Genomic analysis of L5178Y tk+/- cells and their induced tk-/- mutant colonies

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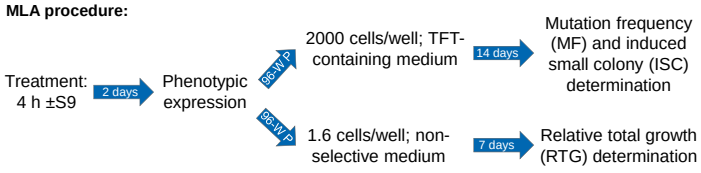
Introduction and Goal

- The genetic basis that underpins the mouse lymphoma assay (MLA) is ostensibly well understood; inactivation of the functional *thymidine kinase* (*tk*+) allele in L5178Y cells via mutation or deletion induces trifluorothymidine (TFT) resistance and $tk^{-/-}$ mutants can be selected for against a background of $tk^{+/-}$ wild-type cells via TFT-mediated enrichment (Clements. 2000).
- However, despite its widespread use over the past 20 years for hazard identification purposes, few studies have sought to characterise in greater detail the colonies of cells which propagate as a result of mutagenesis in the assay.
- The advent of whole genome sequencing has the potential to enhance mutagenicity testing since genetic changes can now be investigated with high level of detail at the genome level.
- Next generation sequencing (NGS) technology was used to sequence the genomes of:
  a) an L5178Y cell line frequently used in MLA studies.
  b) $Tk^{-/-}$ mutant colonies induced by two prototypical mutagens in the assay.
- The goal of this pilot study was to exploit NGS technology with a view to shedding new light on the genome of cells used in the MLA, as well as the changes induced as a result of the exposure to mutagens.

Materials and Methods

- **Cell line:** L5178Y $tk^{+/-}$ clone 3.7.2C IVGT (Public Health England, UK). Spontaneously-occurring $tk^{-/-}$ mutant cells were purged from the population via a cleansing procedure that used methotrexate to select against *tk*-deficient cells and thymidine, hypoxanthine and guanine to ensure optimal growth of *tk*-proficient cells.
- **MLA:** carried out according to established standards (Moore *et al.,* 2006). Briefly, cells (10^6) were exposed to either methyl methanesulfonate (MMS; 10 and 20 µg/ml) in the absence of S9 or 7,12-dimethylbenz[a]anthracene (DMBA; 1 and 1.5 µg/ml) in the presence of S9 for 4 hours in a shaking incubator set at 37°C. DMSO was the solvent control in each case.
- **MLA procedure:**



- ***Tk*$^{-/-}$ mutant colony harvesting:** following MF and ISC determination, small and large $tk^{-/-}$ mutant colonies were randomly selected and removed from the wells for processing.
- **DNA extraction and sequencing:** Colonies were processed via the QiaAMP® DNA mini kit (Qiagen, USA). Genomic DNA extracted from colonies was processed via the Ovation® Ultralow DR Multiplex System kit (Nugen, USA). All libraries were sequenced on an Illumina HiSeq-2500 with runs of 2 × 100 bases. Average sequencing depth for the L5178Y cell line was 58-fold and between 8 to 31-fold for the mutants.
- **Bioinformatics:** The paired-ends were trimmed and cleaned using Trimmomatic (Bolger et al, 2014) and aligned to the GRCm38 version of the mouse genome using the BWA MEM (Li H, 2013) algorithm. SNPs were called using the software Freebayes (Garrison E & Marth G, 2012):
  - **L5178Y heterozygous sites**: Stringent quality filtering was applied to identify reference heterozygous sites in the L5178Y cell line.
  - **Loss of heterozygosity**: Heterozygosity at the reference sites in the mutants was determined by running Freebayes with very permissive parameters, so as to avoid false negative heterozygosity calls.

Heterozygous point mutations in L5178Y

Table ①

Subset	All chromosomes	Chromosome 11
Total	2,079,744	221,108
Shared with Mouse Genome Project	1,803,902	205,952
Exclusive to L5178Y	275,842	15,156

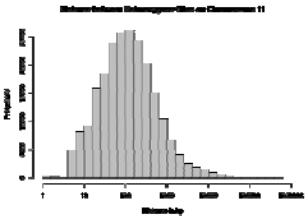
Survey of heterozygous sites in L5178Y:
Table ①: Survey of SNPs located in the L5178Y genome.
Table ②: Mutations exclusive to L5178Y, which are predicted to impact DNA repair enzymes, according to the SIFT algorithm (Ng P & Henikoff S, 2001).

Table ②

Gene	Reference amino acid	Position	Mutant amino acid	SIFT score	Gene function
Asf1a	D	57	N	0.0006	Histone chaperone ASF1A
Ino80	S	270	P	0.0176	Chromatin remodeling complex
Fbxo6	R	76	K	0.028	Putative; control of the cell cycle
Brca2	N	737	T	0.0424	DNA repair associated
Rfc1	R	372	H	0.0023	Replication factor C
Lig1	Y	671	H	0.0252	DNA ligase
Herc2	R	1744	L	0.0028	Retention of repair proteins on damaged chromosomes

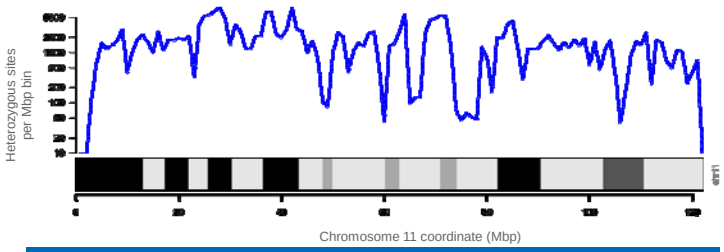
Heterozygosity on chromosome 11

Figure ①



Survey of heterozygous sites in L5178Y:
Figure ①: The distance between heterozygous sites on chromosome 11 limits the resolution with which chromosome loss can be determined
Figure ②: Number of heterozygous sites per 1 Mbp bin on chromosome 11

Figure ②



Mapping loss of heterozygosity on chromosome 11

Figure ③

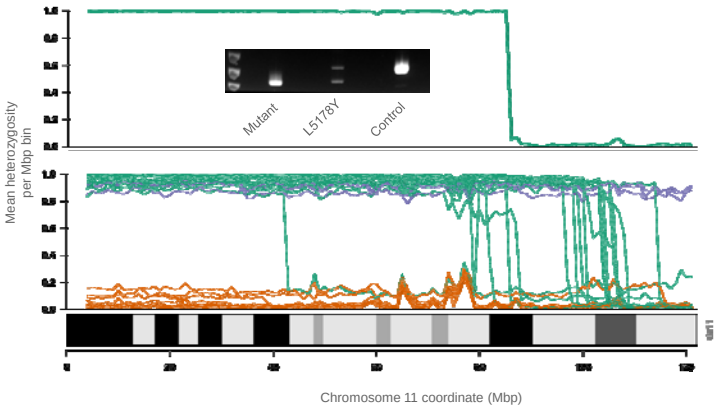


Figure ③: Using heterozygous sites in L5178Y to map loss of heterozygosity in mutant colonies

- **Upper Panel:** Example of abrupt loss of heterozygosity (LOH) due to breakage. Inset: Confirmation of LOH by PCR with Agl2 primers (Liechty et al. 1996)
- **Lower Panel:** LOH mapping for all colonies: mauve - no LOH; green - LOH; orange - loss of whole chromosome.

Summary and Conclusion

- Analysis of the L5178Y $tk^{+/-}$ cell line's genome revealed numerous pre-existing small polymorphisms, many of which are shared with various mouse strains.
- Several novel mutations in L5178Y were identified, which could explain the sensitivity of this cell line to mutation.
- Loss of heterozygosity at pre-existing heterozygous sites can be observed in many mutant colonies by NGS sequencing.
- Mapping LOH to allows breakpoints on chromosome 11 to be identified, and the density of heterozygous sites along the chromosome defines the resolution of this method.
- In conclusion, NGS-mediated genomic analysis of $tk^{-/-}$ mutant cells may shed new light on the genetic basis of the MLA, while also potentially providing additional evidence for the risk assessment of mutagenic compounds.

References

- Clements J. 2000, The mouse lymphoma assay, *Mutat. Res.* 455: 97-110.
- Moore M *et al.,* 2006, Mouse Lymphoma Thymidine Kinase GeneMutation Assay: Follow-up Meeting of the International Workshop on Genotoxicity Testing-Aberdeen, Scotland, 2003-Assay Acceptance Criteria, Positive Controls, and Data Evaluation, *Environ. Mol. Mutagen.* 47: 1-5.

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