CHAPTER 6. PILOT MISMATCH REPAIR SCREEN

6.1 Introduction

Before the mutation load of the 10 Gray irradiated cells was known (Chapter 5), a small-scale experiment was designed to test the possibility of screening DNA mismatch repair mutants generated by 10 Gray gamma irradiation in the NGG5-3 *Blm*-deficient cells (Figure 6-1). In this experiment, a recombinant Moloney Murine Leukemia Virus (MMuLV) containing the positive/negative selection marker *PuroΔtk* was used. The negative selection cassette carried by the provirus facilitates selection for deletions after irradiation because a deletion that removes the *PuroΔtk* cassette can be directly selected in FIAU. Following infection, puromycin selection was implemented to isolate infected cells, which retained the *PuroΔtk* gene thus were resistant to puromycin. Then the cells were irradiated and selected in FIAU media to ensure all surviving cells lost the functional *PuroΔtk* cassettes. In principle, FIAU selection avoided the presence of non-mutated cells in the survival pool. However, the *PuroΔtk* cassette can also be lost by LOH, which is elevated in the *Blm*-deficient cells and is likely to be elevated further by irradiation. The clonal survival in FIAU was assessed and $FIAU^R$ clones were examined for deletions using a 200 kb resolution CGH array. While this assessment was in progress, the population of FIAU resistant clones were cultured for long enough to accumulate LOH events and were then selected in 6-thiogunine for MMR mutants. $6TG^R$ clones were isolated and also analysed by array CGH technology.

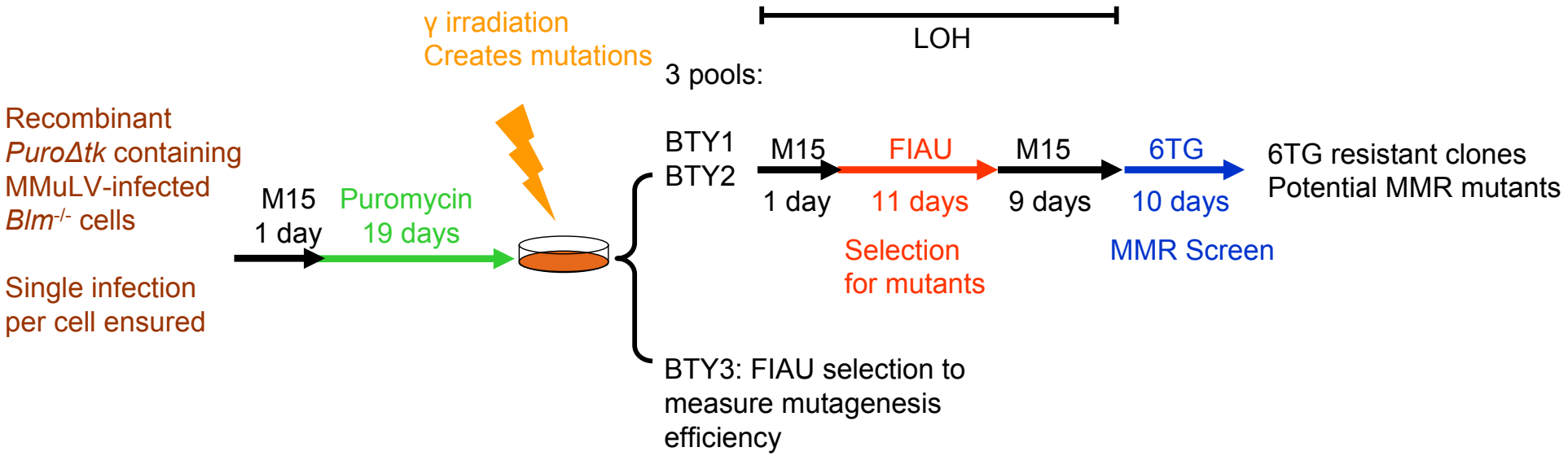

Figure 6-1 Pilot screen on *Blm*$^{-/-}$ cells carrying selected mutations generated by gamma irradiation

The *Blm*-deficient cells were infected with a *PuroΔtk* containing recombinant MMuLV. Experiments were controlled to ensure each cell had a single infection, which means one copy of the *PuroΔtk* gene was present. Single infected clones were generated after the puromycin selection. These clones were expanded followed by a 10 Gray irradiation. Cells were then split into three pools BTY1, 2 and 3. Pools BTY 1 and BTY2 were cultured in FIAU to obtain cells, which mainly had lost the functional *PuroΔtk* cassette. Then these cells were cultured for another 9 days in M15 media to allow LOH to occur. Cells were screened in 6TG for MMR-deficient mutants. Pool BTY3 was selected in FIAU to measure the complexity of the mutation pool.

6.2 Results

6.2.1 The procedure of the pilot screen

The *Blm*-deficient cells were infected with a *PuroΔtk* recombinant retrovirus (MMuLV). The *PuroΔtk* cassette provides infected cells with the resistance to puromycin and the sensitivity to FIAU. By infecting three million cells with 50 μL viral supernatant (virus titer is 10^4 cfu/mL), each infected cell was ensured to contain one copy of the *PuroΔtk* gene and five hundred infected clones were recovered. These infected clones were cultured in puromycin to avoid loss of the *PuroΔtk* gene, which can be resulted from spontaneous mutation and LOH. These cells were then expanded to 2×10^7 followed by a 10 Gray irradiation. After the irradiation, cells were then equally split into three pools BTY1, 2 and 3 and maintained as pools. All the three pools were cultured in FIAU media to isolate mutants which lost *PuroΔtk* function.

The cells in the pool BTY3 were used to measure the complexity of the mutation pool (6.2.2) and the efficiency of irradiation as a mutagen (6.2.4). After FIAU selection, cells in the pool BTY3 were stained by methylene blue and surviving cells were counted. Before the staining, five $FIAU^R$ clones were picked and expanded for a copy number variation analysis using array CGH.

The cells in the pools BTY1 and BTY2 were cultured for another 9 days in M15 media to allow LOH to occur. Cells were then screened in 2 μM 6TG at various cell densities (Table 6-1) for 10 days and eight clones were recovered (named as ITC1–8). General analysis of these mutant clones will be described in the section 6.2.5.

A parallel experiment under the same experimental condition was conducted on ES cells with a non-mutated *Blm* locus, the BAC acceptor cell line ZK6. The mutant pools generated in the ZK6 genetic background were called ATY1, 2 and 3. Five clones of pool ATY3 were also picked for copy number variation analysis.

Table 6-1 $6TG^R$ clones isolated from pools BTY1 and BTY2

Pools	Cell density (cells/90 mm plate)	$6TG^R$ clone ID
BTY1	2×10^6, 5 plates	ITC1–6
	5×10^6, 1 plate	-
	10^7, 1 plate	ITC7
BTY2	2×10^6, 5 plates	-
	5×10^6, 1 plate	-
	10^7, 1 plate	ITC8

The mutation pools BTY1 and BTY2 were selected in 6TG at a series of cell densities. Total 25×10^6 cells of each pool were selected. Eight $6TG^R$ clones were isolated, seven from the pool BTY1 and one from the pool BTY2.

6.2.2 The complexity of mutant pools

In this pilot experiment, the multiplicity of infection was controlled so that each cell would only be infected once because the number of plated cells (10^6 of both the wild type cells and the *Blm*-deficient cells) overwhelmed the active virus particles (500). The wild type (ZK6) and *Blm*$^{-/-}$ cells (NGG5-3) infected with recombinant virus were named AT and BT, each containing 500 single infection events. AT and BT cells were kept in puromycin for 19 days before 2×10^7 of each of them were irradiated at 10 Gray. Immediately after irradiation the cells were divided into three pools.

The complexity of each mutant pool was assessed. The 10 Gray survival frequency and FIAU-resistant colony number were calculated for the ATY3 and BTY3 pools (Table 6-2). The FIAU-resistant background was also assessed. Although LOH events could be reduced by maintaining puromycin selection until irradiation, it was not possible to select for FIAU function. One major cause of background is the low fidelity of reverse transcriptase (Monk *et al.* 1992). Thus, the *Δtk* cassette might carry point mutations causing cells to be resistant to both puromycin and FIAU. The puromycin and FIAU double resistant background was assessed in ATY3 and BTY3 pools. About 0.7% of infected wild type cells and 0.4% of infected *Blm*$^{-/-}$ cells should be puromycin^R and FIAU^R due to spontaneous mutation, accounting for 10 to 20 of the surviving FIAU-resistant clones. Excluding the puromycin^R and FIAU^R backgrounds, 14% of ATY3 clones and 25% of BTY3 clones are FIAU-resistant. About 96% of FIAU^R clones had lost FIAU sensitivity due to mutation or loss of the *Δtk* cassette.

6.2.3 Genomic changes after irradiation and FIAU selection

Five clones from each pool of ATY3 and BTY3 were picked for copy number variation analysis before methylene blue staining. The reference DNA was from either the ZK6 cell line (for analysis of ATY3 clones) or the *Blm*-deficient NGG5-3 cell line (for analysis of BTY3 clones). DNA from ZK6 and NGG5-3 was extracted from the passage immediately before the irradiation. The results (Table 6-3) showed that eight out of ten clones (3 from ATY3 and 5 from BTY3) carried at least one deletion after irradiation at 10 Gray. Every FIAU^R *Blm*-deficient clone contained 2 to 11 deletions ranging from 0.1 Mb to 17 Mb, although the one-BAC-sized deletions (0.1 Mb) are not convincing data.

Table 6-2 FIAU^R cells in ATY3 and BTY3

Mutation pool/ Genotype	ATY3/ WT (AB1)		BTY3/ *Blm*$^{-/-}$ (NGG5-3)	
Irradiated cell plated	6.6×10^6	5×10^5	6.6×10^6	5×10^5
10 Gray survival frequency	-	0.03%	-	0.06%
Surviving cells after Irradiation	2000	155	4000	303
Number of FIAU^R clone	287	-	984	-
FIAU^R background	14%	-	25%	-
(Puro and FIAU)^R background *	0.7%	-	0.4%	-
Number of (Puro and FIAU)^R cells	10	-	20	-

FIAU-resistant clones in the ATY3 and BTY3 pools are analysed. Pools ATY3 and BTY3 had 6.6×10^6 cells prior to gamma irradiation. In this experiment, survival frequencies of 10 Gray irradiation was measured by counting surviving colonies of 5×10^5 cells. These survival frequencies were then used to estimate the number of surviving cells of each pool. Two thousand wild type cells and four thousand *Blm*-deficient cells survived in each pool. After FIAU^R colonies were stained, 287 and 984 FIAU^R colonies were in plates of ATY3 and BTY3, respectively. * The background of puromycin and FIAU double resistant cells in pools was measured from the AT (Passage 4) and BT (Passage 4) cells, which were used to generate ATY1–3 and BTY1–3 mutation pools on the same day.

Table 6-3 Summary of chromosome changes of random picked clones from pool ATY3 and BTY3

Genotype	Clone	Number of deletion	Deletion range (Mb)
Wild type (ZK6) Pool ATY3	A1	0	-
	A2	7	0.1(6), 2.2
	B1	4	1(2), 1.6, 2
	C1	0	-
	D1	1	6
Blm$^{-/-}$ (NGG5-3) Pool BTY3	B2	2	0.1, 14
	C2	7	0.1(2), 0.5, 4.1, 1.8, 1.9, 6.9
	D2	3	0.1, 0.2, 5.5
	D11	6	0.1(4), 0.7, 14
	D12	11	0.1(6), 0.7, 1.0, 17, 15, 12

Five FIAU^R clones from each ATY3 and BTY3 pools were analysed using CGH arrays. Numbers and lengths of the deletions are described here. The number in the brackets indicates the frequency of the deletion.

6.2.4 Efficiency of gamma irradiation

The ratio of both FIAU^R and (puro and FIAU)^R colonies in the populations before and after 10 Gray irradiation were measured in multiple experiments using the parental cells AT and BT (Table 6-4). Before irradiation, about 3-4% of both AT and BT cells were FIAU-resistant. This number was roughly the same as the ratio of puromycin and FIAU double resistant cells. At this stage, all puromycin and FIAU double resistant events arose from replication errors of reverse transcriptase on the *Δtk* cassette while FIAU^R clones likely arose from LOH events. After the irradiation at 10 Gray, FIAU-resistant cells accounted for 13–14% of the surviving cells in both AT and BT cells. Assuming that the proportion of spontaneous FIAU^R and puro^R+FIAU^R cells remains the same before and after irradiation, the effect of irradiation can be assumed to represent the increase in events over this background. The data in Table 6-4 illustrates that the propoition of FIAU^R and puro^R+FIAU^R clones increased after irradiation to 10–15% of the population. This is somewhat lower than that suggested by the data in Table 6-2. This difference could be a variation between observation of a single experiment and the mean value of several experiments. The proportion of the puro and FIAU double resistant clones is about 10%, up from 3–4% before irradiation. This increase may be caused by deletions at the *Δtk* part of the puro*Δtk* gene or LOH events of a pre-existing mutation of a gene on the FIAU metabolism pathway, like what was discussed in Chapter 3. The proportion of FIAU^R cells rose from 3–4% to 13–14%. The same cause of the appearance of the puro and FIAU double resistant clones contributes to this increase. In addition, it seems 3–4% of the population are puromycin-sensitive and FIAU-resistant. These cells account for the real loss of the puro*Δtk* gene, which can be resulted from the irradiation-induced large deletions or LOH events that replaced part of the chromosome containing the puro*Δtk* gene with a part of the homologous chromosome.

6.2.5 Clone ITC8 was 6TG-resistant

After the pilot MMR screen, eight 6TG^R clones (ITC1–8) were isolated and expanded. Their drug resistance profiles were re-tested (Figure 6-2). Clones ITC1–7 were sensitive to 6TG, although a small number (1–20) of 6TG^R colonies can be seen in ITC4, 6 and 7. These single colonies may indicate weak 6TG resistance of clone ITC4, 6 and 7. Clone ITC8 appeared resistant to 6TG. This clone was analysed by array CGH technique (section 6.2.6).

Table 6-4 The ratio of FIAU-resistant and (Puro and FIAU) double resistant colonies before and after irradiation

		FIAU^R	(Puro and FIAU)^R	N	Passages
AT	**Before IR**	3.3 ± 1.3%	3.1 ± 1.8%	n=2	5–6
	After IR	13 ± 3%	10 ± 1%	n≥5	4–6
BT	**Before IR**	3.9 ± 0.5%	3.8 ± 0.3%	n=2	5–6
	After IR	14 ± 3%	11 ± 2%	n≥5	4–6

The frequencies of FIAU-resistant cells and puromycin- and FIAU-double resistant cells in the AT and BT cells infected by the *PuroΔtk* containing MMuLV were measured before and after 10 Gray irradiation (IR). The frequencies are indicated as the mean value of frequencies ± standard deviation.

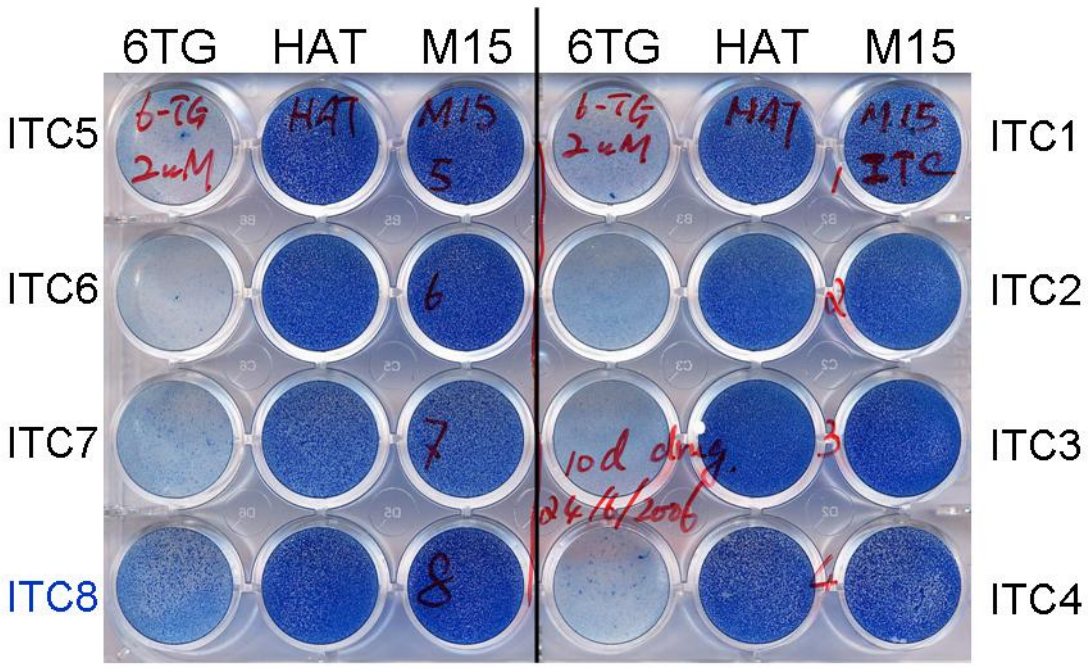

Figure 6-2 Phenotype confirmation of ITC clones

Cells from each of the clones ITC1–8 were distributed evenly into 3 wells, cultured in either normal media (M15), HAT media or 6TG media (2 μM) for 10 days. Then all the wells were stained by methylene blue (2%). Clones ITC1–7 were sensitive to 6TG, although a small number (1–20) of 6TG^R colonies can be seen in ITC4, 6 and 7. Clone ITC8 was proved resistant to 6TG. All of the eight ITC clones are HAT resistant (*Hprt*$^{+/-}$ or *Hprt*$^{+/+}$).

6.2.6 ITC8 carries a homozygous deletion covering the *Msh2* and *Msh6* loci

Array CGH analysis was conducted on ITC8 DNA with reference DNA from the NGG5-3 *Blm*$^{-/-}$ cells (Figure 6-3). A 1.8 Mb deletion on chromosome 17 with a Log_2(ratio) of -1.7 was detected. This ratio suggests that this is a homozygous deletion. This deleted region contains the DNA mismatch repair genes *Msh2* and *Msh6*. The deletion is complex and does not appear to be homozygous across the whole region. It appears that in one chromosome, 11.4 Mb distal region (including genes *Msh2* and *Msh6*) of chromosome 17 was deleted; and in the other chromosome, 2.8 Mb DNA including genes *Msh2* and *Msh6* was deleted. This 2.8 Mb region in the middle of the 11.4 Mb region is homozygously deleted. The isolation of this mutant clone and its configuration by array CGH confirmed various aspects of the experimental design: 1. It confirmed irradiation is efficient to generate mutations in ES cells; 2. It confirmed the screening conditions to isolate MMR gene deficient mutants from mutation pools; 3. It confirmed array CGH technique is adequate to identify deletions at MMR loci. Therefore a genome-wide MMR screen was designed and implemented (Chapter 7). However, it was noticed that the deletion in the mutant clone ITC8 is not homozygous but a complex, suggesting not only homozygous deletions caused by LOH, but also mutation complexes can be identified in the large-scale MMR screen.

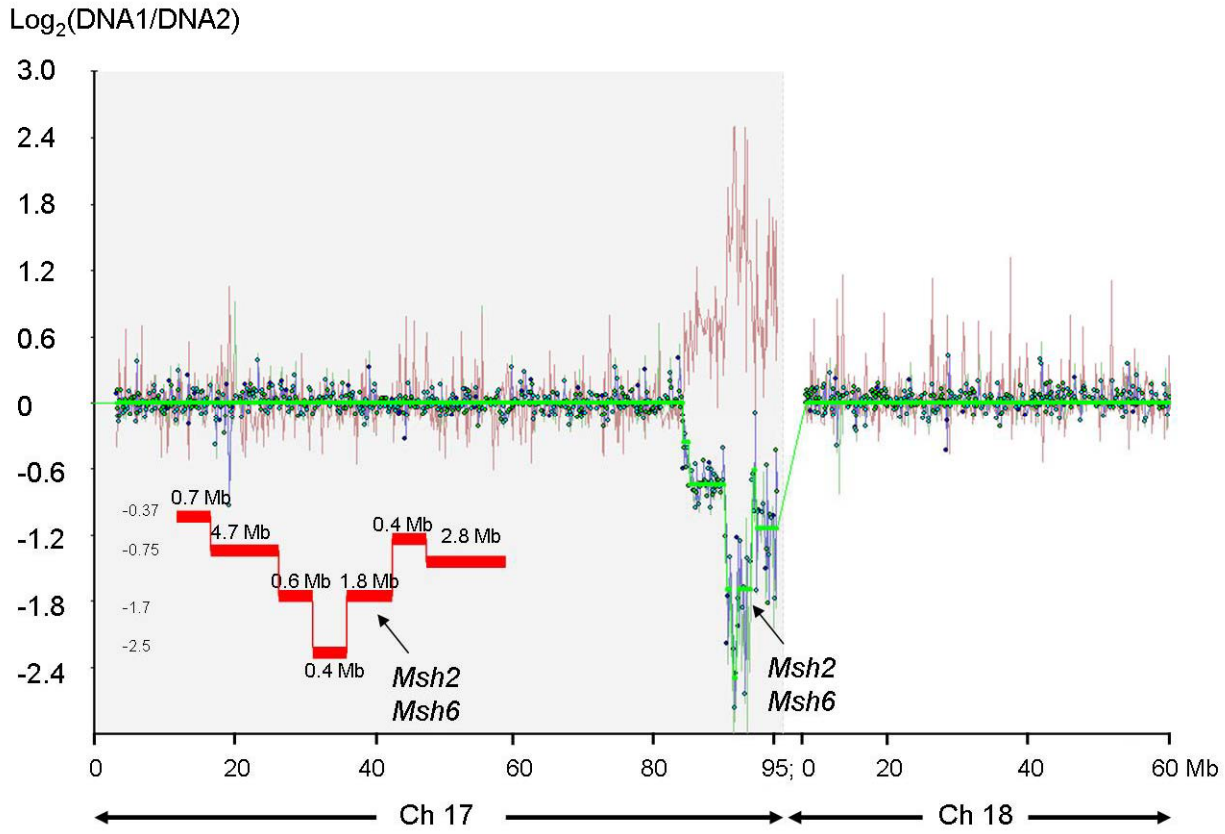

Figure 6-3 Array CGH analysis of 6TG^R clone ITC8

The array CGH profile is shown for part of chromosome (Ch) 17 and 18. The Log_2(ratio) of the mutant DNA to the *Blm*-deficient cell DNA was calculated for each BAC probe and plotted in this region. A blue line connects the nearest BACs to each other. A light green line indicates the average Log_2 (mutant DNA signal/reference DNA signal). A dark red line indicates the average Log_2 (reference DNA signal/mutant DNA signal). The red bars are a schematic illustration of the deletion. At -1.7 of the Log_2 ratio, a 1.8 Mb deletion contains the *Msh2* and *Msh6* genes.

6.3 Discussion

At the time of designing this pilot screen, it was not known that nearly 100% of surviving cells treated by 10 Gray irradiation contain mutations. Therefore it was needed to test the possibility and the procedures of isolating MMR-deficient mutants in an irradiation-induced mutation library. This pilot study used the *PuroΔtk* containing recombinant MMuLV to facilitate a preselection for mutants. Five hundred single infected cells were isolated and expanded. These cells were irradiated at 10 Gray and selected in FIAU for the mutations destroying the *Δtk* cassette at the proviral loci. In the parallel experiments on wild type cells, three out of five FIAU-resistant cells contain deletions, ranging from one deletion to seven deletions in each mutated cell. All five randomly chosen $FIAU^R$ cells contain deletions, ranging from 2 to 11 deletions per cell. These data confirmed that FIAU selected *Blm*-deficient cells all carry deletions thus were ready for MMR screen. However, it could not be assumed that all mutations only occurred around the proviral loci because we know now that the average size of all deleted regions in one survival cell may be as long as 10 Mb and the average number of deletions per cell is about two (Chapter 5). The proportion of $FIAU^R$ and $(puro+FIAU)^R$ cells were measured before and after irradiation at 10 Gray. About 13–14% of the cells surviving irradiation are FIAU-resistant, up from 3–4% before irradiation. Three to four per cent surviving cells are $puromycin^S$ and $FIAU^R$, which account for the real loss of the *PuroΔtk* cassette, probably due to deletion or LOH.

After FIAU selection, these *Blm*-deficient mutant cells were cultured for another nine doublings to accumulate LOH events and generate homozygous mutations. Eight $6TG^R$ clones were isolated. One of the eight mutants, ITC8, is strongly resistant to 6TG. Using array CGH, the muant clone ITC8 was analysed and both copies of the *Msh2* and *Msh6* genes were found to have been lost. This indicated a high likelihood of isolating homozygous deletions within the DNA mismatch repair pathway. In addition, the array CGH experimental data demonstrated that it was qualified to detect large chromosome copy number variations generated by irradiation. As this was a pilot screen, array CGH analysis on the other mutants was not performed.

About 14% ((287-10)/2000) of the wild type cells and 24% ((984-20)/4000) of the *Blm*-deficient cells, both containing the *PuroΔtk* cassette, lost the function of the *Δtk* cassette after 10 Gray irradiation (Table 6-2). That means around 10–20% of the mouse genome (30–60Mb) was affected by 10 Gray gamma irradiation. This number is too high to believe. To further validate the possibility, several experiments can be designed. One of these is to

use single targeted cells with the *PuroΔtk* positive/negative selection cassette to conduct similar experiments. Different targeted loci can be used but experiments have to be conducted separately. These targeted cells can be irradiated then selected for loss-of-function of the *Δtk* cassette. This will avoid the complexity of measuring mutagenesis efficiency in a pool. Secondly, array CGH with a higher resolution can be used. The array CGH I used was a 200 kb resolution array CGH. This array cannot provide more accurate data for deletions and duplications. Currently I assume that irradiation generates a huge number of small deletions and other mutations, which are beyond the current detection limit of array CGH. By using arrays with a resolution of 50 bp or 5 kb, more small deletions and duplications should be identified. Thirdly, the average length of deleted regions per cell and the average length of amplified regions per cell were obtained from 3–6 samples at each dose (Chapter 5). The sizes of these sample groups were not big enough to make these data convincing. Thus, more survival clones need to be analysed to validate these data.

This pilot screen demonstrates several facts of successfully using irradiation mutagenesis to conduct a genome-wide recessive MMR screen. 1. 10 Gray irradiation can efficiently generate mutations in *Blm*-deficient ES cells; 2. the *Blm*-deficient cells are efficient in generating homozygous mutations. 3. MMR-deficient mutants can be isolated by 6TG selection. 4. 200 kb resolution array CGH can be used to identify homozygous deletions generated by irradiation. However, there are some notable drawbacks of irradiation. Combining these results with data from Chapter 5, I found that irradiation can generate multiple duplications and deletions in a single cell. This will increase the difficulty of identifying which gene is responsible for the 6TG resistance. Especially, large heterozygous deletions in one allele may be coupled with small mutations in the other allele. When the small mutations are not identified by array CGH, the phenotype causing the mutation cannot be detected. Thus, in the genome-wide screen, expression array technique may be used to complement the analysis using array CGH. Expression array can generate data of transcriptional variations. It can identify which genes lose their transcripts even though these genes are not regarded as deleted according to array CGH. Nonetheless, an expression array analysis also has some drawbacks in identifying 6TG resistance related mutations. As the transcription of downstream regulated genes can be shut down by the effect of other mutations, downstream regulated genes may be regarded as mutated. A possible way to solve this problem is to combine data from both array CGH and expression. It is highly likely that genes which are deleted and transcriptionally silenced in a mutant retain 6TG resistance, causing mutations at MMR loci.