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A Study of Radiation-Induced Instability for the Gene Locus Associated with Intellectual Disorders or Developmental Delays

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Abstract

Multiplex Ligation-Dependent Probe Amplification (MLPA) was used to study the integrity of the chromosomes for two WIL2-derived lymphoblastoid cell lines (TK6 and WTK1) in the presence and absence of ionizing radiation. WTK1 cells contain a p53 mutation, whereas the TK6 cell line has the native p53 tumor-suppressor gene. Each cell line was isolated pre- and post-irradiation (2 and 3 Gy) and analyzed by MLPA. Using probes that target specific regions on chromosomes associated with a distinct subset of microdeletions and microduplications either established or thought to be responsible for intellectual disability or developmental delay, we have demonstrated that WTK1 and TK6 are not impacted in the same way by irradiation. Instead, each cell line presents its own unique MLPA profile. The most notable differences are the appearance of nine unique probe signals only seen in WTK1 cells. These results are important in the study of how different cell lines can be affected in significantly different ways depending on the presence or absence of wild type p53.

Keywords

Ionizing Radiation, Multiplex Ligation-Dependent Probe Amplification (MLPA), Intellectual Disability (ID), Developmental Delay (DD), p53 Tumor Suppressor

1. Introduction

p53 is a critical cell-cycle check-point protein that regulates the G1 phase of the cell cycle and is directly responsible for maintaining genomic DNA stability during the DNA damage repair stage. It also coordinates the repair of damaged

DNA and the removal of DNA lesions before the cell enters S-phase. Any problems that might occur during the G1 cell cycle arrest may therefore impact normal repair to the damaged DNA that occurs prior to entering S-phase. The p53 gene has been identified as one of the most frequently mutated genes in human cancers [1] [2] [3] [4], but the question of how far-reaching the effects of these mutations are on cell integrity is still being studied. The p53 regulatory protein is crucial in cellular response to DNA damage; it plays a direct role in the DNA repair pathways, affecting the activities of a number of diverse regulatory proteins that, collectively, control the early stages of the cell cycle [5]-[19]. Mutations or removal of the p53 protein may lead to genomic instability, resulting in abnormal numbers of chromosomes, gene amplifications, chromosomal rearrangement, deletions, insertions, accumulation of double-strand breaks, and gene amplifications [20]-[26]. Apoptosis, or controlled cell death, also appears to be affected by p53 mutations, as cell lines with these alterations have a reduced frequency of apoptotic death, which could in turn lead to the accumulation of gene mutations and progression of tumorigenesis [27] [28] [29] [30].

Two closely related WIL2-derived lymphoblastoid cell lines were utilized in this study. The WTK1 cell line is a TP53-knockout mutant derived from the WIL2 parent cell line; the p53 gene in the WTK1 cell line has a substitution at codon 237, which leads to a mutation in the p53 protein from methionine to isoleucine. This cell line will only overexpress this mutant p53 protein; no wild type p53 will be present. The TK6 cell line is also derived from the WIL2 parent cell line but is a thymidine kinase heterozygote cell line that is wild-type for p53 [31] [32] [33] [34]. Previous studies on the effects of ionizing radiation on the two cells lines show that the WTK1 cell line is more resistant to radiation-induced killing and that there is significantly less apoptosis in WTK1 when compared to TK6. Mutability was also shown to be drastically different, with TK6 exhibiting a 10-fold decrease when compared to WTK1 [35] [36].

The aim of this study was to begin to elucidate the effects of ionizing radiation, in the presence and absence of the p53 tumor suppressor protein, on specific regions of the chromosomes that are associated with a number of microdeletion and microduplication syndromes. As stated by MRC Holland, the supplier of the P064 probe set, most patients with these syndromes present with intellectual disability, developmental delay, and/or congenital abnormalities (which in the past could be referred to as mental retardation). We have employed the use of Multiplex Ligation-Dependent Probe Amplification (MLPA) in combination with a specific probe mix (P064) to ascertain if there are copy number variations for chromosomal sequences in both the TK6 and WTK1 cells. MLPA is a variation of the multiplex polymerase chain reaction. It permits amplification of multiple targets with only a single primer pair and has become a preferred method for detecting deletions and amplifications in human genes, because it requires far less DNA and is not as time-consuming as other techniques such as Southern blots and fluorescent *in situ* hybridization [37] [38] [39] [40].

2. Materials and Methods

2.1. Cell Lines and Cell Culturing

Cells used were the thymidine kinase heterozygote cell line TK6 and the TP53 mutant cell line WTK1. Both cell lines were each cultured in a 75 cm^2 tissue culture flask at 37˚C with 8% CO_2 and maintained at a cell concentration between 2 × 10^5 and 1 × 10^6 cells/mL. The culture medium consisted of RPMI 1640 and 10% v/v heat-inactivated horse serum (Gibco®, a ThermoFisher Scientific Company, Waltham, MA, USA).

2.2. Irradiation of Cell Lines and Preparation for MLPA Analysis

The two lymphoblastoid cell lines (TK6 and WTK1) were treated with ionizing radiation (3 Gy vs. 0 Gy control) using a ^{137}Cs biological irradiator (Gammacell-1000 Unit). Each cell line was irradiated 0.3 cm from the surface with a dose rate of 6.41 Gy/min for 28 seconds to provide a total of 3 Gy exposure. After irradiation, cells were incubated for 48 hours at 37˚C under 8% CO_2, followed by isolation for MLPA analysis. Whole genomic DNA was isolated from both cell lines using a DNeasy Blood and Tissue Kit (Qiagen). Approximately 5 million cultured cells (WTK1 or TK) were centrifuged separately for 5 minutes at 300× g. After centrifugation, 200 µL of PBS (50 mM potassium phosphate, 150 mM NaCl, pH 7.2) was used to resuspend the pellet. Once all cells were in free suspension, 20 µL of Proteinase K (600 mAU/mL) and 4 µL of RNase A (100 mg/mL) were added, and the cells were incubated at room temperature for 2 minutes.

After the incubation period, 200 µL of AL buffer (Qiagen) was added, the suspension was mixed by vortexing, and the cells were incubated again for 10 minutes at 56˚C. The sample tube was left to cool to room temperature before 200 µL 95% molecular grade ethanol was added, and the sample was vortexed until it was thoroughly mixed. The sample was then placed in a DNeasy spin column and centrifuged at 6000× g for 1 minute. The flow through and collection tube were discarded and the DNeasy spin column was placed in a new tube. 500 µL of AW1 buffer (Qiagen) was added to the tube and centrifuged at 6000× g for 1 minute. The DNeasy spin column containing the DNA was placed in a new collection tube and 500 µL of AW2 was added and centrifuged at 20,000× g for 1 minute. The DNeasy column was then put into a clean tube and 200 µL of AE buffer (Qiagen) was added to the membrane and left to incubate at room temperature for 1 minute. The sample was then eluted by centrifuging at 6000× g for 1 minute.

2.3. Preparation of Genomic DNA for MLPA Analysis

A total of 50ng of genomic DNA from either WTK1 or TK6 cellular extracts in a volume of 5 µL was used for each MLPA reaction. Dilutions were carried out using AE buffer (Qiagen, 10 mM Tris-HCl, 0.5 mM EDTA, pH 9.0). The DNA was put into a 0.2-µL PCR tube and denatured at 98˚C for 5 minutes. Samples

were cooled to room temperature before continuing with the hybridization step.

A hybridization master mix consisting of 1.5 µL of buffer (KCl, Tris-HCl, EDTA, PEG-6000, oligonucleotides (pH 8.5)) and 1.5 µL SALSA MLPAProbe-mix P064 Microdeletion Syndromes-1B(MRC Holland), for a total volume of 3 µL, was added to the DNA and allowed to incubate for 1 minute at 95˚C. Hybridization was then carried out for 20 h at 60˚C.

2.4. PCR Amplification

A ligation master mix was made containing 25 µL sterile H_2O, 3 µL NAD (of bacterial origin) pH 3.5, 3 µL Tris-HCl, non-ionic detergents, $MgCl_2$ (pH 8.5), and 1 µL Ligase-65 enzyme, for a total volume of 32 µL. Samples to be ligated were placed in a 54˚C water bath. When the samples reached 54˚C, the full volume of the ligation mix was added to the reaction tube. The ligation reaction was allowed to continue at 54˚C for 15 minutes and then was subsequently heat inactivated for 5 minutes at 98˚C.

Amplification of the ligated probes was carried out by PCR. A polymerase master mix containing 7.5 µL dH_2O, 2 µL SALSA FAM-labeled PCR primer mix, and 0.5 µL SALSA polymerase was added to the reaction tube. Amplification was performed under the following parameters: 35 cycles of 30 seconds 95˚C; 30 seconds 60˚C; 60 seconds 72˚C; 20-minute incubation at 72˚C.

Electrophoresis and fragment analysis were carried out using the ABI-3130 CE capillary sequencer and Coffalyser.Net data analysis software. A specific probe set (P064) was then used to study for the detection of a distinct subset of recurrent microdeletions and microduplications known to be associated with intellectual disorders and development delays. The GS500-250 size marker was used as a size standard in conjunction with a 6-FAM dye.

2.5. Statistical Analysis

Statistical analysis was carried out using Microsoft Excel. Data was collected as a function of probe signal intensity. The total area under the peak for each probe was obtained and normalized using the Q-fragment at position 76, which is a control for sufficient DNA addition and successful ligation. This specific control is used to determine DNA concentration; once the samples were normalized for their concentration, a direct comparison of the adjusted signals for each sample could be made. After normalization, the data were further adjusted so that the datum point with the greatest area under the peak was set at 100% probe signal and all other data points were represented relative to this value. Experimental data sets were then subtracted from the baseline (given by the control data set responses) and data was plotted as a percentage difference from the control sample. In this case, a response greater than the control was shown as a positive, and a response lesser than the control was shown as a negative.

3. Results

By employing the use of Multiplex Ligation-Dependent Probe Amplification

(MLPA), we were able to investigate chromosomal copy number imbalances associated with intellectual development disorders syndromes.

3.1. MLPA Analysis—TK6 Results

Figure 1 shows the MLPA data after 24 and 48 h incubation for 0 Gy exposure to irradiation for the TK6 cell line as a function of probe signal intensity. This set of data serves as the control for this cell line. The percent peak ratio for almost all probes (92.6%) decreases to varying extents, between 1.0% and 45.9%, between the 24h and 48 h incubation samples, potentially due to cellular degradation. By comparing the data in Figure 1 with data gathered under similar conditions post-irradiation, any changes that might have occurred as a result of ionizing radiation can be elucidated.

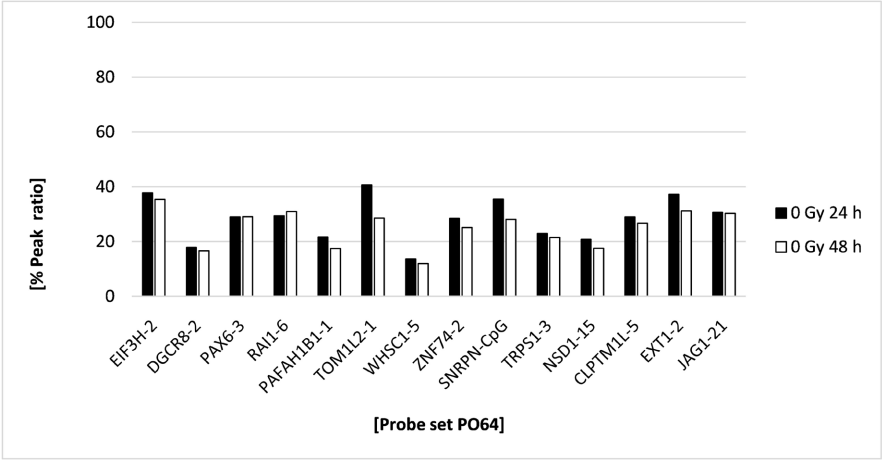
Figure 2 shows the MLPA data for 2 Gy and 3 Gy exposure to irradiation after 24 and 48 h incubation for the same (TK6) cell line as a function of probe signal intensity. All probes in this probe set for 2 Gy irradiation show a large decrease in percent peak ratio between 24 and 48 h incubation, ranging from 72.7% to 90.5%. Many probes in this probe set for 3 Gy irradiation also show a decrease in percent peak ratio between 24 and 48 h incubation, ranging from 0.8% to 64.9%. However, some probes (29.6%) in this probe set for 3 Gy irradiation show an increase in percent peak ratio between 24 and 48 h incubation, ranging from 0.6% to 53.6%. The pre- and post-irradiation data do not show an obvious pattern of copy number imbalance that would be evident in the gain or loss of probe enhancement. However, it is interesting that some probes increase in percent peak ratio between incubation times when exposed to 3 Gy radiation but do not when exposed to 2 Gy radiation. It is unclear what drives this difference.

3.2. MLPA Analysis—WTK1 Results

Figure 3 shows the MLPA data after 24 and 48 h incubation for 0Gy exposure to irradiation for the WTK1 cell line as a function of probe signal intensity for the probe sets that are also found in the TK6 results. This set of data serves as the control for the WTK1 cell line. The percent peak ratio for 37.9% of the probes actually increases to varying extents, between 9.6% and 318.0%, between the 24 h and 48 h incubation samples. The rest of the samples have a decrease in percent peak ratio ranging from 31.1% to 94.3%. These results, where approximately one-third of the samples have an increase in percent peak ratio between 24 and 48 h with no irradiation, is a noticeable change from the TK6 control post-incubation samples.

Figure 4 shows the MLPA data after 24 and 48 h incubation for 0 Gy exposure to irradiation for the WTK1 cell line as a function of probe signal intensity for the two probes that are not found in the TK6 results. These two probes—NSD1-24 and METTL16-1, indicate a potential fundamental difference between the two cell lines in the absence of irradiation.

By comparing the data in Figure 3 and Figure 4 with data gathered under similar conditions post-irradiation, any changes that might have occurred as a



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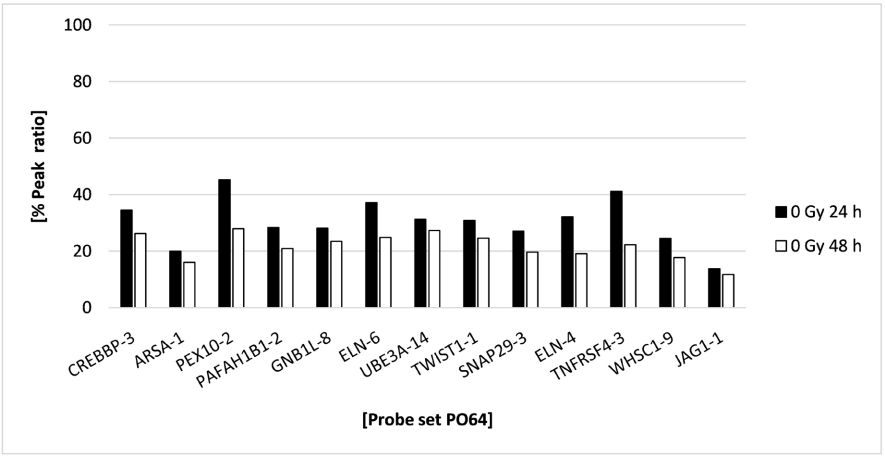
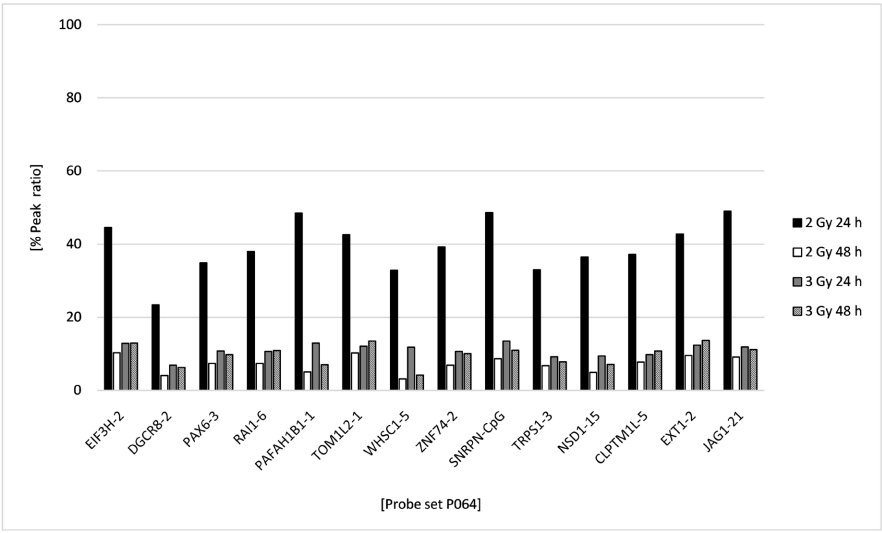


(b)

Figure 1. The data after 24 (black bars) and 48 (white bars) h incubation for 0 Gy exposure to irradiation for the TK6 cell line as a function of probe signal intensity. This is the control for this cell line. Each set of bars represents different probes in the P064 probe set.



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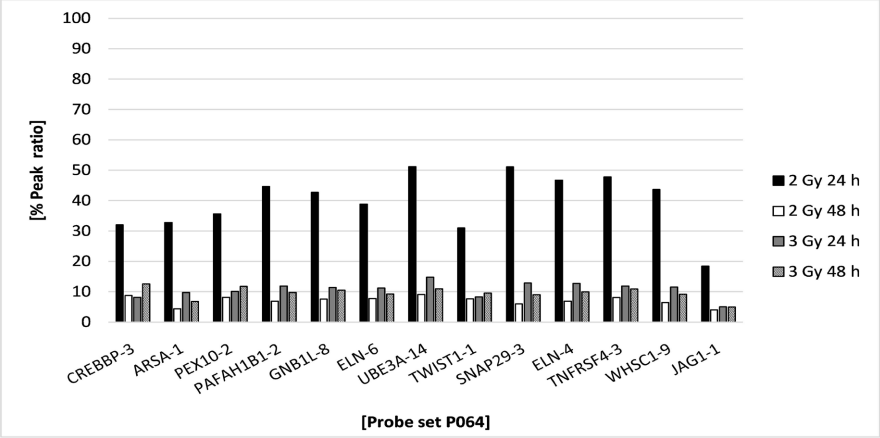

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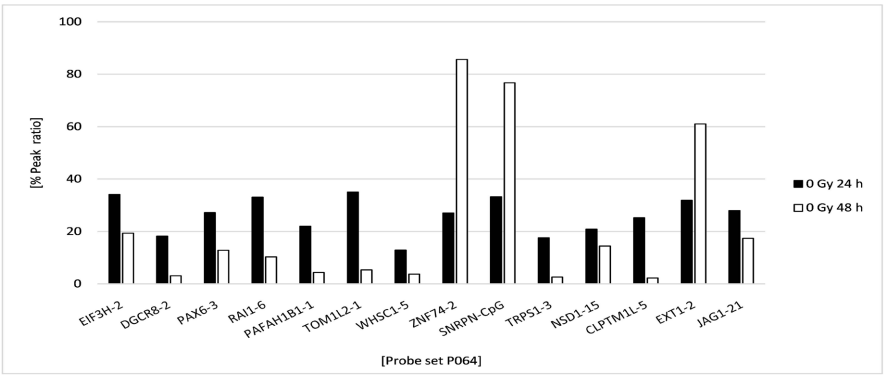
Figure 2. The data after 24 (black and gray bars) and 48 (white and hashed bars) h incubation for 2 Gy (black and white bars) and 3 Gy (grey and hashed bars) exposure to irradiation for the TK6 cell line as a function of probe signal intensity. Each set of bars represents different probes in the P064 probe set.



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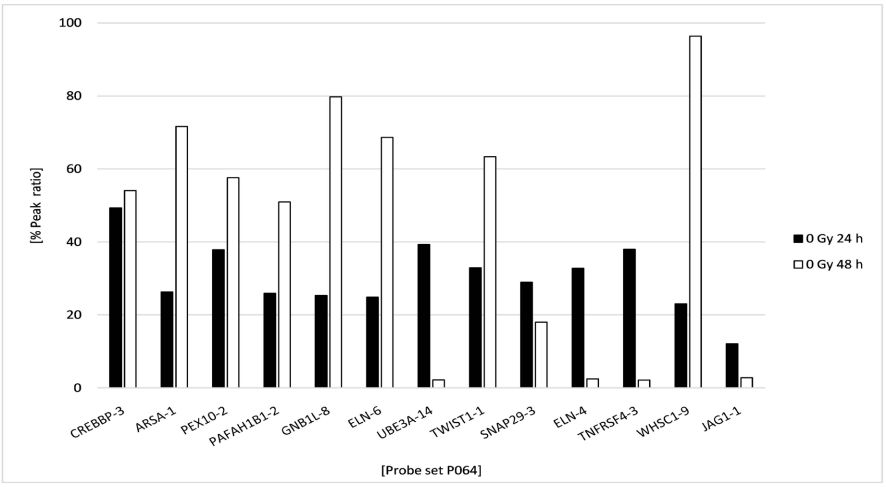

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Figure 3. The data after 24 (black bars) and 48 (white bars) h incubation for 0 Gy exposure to irradiation for the WTK1 cell line as a function of probe signal intensity. This is the control for this cell line. Each set of bars represents different probes in the P064 probe set that are also identified in the control and post-irradiation TK6 results.

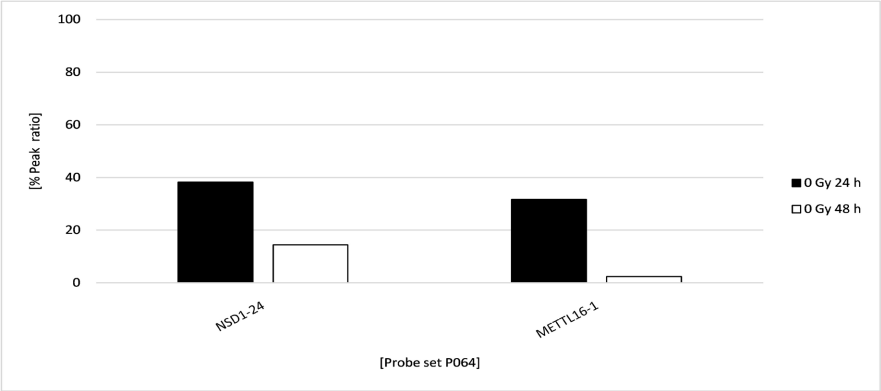


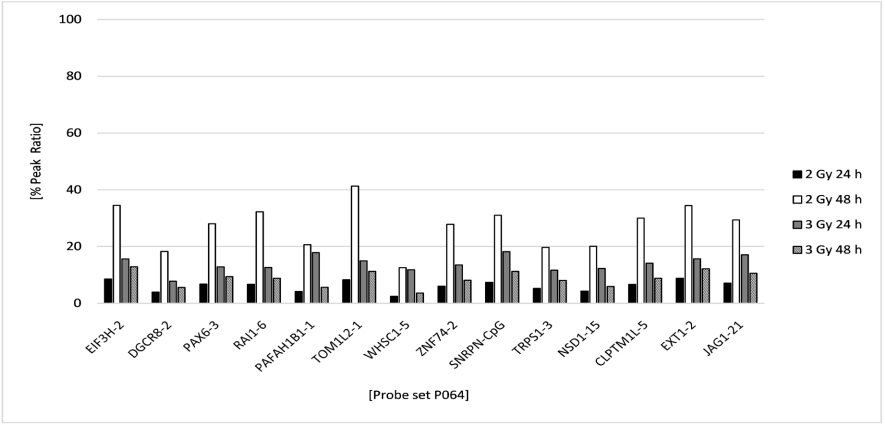
Figure 4. A subset of the data after 24 (black bars) and 48 (white bars) h incubation for 0 Gy (the control) exposure to irradiation for the WTK1 cell line as a function of probe signal intensity. Each set of bars represents different probes in the P064 probe set that are not identified in the control or post-irradiation TK6 results.

result of ionizing radiation can be elucidated in the same way as was done for the TK6 results. **Figure 5** and **Figure 6** show the MLPA data for 2 Gy and 3 Gy exposure to irradiation after 24 and 48 h incubation for the same (WTK1) cell lineas a function of probe signal intensity. **Figure 5** shows all probes for this cell line post-irradiation that are also found in both the non-irradiated and post-irradiated TK6 samples—in other words, these probes were found in the WTK1 irradiated samples as well as both pre- and post-irradiated samples for the TK6 cell line. The first two probes (NSD1-24 and METTL16-1) shown in **Figure 5** (post-irradiation) are also found in the WTK1 control (pre-irradiation) samples. However, there are seven additional probes that are only found in the post-irradiated WTK1 samples (**Figure 6**).

For the 2 Gy exposure for the WTK1 cell line, all probes in this probe set show a large increase in percent peak ratio when comparing between 24 and 48 h incubation time, ranging from 266.6% to 688.9%. For the 3 Gy exposure for this cell line, all of the probes that are also found in the TK6 results show a decrease in percent peak ratio when comparing between the incubation times, ranging from 1.4% to 69.5%. However, many (77.8%) of the 3 Gy exposed samples that are unique to the WTK1 cell line post-irradiation show an increase in percent peak ratio when comparing between 24 and 48 h incubation time, ranging from 128.3% to 5611.5% (**Figure 6**).

3.3. MLPA Analysis—Specifics of TK6 and WTK1 Results

For all probes, both in the 24 h incubation control (that underwent no irradiation) and in the 2 Gy 24 h post-irradiation, the percent peak ratios for the mutant TK6 cell line are always higher than the percent peak ratios for the p53 mutant WTK1 cell line under the same conditions (**Figures 1-4**). However, the 2 Gy 48 h post-irradiation samples showed the opposite—the WTK1 cell line had higher percent peak ratios for all probes than was demonstrated by the wild-type TK6 cell line under the same conditions.



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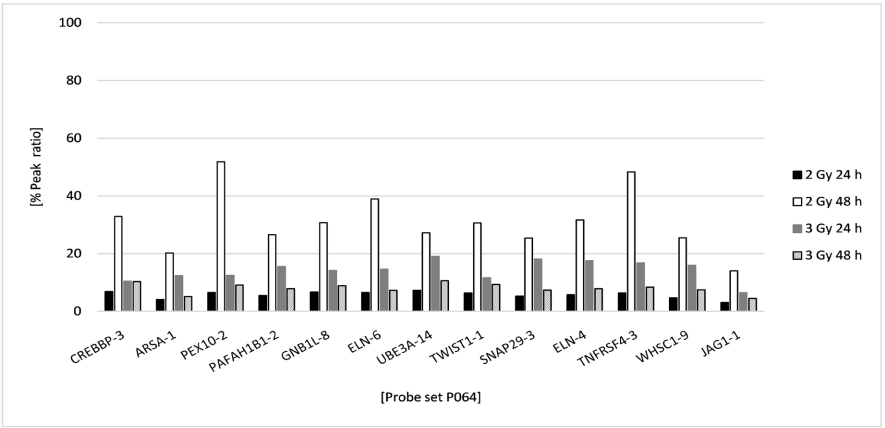

(b)

Figure 5. A subset of the data after 24 (black and gray bars) and 48 (white and hashed bars) h incubation for 2 Gy (black and white bars) and 3 Gy (gray and hashed bars) exposure to irradiation for the WTK1 cell line as a function of probe signal intensity. Each set of bars represents different probes in the P064 probe set that are also found in the TK6 results.

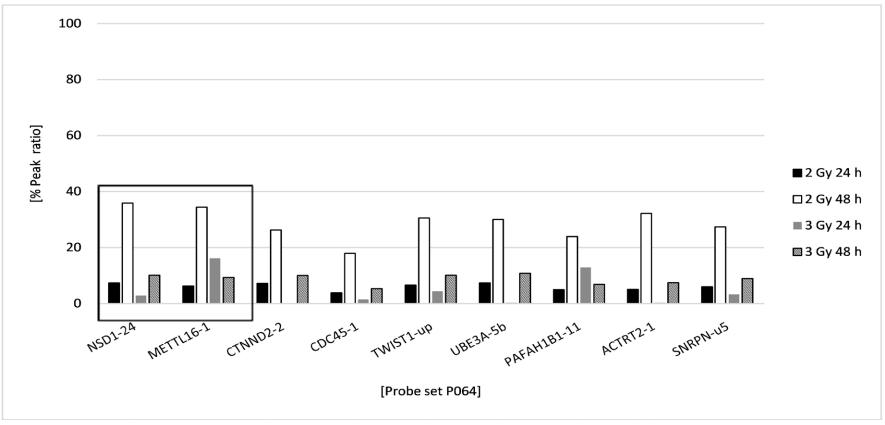

Figure 6. A subset of the data after 24 (black and gray bars) and 48 (white and hashed bars) h incubation for 2 Gy (black and white bars) and 3 Gy (gray and hashed bars) exposure to irradiation for the WTK1 cell line as a function of probe signal intensity. Each set of bars represents different probes in the P064 probe set that are not identified in the TK6 results. The data that is boxed in is also identified in the WTK1 control results (as shown in **Figure 4**).

4. Discussion

This study focused on determining the effects of ionizing radiation on two closely related cell lines: TK6 (which expresses the wild-type p53) and WTK1 (which expresses the p53 mutant). Specifically, we wanted to investigate the copy number variations between these two cell types to determine the level of impact that ionizing radiation, in the presence and absence of wild-type p53 protein, had on the following 20 microdeletion and microduplication syndromes: 1p36 deletion syndrome; Wolf-Hirschhorn syndrome; Cri-du-Chat syndrome; Saethre-Chotzensyndrome; Williams-Beuren syndrome; Williams-Beuren duplication syndrome; Langer-Giedion syndrome; WAGR syndrome; Prader-Willi syndrome; Angelman syndrome; Rubinstein-Taybi syndrome; Miller-Dieker syndrome; Lissencephaly-1; Smith-Magenis syndrome; Potocki-Lupski syndrome; Alagille syndrome; DiGeorge syndrome; 22q11.2 microduplication syndrome; and Phelan-McDermid syndrome.

WTK1 contains the mutant p53 (M237I) at the thymidine kinase (tk) locus [41]. The p53 gene has been shown to be one of the most mutated genes as it relates to cancer. When comparing the level of mutability with the wild type p53 TK6 cells, it was shown that there is a 10-fold rate of hypermutability at the tk locus in the WTK1 cell line. p53 has also been shown to play a major role in maintaining genetic stability. p53 is a transcription factor that functions as a tumor suppressor, and p53 mutants have been shown to have loss of DNA binding function that prevents them from carrying out their regulatory role [42]. In addition, disruption of the interactions between the oncoprotein Mdm2, which promotes the rapid degradation of p53, with certain p53 mutants, disrupts the p53 degradation pathway. This would indicate that mutant p53 is able to engage in aberrant interactions with other cellular factors. In fact, this has been shown to be the case, and typically results in gain-of-function phenotypes [43] [44] [45].

The application of Multiplex Ligation-Dependent Probe Amplification (MLPA) on these cell lines has provided a wealth of insight into the impact of ionizing radiation on chromosomal instability. Our TK6 cell line results show that the same probes are being amplified both pre- and post-irradiation, although to varying degrees. Pre-irradiation, the percent peak ratio values for most probes is above 20%, indicating that perhaps the cells are pre-G1 phase or are simply not repairing latent damage to those regions. After being irradiated with 2 Gy radiation, there is significant increase in percent probe ratio for all probes, indicating that the irradiation has led to microdeletions and microduplications of these probed areas. However, after 48 hours, the percent probe ratio is below even that of the control, indicating that cells with the p53 protein have potentially repaired these microdeletions and microduplications. This is not true after 3 Gy irradiation, as incubation of those regions leads to no change in percent probe ratio. It is possible that 3 Gy irradiation, even in the presence of p53, irreversibly damages those areas.

Table 1. The nine probes unique to WTK1 (pre- and/or post-irradiation) with chromosome positions and syndrome. The probes indicated with an asterisk are found in both the pre- and post-irradiation WTK1 samples; all others are only found post-irradiation.

Gene	Chromosomal position	Syndrome(s) detected
NSD1-24*	5q35.2; 5q35.3	Sotos syndrome
METTL6-1*	17p13.3	Miller-Dieker syndrome, Lissencephaly-1
CTNND2-2	5p15.2	Cri-du-Chat syndrome
CDC45-1	22q11.21	DiGeorge/22q11.2 duplication syndrome
TWIST1-up	7p21.1	Saethre-Chotzen syndrome
UBE3A-5b	15q11.2	Prader-Willi/Angelman syndrome
PAFAH1B1-11	17p13.3	Miller-Dieker syndrome, Lissencephaly-1
ACTRT2-1	1p36.32	1p36 deletion syndrome
SNRPN-u5	15q11.2	Prader-Willi/Angelman syndrome

Conversely, for the WTK1 cells there are a number of probes that have a significantly higher percent peak ratio pre-irradiation than are seen in the TK6 cell line control. Some of these peak ratios increase and some decrease after incubation; p53 affects many different cellular pathways, so these changes can indicate different fluctuations in the absence of wild-type p53. Post-2 Gy irradiation, there is significant microdeletion and microduplication identified after 24 hours; there is moderate repair after 48 hours incubation, but the levels do not return to those seen pre-irradiation in the control sample. The 3 Gy irradiation, however, yields similar results to the TK6 3 Gy irradiation.

Most interestingly, there are notable copy number changes for a number of the syndromes probed (Table 1). Of those, two probes—the one associated with Miller-Dieker and Lissencephaly-1 syndromes (METTL6) and the one associated with Sotos syndrome (NSD1)—are only found in the WTK1 cells both pre- and post-irradiation. This suggests that the presence of METTL16 and NSD1 could be in part due to the absence of wild-type p53. Other probes are only found in the mutant cell line post-irradiation, indicating that the presence or absence of wild-type p53 can alter how specific regions of the chromosomes that are associated with a number of microdeletion and microduplication syndromes are affected by trauma such as radiation. Of these probes, one detects Cri-du-Chat syndrome, two detect Prader-Willi/Angelman syndrome, one detects DiGeorge/22q11.2 duplication syndrome, one detects Saethre-Chotzen syndrome, one detects 1p36 deletion syndrome, and one detects Miller-Dieker and Lissencephaly-1 (as does the METTL6 probe mentioned above). The clinical implications of these results and what mutations of p53 may mean for these syndromes, along with how (and whether) they are related to intellectual disability, developmental delay, and/or congenital abnormalities, are unclear and would warrant future study.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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