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Final Report

A Form 241.3 was already submitted on the 5/27/2004 (OSTI ID 824491).
However, the final report uploaded to the form was not saved in a PDF format.

A new Form 241.3 was submitted electronically (on 28/09/04). Here is a new final report (PDF document) uploaded with the new form. This report contains few modifications related to the final analysis of the quantitative PCR results (see Table 6 and figure 3).

The Adaptive Response in p53 Cancer Prone Mice: Loss of heterozygosity and Genomic Instability.

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The *Trp53* gene is clearly associated with increased cancer risk. This, coupled with the broad understanding of its mode of action at the molecular level, makes this gene a good candidate for investigating the relationship between genetic risk factors and spontaneous cancer occurring in a mouse model exposed to low dose, low dose rate radiation. We and other investigators have shown that adaptive responses to low dose/dose rate exposure can alter cancer incidence and latency, as well as overall lifespan. These end points were used to assess and compare changes in risk in both normal and cancer prone mice heterozygous for a genetic defect in the *Trp53* gene. In this contract, to better understand the molecular processes that influence cellular risk, modern tools in molecular biology were used to evaluate the loss of heterozigozity (LOH) at the *Trp53* locus, and chromosomal instability in the *Trp53* +/- cells from mice exposed to chronic low dose gamma radiation. These biological end points are known to be associated with increased radiation-induced cancer risk.

Male mice carrying a single defective copy of the *Trp53* gene (B6.129S2-Trp53^{tm1Tyj}) were obtained from the Jackson Laboratory (Bar Harbor, ME) and were crossed with 129X1/SvJ female mice (*Trp53* +/+). The resulting F1 progeny were genotyped and the female *Trp53* heterozygotes were selected for our experiments. The mice were irradiated with doses of gamma-radiation delivered at a low dose rate of about 0.7 mGy/hr. Groups of mice were exposed to 0.33 mGy per day for 15, 30, 45, 60, 67 and 75 weeks (exactly 73, 143, 217, 295, 331 and 366 days respectively) equaling total body doses of 2.4, 4.7, 7.2, 9.7, 10.9 and 12.1 cGy, respectively. The experimental groups were composed of 5 irradiated and 5 unexposed mice.

It is known that the presence of a single defective copy of the *Trp53* gene increases cancer risk in these mice. However, *in vivo* exposure to low dose and low dose rate radiation increased cancer latency thereby increasing life expectancy. We hypothesized

that: 1) These mice might have spontaneous chromosome instability, and 2) that this low dose adaptive exposure would reduce the chromosomal instability. This instability was investigated using spectral karyotyping (SKY). Bone marrow cells from 5 irradiated mice (total body dose of 10.9 and 12.1 cGy) and 5 control mice were collected for metaphase harvest. Also, bone marrow cells from two additional wild type mice (one unexposed and one irradiated), littermates of the 75 week group heterozygous mice, were used for this analysis. Briefly, the cells were incubated at 37°C for 4 hours in RPMI containing 25% heat-inactivated FBS and 0.1 μg/ml colcemid. The cells were given a hypotonic treatment of 0.075M KCl containing 0.1 μg/ml colcemid for 20 minutes at 37ºC.

An average of 100 metaphases per mouse were karyotyped using SKY. The results are summarize in Table 1. The Trp53 heterozygous mice (cancer prone) do not show apparent genomic instability at the level of structural chromosomal aberrations. In all mice studied, only numerical aberrations were observed in 5 to 20% of the cells from both unexposed and irradiated mice, chromosomal loss being more frequent than chromosomal gain. The numerical aberrations observed in all 75 week irradiated mice were not significantly different (+/- vs +/+). There seem to be an age related increase in numerical aberrations as mice grow old. The results indicate that the presence of a defective copy of the *Trp53* gene does not seem to affect spontaneous chromosomal instability or in response to chronic low dose exposure to γ-radiation. Figure 1 show a normal karyotype of a heterozygous *Trp53* bone marrow cell.

In previous studies it was speculated that low dose and low dose rate *in vivo* exposure to γ-radiation induces an adaptive response, which reduces the risk of cancer death generated by subsequent DNA damage from either spontaneous or radiation induced events possibly due to enhanced recombinational repair (Mitchel *et al.* 1997). Reduced

cancer risk due to low dose induced recombination could result from reversion to homozygosity (normal wild type) at *Trp53* gene locus (*Trp53* +/- to *Trp53* +/+) or loss of heterozygosity in unirradiated mice (*Trp53* +/- to Trp53 -/-). This hypothesis was investigated using two modern tools in molecular biology, the quantitative real-time Polymerase Chain Reaction (QRT-PCR) quantification method and the novel Rolling Circle Amplification technique (RCA) (Christian *et al*. 2001). For these purposes, spleenocytes, peripheral blood lymphocytes, and bone marrow cells from all the experimental mice were isolated for cell fixation and DNA extraction.

The defective *Trp53* allele is generated by a deletion of exons 2 to 6 of the gene and integration of a portion of the cloning vector pKONEO DNA into the coding sequence. Therefore, the genotypic changes are monitored based on the detection of the *NEO* allele and the normal *Trp53* allele in the cells.

To evaluate loss of heterozygosity at the Trp53 gene locus in an individual cell, detection of the *NEO* allele and the normal *Trp53* allele using the dual color RCA was utilized. In our hands, this protocol did not give the required sensitivity. The probes and primers sequences that were used in the RCA protocol are listed in Table 2. The allele specific fluorescent signal enumeration was inconsistent and not reproducible. The protocol was modified by including a pepsine treatment prior to the RNase digestion, changing the chromatin denaturation procedure, increasing the probe hybridization and ligation time, increasing the washes stringency before the RCA reaction, and still could not be optimized.

Therefore, the QRT-PCR method was selected to allow us to evaluate the loss of heterozygosity with greater sensitivity and efficiency. A set of 6 primers was designed to

target the *NEO* allele, the normal *Trp53* allele, and a reference gene (*JLY*) in a PCR experiment using the LightCycler instrument (Roche Diagnostics). The probes and primers sequences used in the QRT-PCR protocol are listed in Table 3. More than 800 real-time PCR reactions were conducted on DNA extracted from tissues of the irradiated and unexposed heterozygous mice (see Table 4 for the samples description and Table 5 for the PCR reaction conditions).

Labeling and detection of each specific PCR amplicon using the SYBR Green fluorescent dye provided real-time analysis of amplified allelic sequences (LightCycler-FastStart DNA Master SYBR Green I kit from Roche Diagnostics). A typical QRT-PCR amplification curves for the detection of the *Trp53* allele in samples of different DNA concentrations is represented in Figure 2. The curve is an indicator of the SYBR Green fluorescence intensity, which is directly proportional to the number of amplified copies of the allele (PCR amplicons). The crossing point value (Cp) corresponds to the cycle number where the fluorescence crosses the background threshold and correlates directly with the original number of copies of the allele targeted in the DNA sample. The LightCycler quantification software evaluates the Cp values.

A standard curve representing the Cp values as a function of different concentrations of heterozygous control DNA was constructed for both the *Trp53* and the *NEO* alleles. The DNA samples were analyzed in triplicates and the mean Cp values were calculated. The concentration of each allele in the sample was measured using the standard curves. The *Trp53/NEO* concentration ratios, the percentage of the *Trp53* allele per sample and the mean % of the *Trp53* allele per group of irradiated mice and their unexposed littermates were also evaluated (Table 6 and Figure 3). At 30 weeks, the mean % of the normal *Trp53* allele in the DNA samples is close to 50%. This value correlates with a heterozygous genotype in a major proportion of the bone marrow cells. For the 45, 60, 67 and 75 weeks groups, the mean percentage of *Trp53* varies between 45 to 67% in the

DNA samples from the unexposed mice, and varies between 50 to 63% in the irradiated mice. It appears that there is no significant difference in the number of copies of the normal *Trp53* allele in the DNA of the mice that received different radiation treatment compared with their unexposed littermates. Thus, the chronic low dose exposure has not clearly induced at the molecular level, a change in the genotype of the cells.

The chronic low dose exposure to gamma radiation did not modify the genomic stability of the cells and the loss of heterozygozity. Those biological endpoints are involved in radiation-induced cancer risk. This research provides important information regarding the health effects and cancer risk of low doses of low LET radiation and should support the development of a biologically based model for risk assessment and subsequent radiation protection policy. The molecular genetic work supported by this US DOE grant was part of a larger research project to determine cancer risk and lifetime survival of a cancer prone *Trp53* heterozygous mouse following low dose gamma radiation exposure. The results of this study will be included in the final manuscript submission for the cancer risk study. DOE will be acknowledged accordingly in the final research article

References

Mitchel REJ, Azzam EI, de Toledo SM (1997) Adaption to ionizing radiation in mammalian cells, in stress-inducible processes in higher eukaryotes, T.Koval (editor), (Plenum Press, New York, 1997) pp.221-243.

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Table I SKY analysis of metaphases derived from bone marrow cells of unirradiated and gamma irradiated *Trp*53 heterozygous and wild type mice.

Mouse	Genotype	Weeks	Total body dose (cGy)	Total number analyzed	Structurally aberrant metaphases (%)	Numericaly aberrant metaphases (%)
1	+/-	67	0	107	2.8 (3 RL)	4.6
2	+/-	67	0	109	0	6.4
3	+/-	67	0	87	0	4.6
4	+/-	67	10.9	94	0	8.5
5	+/-	67	10.9	103	0	8.7
6	+/-	67	10.9	100	2 (2 t)	10
7	+/-	75	0	97	1 (1 del)	14.4
8	+/-	75	0	102	0	11.8
9	+/-	75	12.0	93	0	17.6
10	+/-	75	12.0	102	0.9 (1 RL)	17.6
11	+/+	75	0	50	0	20
12	+/+	75	12.0	58	0	19

(A total number of 1102 metaphases were analyzed)
RL; Robertsonian-like translocation, t; Translocation, del; Deletion.

Table 2: The probes and primers oligonucleotides sequences used in the RCA experiment and their gene location.

Sequence name	Gene location	Oligonucleotide sequence
NEO probe	pKONEO vector	5'-CTA TTC ggC TAT gAC TTT TTT TTT ATT Tag gTg ACA CTA Tag TTT TTT TTC CCT ATA gTg AgT CgT ATT ATT TTT TTT gCT Tgg gTg gAg Agg –3'
Trp53 probe	Exon 5	5'-CAC ACC TCC AgC Tgg TTT TTT TTA TTA ACC CTC ACT AAA ggg ATT TTT TTT CCC TAT AgT gAg TCg TAT TAT TTT TTT TgT TgT ggg TCA gCg C –3'
T7 primer	na	5'-TAA TAC gAC TCA CTA Tag gg –3'
SP6 primer	na	5'-TAT TTA ggT gAC ACT ATA g –3'
T3 primer	na	5'-ATT AAC CCT CAC TAA Agg gA –3'

na; non applicable

Table 3: The oligonucleotides sequences used in the QRT-PCR experiment and their gene location.

Sequence name	**Gene location**	**Oligonucleotide sequence**
NEO forward primer	pKONEO vector	5'-gATCggCCATTgAACAAgAT-3'
NEO reverse primer	pKONEO vector	5'-CCTCgTCCTgCAgTTCATT-3'
Trp53 forward primer	Intron 3	5'-AAgTTCgAggCCATCTCTgA-3'
Trp53 reverse primer	Intron 3	5'-gACTgggACCTTTCCTTCTT-3'
JLY forward	na	5'-CAgATTTgCCACCgCAC-3'
JLY reverse	na	5'-ACggTCTggCTCTTCAC-3'

na; non available

Table 4: The DNA samples studied using the QRT-PCR method.

Week group	Dose exposure (cGy)	DNA tissue sample	Number of mice analyzed for *NEO*	Number of mice analyzed for *Trp53*
30	0	Bone marrow	5	3
30	4.7	Bone marrow	5	3
45	0	Bone marrow	4	4
45	7.2	Bone marrow	4	3
60	0	Bone marrow	5	5
60	9.7	Bone marrow	4	4
67	0	Bone marrow	3	3
67	10.9	Bone marrow	3	3
75	0	Spleenocytes	2	2
75	12.1	Spleenocytes	2	2

Table 5: The QRT-PCR conditions used for the specific amplification and quantification of each target.

	Gene target		
PCR reaction steps	***Trp53***	***NEO***	***JLY***
Denaturation	Hold 95ºC for 10 min		
Temperature cycle repeat for 45 times:	95ºC for 10 sec 57ºC for 6 sec 72ºC for 8 sec	95ºC for 10 sec 63ºC for 6 sec 72ºC for 8 sec	95ºC for 10 sec 68ºC for 5 sec 72ºC for 15 sec
Hold 65ºC for 30 sec			
Melting curve from 65ºC to 95ºC at 0.1ºC-sec with optical chanel on			

Table 6: The *Trp53/NEO* concentration ratios and percentage of the *Trp53* normal allele in the DNA samples.

Weeks	Mice	Treatment	Ratio *Trp53* to *NEO*	% *Trp53*
30	1	Control	0.82	45.1
	2		1.21	54.6
	3		1.54	60.7
	4	4.7 cGy	1.16	53.7
	5		1.03	50.7
	6		1.23	55.2
45	7	Control	1.39	58.2
	8		1.70	63.0
	10		1.63	61.9
	11	7.2 cGy	1.50	59.9
	12		0.67	40.0
	13		1.12	52.9
60	14	Control	0.64	38.9
	15		0.71	41.6
	16		0.60	37.6
	17		0.94	48.4
	18		1.56	60.9
	19	9.7 cGy	1.26	55.8
	20		0.94	48.4
	21		0.86	46.2
	22		1.13	53.1
67	23	Control	1.82	64.6
	24		3.98	79.9
	25		1.34	57.3
	26	10.9 cGy	1.32	56.9
	27		1.03	50.7
	28		2.85	74.1
75	29	Control	1.10	52.6
	30		1.08	51.9
	31	12.1 cGy	1.73	63.4
	32		1.94	65.9

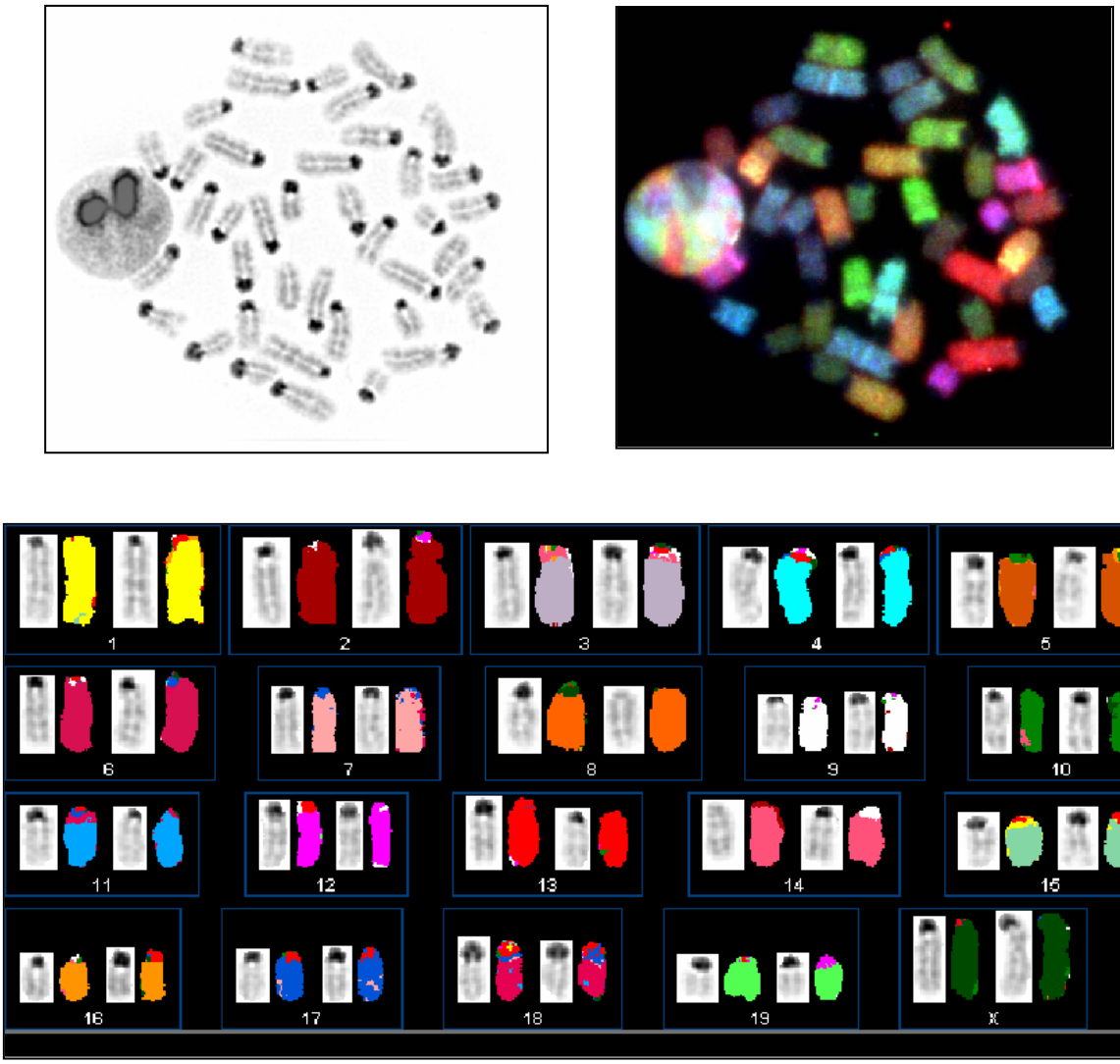

Figure 1: SKY analysis of a bone marrow cell from a *Trp53* heterozygous mice irradiated for 331 days at a dose of 0.33 mGy per day (total body dose of 109 mGy). The inverted DAPI banding images, the RGB (Red-Green-Blue), pseudo-color and are illustrated.

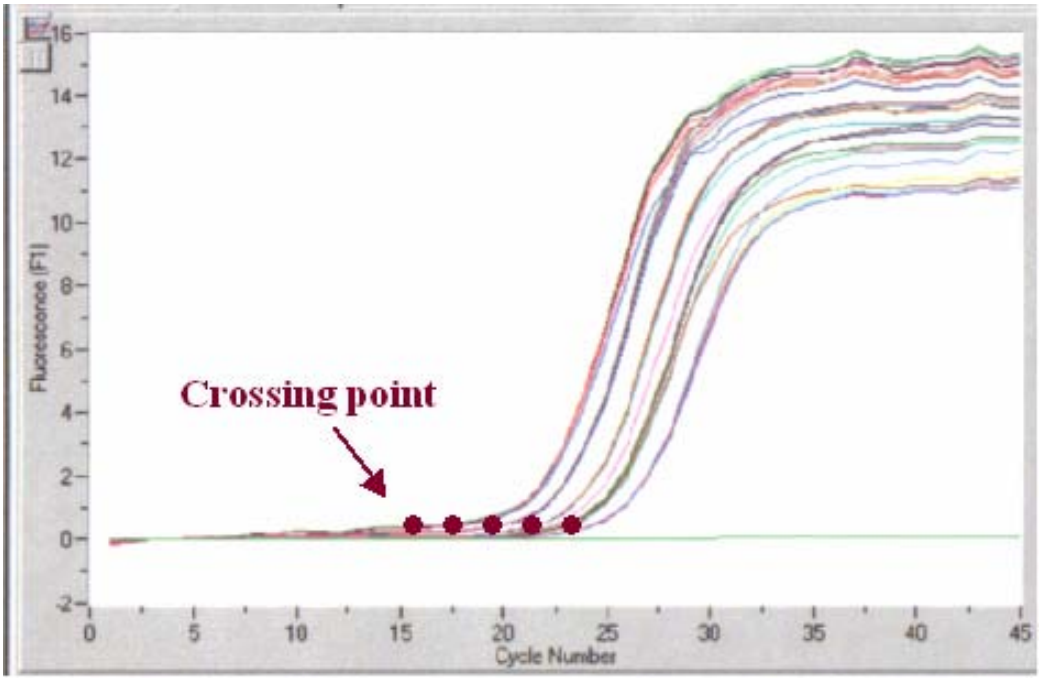

Figure 2: Amplification curve of the normal *Trp53* allele showing different crossing point values corresponding to different number of copies of this gene in the DNA samples.

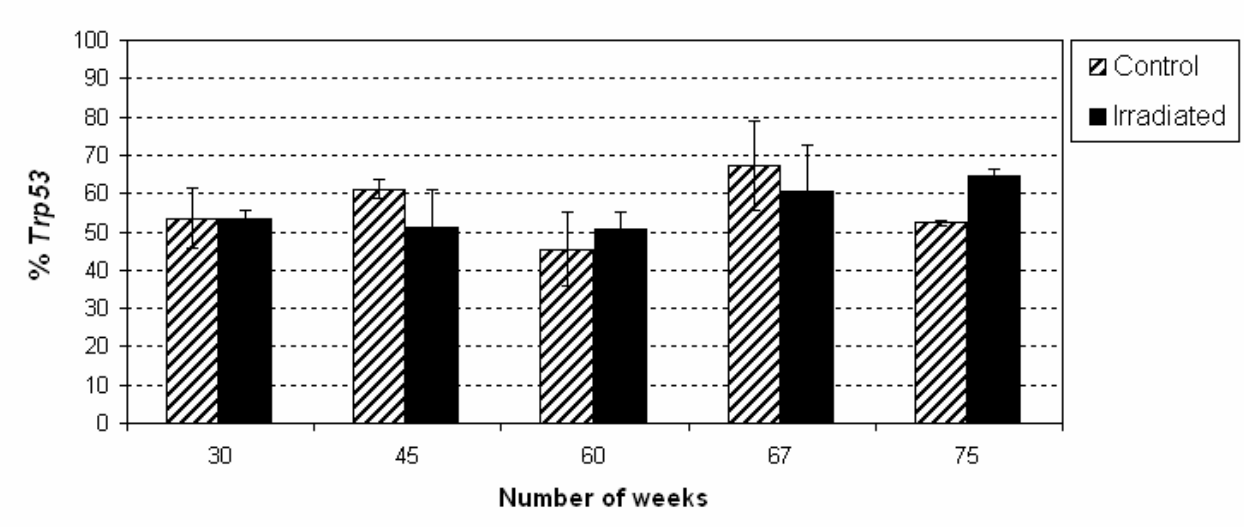

Figure 3: Average % of the normal *Trp53* allele in the bone marrow DNA sample of the irradiated mice following different chronic low dose exposure period and their unexposed (control) littermates.