Assessing potential novel therapeutic targets identified through analysis of true cancer-specific DNA methylation changes

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Background

Medulloblastoma is the most common malignant paediatric brain tumour, with a 5-year survival rate of 70%.[1] Current treatments for the disease have been associated with long-term side effects which decrease quality of life; there is therefore a need for novel non-toxic treatments.

Synthetic lethal (SL) genes have been identified as a potential target for non-toxic therapies. These are genes which are only required for cell survival in the presence of another mutated gene. If the mutated gene is cancer-causing, then targeting the SL gene should allow specific killing of cancer cells which bear the cancer-causing mutation.

Bioinformatic approaches, using DNA methylation and gene expression data, have allowed identification of potential SL genes in specific subgroups of medulloblastoma (WNT, SHH, Group 3 and Group 4).[2] DNA methylation can switch genes 'off' and is acquired at a large number of genes when cells proliferate extensively. Genes which fail to acquire aberrant methylation (and are therefore potentially 'on') in the presence of a specific cancer driver suggests that the gene may be required in the presence of the specific mutation and is therefore a potential SL gene.

For functional confirmation of potential SL genes, suitable cell line models must be identified which SL genes can be tested in.

Aims

To establish cell line model systems that can be used for subsequent functional analysis of potential SL genes by;

- Confirming that medulloblastoma cell lines mirror differential methylation status observed in primary samples by COBRA analysis
- Confirming the negative association of DNA methylation on gene expression using qRT-PCR

Methods

COBRA Assay

COBRA (combined bisulfite restriction analysis) assays allow for the quantification of the methylation status of a gene. This is completed in steps. A **bisulfite treatment** is first carried out on the DNA sample. Any unmethylated cytosine residues are converted to uracil, whereas methylated cytosines remain as cytosines. The DNA is then amplified by **PCR**. The resultant DNA contains thymine in place of originally unmethylated cytosines and cytosines in place of originally methylated cytosines. (Fig.1)

These steps allow methylated DNA to retain CpG-containing restriction enzyme sites. A **restriction digest** is then carried out with enzymes which will now only cut as sites which were originally methylated. DNA fragments are produced which can then be quantified by use of **gel electrophoresis.**

RT-qPCR

RT-qPCR was carried out on the same genes to quantify expression to investigate whether this negatively correlated with methylation status.

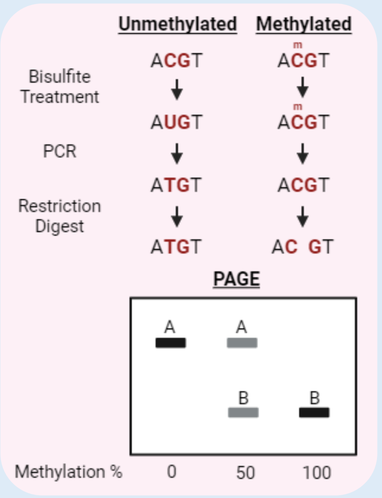

Figure 1 – COBRA assay process.

Results and Discussion

Two genes were identified in two cell lines which show a negative correlation between methylation and expression, and therefore have potential to be used as model cell lines for investigating synthetic lethality. (Table 1)

SYK shows a negative correlation in the DAOY cell line between methylation and expression which reflects the characteristics of a SL gene.

RASIP1 shows negative correlation between methylation and expression in the D283 cell line. As well as this, the UW228 cell line could be used as a control in a RASIP1 model.

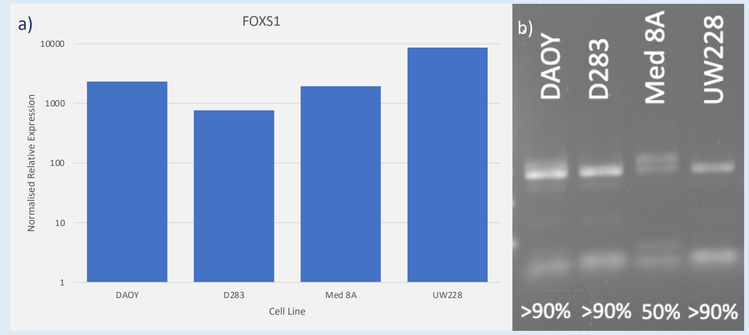

Figure 2 – Example of qRT-PCR data and COBRA assay. Figure 2 (a) shows qRT-PCR data for FOXS1 in each cell line, shown on a log scale graph, normalized to housekeeping gene GAPDH then multiplied by $x10^7$. Figure 2(b) shows a COBRA assay to assess DNA methylation of the FOXS1 gene performed using Hinf1 restriction enzyme.

Both SYK and RASIP1 were identified as potential SL genes from primary medulloblastoma samples which belong to the WNT subgroup. None of the cell lines used in this experiment are a member of the WNT subgroup - further investigation is required to confirm if these are acting as SL genes in these cell lines.

No other genes reflect the SL methylation and expression status seen in primary samples. For example, FOXS1 (Fig 2) was identified as a SL gene from a primary sample in the SHH subgroup. This is not reflected in either the DAOY or UW228 cell lines (both of which belong to the SHH subgroup). The relationship between methylation and expression is consistent in D283 and Med8A cell lines but does not reflect SL status, or a status which could be used as a control in further investigations.

In some instances, the relationship between methylation and expression is not biologically consistent. The investigated differentially methylated regions (DMR) of both CRIP2 and PNPLA2 were not directly at the transcription start site (TSS) of these genes. This may mean less influence on expression of the gene by methylation. Another explanation may be existence of alternative TSS which means a gene can be expressed despite high methylation.

	Gene Name																				
	FOXS1			PNPLA2			CRIP2			SYK			RASIP1			LMX1A			KIF12		
Cell Line	M	E	NC?	M	E	NC?	M	E	NC?	M	E	NC?	M	E	NC?	M	E	NC?	M	E	NC?
DAOY	60%	Med	×	75%	Med	×	80%	High	×	5%	High	✓	0%	Low	×	0%	Low	×	55%	Low	×
D283	73%	Low	×	85%	High	×	80%	High	×	35%	High	×	0%	Med	✓	50%	Low	×	85%	High	×
Med8A	33%	Med	×	90%	High	×	90%	High	×	80%	High	×	25%	Low	×	40%	Neg	×	85%	High	×
UW228	77%	Med	×	90%	High	×	80%	High	×	25%	Med	×	90%	Low	✓	15%	Neg	×	80%	Neg	✓

Table 1 – Methylation and expression status of potential synthetic lethal genes. Column M represents the methylation status of each gene in the corresponding cell line, this is the calculated average of two COBRA assays performed with two different restriction enzymes. Green corresponds to low methylation (<25% average), red corresponds to high methylation (>75% average), orange represents values between. Column E represents the expression status of each gene in the corresponding cell line. Green represents high expression levels, orange represents medium expression levels, light red represents low expression levels and dark red indicates there was no detected expression of the gene. This is decided from analysis of qRT-PCR data which has been normalized and is relative to GAPDH then multiplied by $x10^7$. Log values >10000 are labelled as high expression, log values <1000 are labelled low expression. Between these values are medium expression levels. The NC column indicates whether there is a negative correlation between methylation and expression. PNPLA2 and CRIP2 are highlighted blue to indicate the DMR is not at the TSS.

Conclusion

Further research is required for the majority of the potential SL genes which were investigated. Methylation and expression of these genes could be investigated in other medulloblastoma cell lines to identify more optimal cell line models.

In the case of SYK and RASIP1, if DAOY and D283 are identified as model cell lines, respectively, then knockout experiments could take place to see if this will induce specific cell death of the medulloblastoma cells. If cell death occurs, this allows opportunity for therapeutics to be developed which could target these SL genes to allow for specific non-toxic killing of medulloblastoma cells.

Acknowledgements

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References

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2. Schwalbe, E.C., H, L., Lafta, F. *et al.* Integration of genome-level data to allow identification of subtype-specific vulnerability genes as novel therapeutic targets. *Oncogene* 40, 5213–5223 (2021).