were not modulated by radiation (not shown) and four lists of genes either suppressed or induced by radiation in one of the cell lines compared with the other (Supplemental Table S4). “AS vs DZ w/o IR” corresponds to genes that are suppressed by radiation and that are upregulated in control-transfected SK-N-AS cells compared to SK-N-DZ cells in the absence of radiation. “DZ vs AS w/o IR” is a list of genes that are suppressed by radiation and that are upregulated in control-transfected SK-N-DZ cells compared to SK-N-AS cells in the absence of radiation. “AS vs DZ IR” corresponds to genes that are induced by radiation and upregulated in control-transfected SK-N-AS cells compared to SK-N-DZ cells at 1h after radiation. “DZ vs AS IR” corresponds to genes that are induced by radiation and upregulated in control-transfected SK-N-DZ cells compared to SK-N-AS cells at 1h after radiation. ENSEMBL designations for the differentially expressed genes in Supplemental Table S4 were used in g-profiler to generate Supplemental Figure S5.

Comparison of gene expression in SK-N-AS cells vs. SK-N-DZ cells transfected with pre-miR 34a generated a list of genes that were not modulated by radiation (not shown) and four lists of genes either suppressed or induced by radiation in one of the cell lines compared with the other (Supplemental Table S5). “AS vs DZ w/o IR” corresponds to genes that are suppressed by radiation and that are upregulated in SK-N-AS cells compared to SK-N-DZ cells, both transfected with pre-miR 34a and in the absence of radiation. “DZ vs AS w/o IR” is a list of genes that are suppressed by radiation and that are upregulated in SK-N-DZ cells compared to SK-N-AS cells, both transfected with pre-miR 34a in the absence of radiation. “AS vs DZ IR” corresponds to genes that are induced by radiation and upregulated in SK-N-AS cells compared to SK-N-DZ cells, both transfected with pre-miR 34a at 1h after radiation. “DZ vs AS IR” corresponds to genes that are induced by radiation and upregulated in SK-N-DZ cells compared to SK-N-AS cells, both transfected with pre-miR 34a at 1h after radiation. ENSEMBL designations for the differentially expressed genes in Supplemental Table S5 were used in g-profiler to generate Supplemental Figure S6.

Comparison of gene expression in SK-N-AS cells vs. SK-N-DZ cells transfected with pre-miR 1228 generated a list of genes that were not modulated by radiation (not shown) and four lists of genes either suppressed or induced by radiation in one of the cell lines compared with the other (Supplemental Table S6). “AS vs DZ w/o IR” corresponds to genes that are suppressed by radiation and that are upregulated in SK-N-AS cells compared to SK-N-DZ cells, both transfected with pre-miR 1228 and in the absence of radiation. “DZ vs AS w/o IR” is a list of genes that are suppressed by radiation and that are upregulated in SK-N-DZ cells compared to SK-N-AS cells, both transfected with pre-miR 1228 in the absence of radiation. “AS vs DZ IR” corresponds to genes that are induced by radiation and upregulated in SK-N-AS cells compared to SK-N-DZ cells, both transfected with pre-miR 1228 at 1h after radiation. “DZ vs AS IR” corresponds to genes that are induced by radiation and upregulated in SK-N-DZ cells compared to SK-N-AS cells, both transfected with pre-miR 1228 at 1h after radiation. ENSEMBL designations for the differentially expressed genes in Supplemental Table S6 were used in g-profiler to generate Supplemental Figure S7.

3.5 Selected gene and protein expression differences

A subset of genes were selected from the microarray gene expression analysis to confirm findings from prior section. Cells were untransfected, or transfected with control miR or pre-miRNAs that lead to overexpression of miR-34a or miR-1228 for 24h, then irradiated and total messenger RNA isolated at 1h (Supplemental Figure S1B.b) or 24h (Supplemental Figure S1B.e) after irradiation and analyzed by qPCR (Supplemental Figures S8-S10).

A small selection of proteins was also examined by subsequent ELISA analyses in both cell lines at 24h post-irradiation (Supplemental Figure S1B.f). In SK-N-AS cells, transfection with pre-miRNA for miR-34a led to about 1.5-fold increase in expression of inhibitor of NF-kB alpha. Nuclear concentration of some of the NF-kB proteins may thereby be impacted, and we chose p65 and c-Rel for further investigation. While p65 is one of the NF-kB proteins dependent on IKB expression, c-Rel is not and we

anticipated that the differences in p65 vs. c-Rel expression pattern will confirm the role of IKB in modulation of radiation response in SK-N-AS cells. However, levels of expression of both proteins were low and no significant trend in protein quantities could be detected (Supplemental Figures S11 and S12).

Finally, we also conducted ELISA evaluation of c-Myc protein in the two cell lines (Supplemental Figure S13). As expected, SK-N-AS cell line that is not overexpressing MYCN had higher expression of c-Myc protein. In SK-N-DZ cell line, which has MYCN gene amplification, expression of c-Myc was negligible.

4. Discussion

Differences between the N-type and S-type neuroblastoma cell lines such as SK-N-DZ and SK-N-AS, respectively, are very pronounced and their responses to radiation stress are equally heterogeneous. Studies using Chip-Seq to investigate transcription factors regulating gene expression in different neuroblastoma cell lines, including the two used here, identified 37 transcription factors with distinctive pattern of presence in the cell lines SK-N-AS (considered “intermediate type”) and cell line SK-N-DZ (considered a “type I” cell type).

In this work, we exposed the two cell lines to doses of radiation which caused 90% cell killing for each cell line. In most other cancer cell lines, analogous stress exposures lead to marked gene expression differences. In our examination of short-term effects of radiation, we found only subtle differences in gene expression between non-irradiated and irradiated cells of the same type, regardless of miR-1228 or miR-34a overexpression in either cell line. At the same time, gene expression differences between the two cell lines included many hundreds of genes (Figure 2). When we specifically selected radiation associated genes from this list (Supplemental Tables S3-S6) and used them to identify GO Molecular Function and GO Biological Processes (Supplemental Figures S4-S7), none of them were found to be repeatedly associated with radiation exposure, once again suggesting that radiation exposures that cause 90% cell killing have no profound effect on early gene expression.

Low fold-changes in gene expression array studies of neuroblastoma cell lines exposed to different growth and treatment conditions have been observed in the past as well. For example, SK-N-DZ cells grown as neurospheres show altered gene expression for some 11% of the registered genes only if fold changes criterion is set to “anything above 1” [40]. In this work, not a single gene was found to be modulated above 1.5-fold in SK-N-DZ cells in the first hour after radiation. In SK-N-AS cell line, non-transfected or control transfected cell lines showed no changes in gene expression 1h after exposure to 6Gy. Only changes found in SK-N-AS cells were noted in cells transfected with miR-34a, which showed an increase in inhibitor of NF-kB alpha (*NFKBIA*), while SK-N-AS cells overexpressing miR-1228 showed higher expression of U5B small nuclear RNA 1 (*RNU5B-1*) and the C-C motif chemokine ligand 2 (*CCL2*). Transfections of SK-N-AS cells with either pre-miRNA did not lead to any significant change of cell survival. At the same time, gene expression differences between the two cell lines included several thousands of genes (Figure 2), about 1% of all the genes expressed in each cell type. Taken together,

these findings suggests that gene expression regulation in neuroblastoma cell lines depends on stably engaged gene regulation patterns as suggested by genomic DNA-enhancer studies [42]. It is probable that the gene expression patterns such as these found in two different types of neuroblastoma cell lines are not easily altered in response to stress. What the long-term effects of radiation stress may be will require evaluation of gene expression arrays for post-irradiation timepoints beyond 1h.

5. Conclusions

In conclusion, neuroblastoma cells demonstrate subtle gene expression differences at early timepoints after exposure to external radiation, indicating stable gene regulation patterns regardless of tumor cell subtype. While this makes conventional therapy difficult to effectively utilize, it also provides justification for continued attempts to develop new therapeutic modalities of this pediatric cancer. Exploring whether most neuroblastoma cell types show similarly “stress resistant” gene regulation in future studies would further these findings.

Declarations

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: Characterization of NBL cell lines used; Table S2: PCR primers for qPCR; Table S3: Gene expression in non-transected SK-N-AS cells vs. SK-N-DZ cells for genes either suppressed or induced by radiation; Table S4: Gene expression in control transfected SK-N-AS cells vs. SK-N-DZ cells for genes either suppressed or induced by radiation; Table S5: Gene expression in SK-N-AS cells vs. SK-N-DZ cells transfected with pre-miR-34a and either suppressed or induced by radiation; Table S6: Gene expression in SK-N-AS cells vs. SK-N-DZ cells transfected with pre-miR-1228 and either suppressed or induced by radiation; Figure S1: Schematic of experimental design; Figure S2: Comparison of native expression for miR-34a and miR-1228 in neuroblastoma cell lines SK-N-AS and SK-N-DZ demonstrates reverse expression pattern; Figure S3: Relative quantification of overexpression of miR-34a and miR-1228 in neuroblastoma cell lines SK-N-AS and SK-N-DZ used in miRNA overexpression experiments shown in Figure S1; Figure S4: Different pathways in irradiated non-transfected neuroblastoma cell lines; Figure S5: Different pathways in irradiated neuroblastoma cell lines transfected with control miR; Figure S6: Different pathways in irradiated neuroblastoma cell lines transfected with pre-miR-34a; Figure S7: Different pathways in irradiated neuroblastoma cell lines transfected with pre-miR-1228; Figure S8: Expression of NF-kBIB mRNA in native or transfected SK-N-AS cells at 1h or 24h after irradiation; Figure S9: Expression of DNAJB2 gene in in native or transfected SK-N-AS cells at 1h or 24h after irradiation; Figure S10: Expression of DENNA4 gene in in in native or transfected SK-N-DZ cells at 1h or 24h after irradiation; Figure S11: Relative expression of p65 NFkB subunit in cell nuclei of neuroblastoma cell lines SK-N-AS and SK-N-DZ; Figure S12: Relative expression of c-Rel NFkB subunit in cell nuclei of neuroblastoma cell line SK-N-AS; Figure S13: Relative expression of c-Myc protein in cell nuclei of neuroblastoma cell lines SK-N-AS and SK-N-DZ.

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Data Availability Statement: The microarray dataset presented in this study is openly available in NCBI's Gene Expression Omnibus (Edgar, et. al. 2002) and is accessible through GEO Series accession number GSE197124.

Conflicts of Interest: The authors declare no conflict of interest.

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Figures

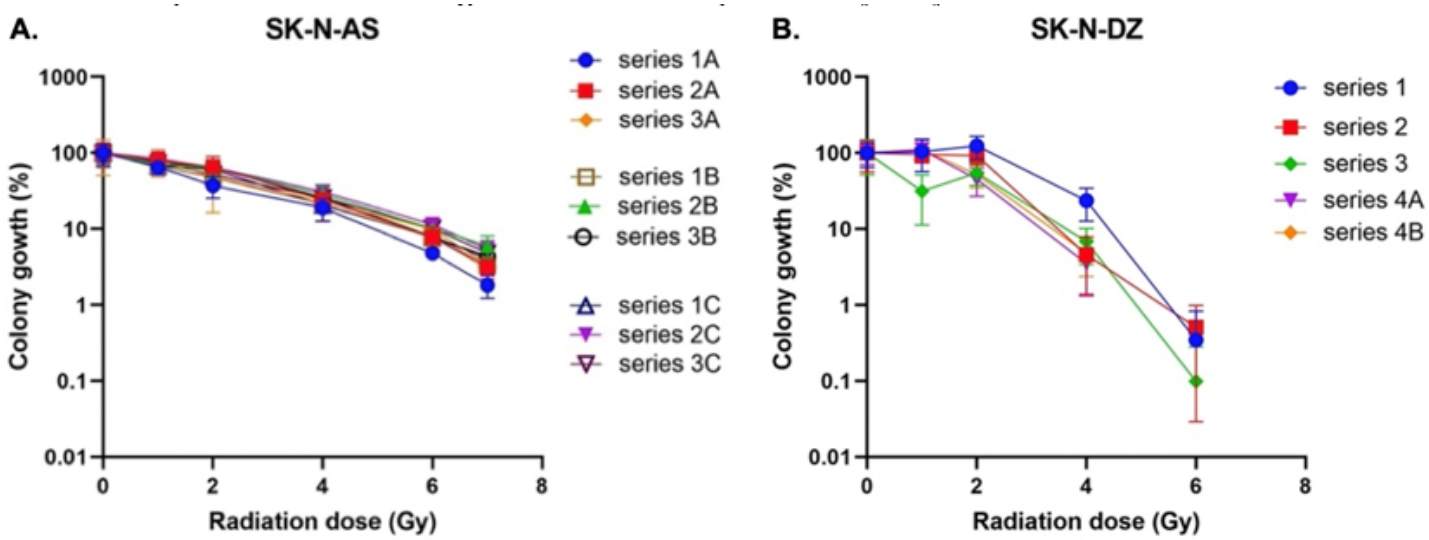

Figure 1

Clonogenic assay results for SK-N-AS (A) and SK-N-DZ (B) cell lines; series with different numbers are different biological replicates, series with different letters after the same number represent technical variations of the same sample (e.g. different multiwell plate formats).

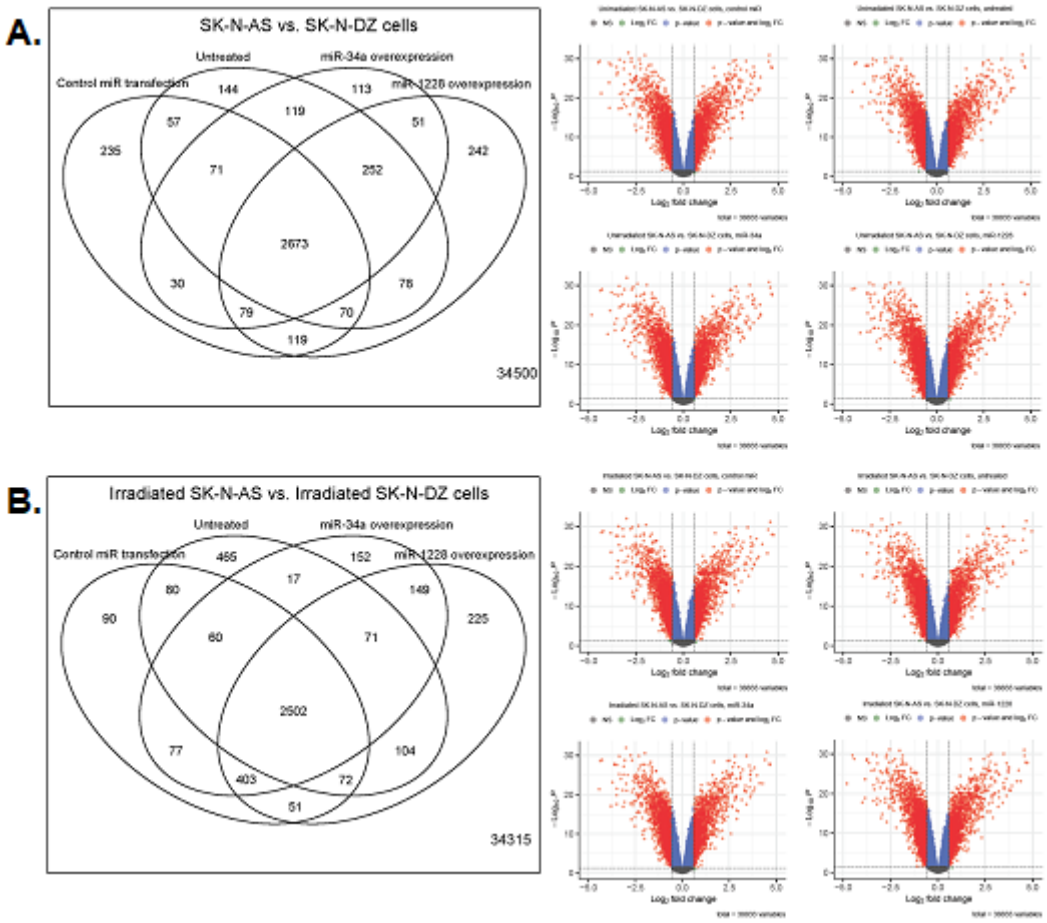

Figure 2

Euler plots for gene expression differences with FC magnitude equal to or greater than 1.5-fold (|log2FC| > 0.58), for different combinations of cell samples indicated above the circles and their corresponding volcano plots. Comparisons of SK-N-AS and SK-N-DZ cells are shown, with non-irradiated (A) and irradiated (B) samples.

Supplementary Files

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- CRitnerOncoSupplemental2022.docx
- TableS3.xlsx
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