Use of Methylation Profiling to Identify Genes involved in Relapse in Adult ALL

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Aims

- Identification of risk genes indicating ALL relapse
- Collect methylation data for chosen genes
- Compare the methylation levels of Presentation and Relapse patient samples

Introduction

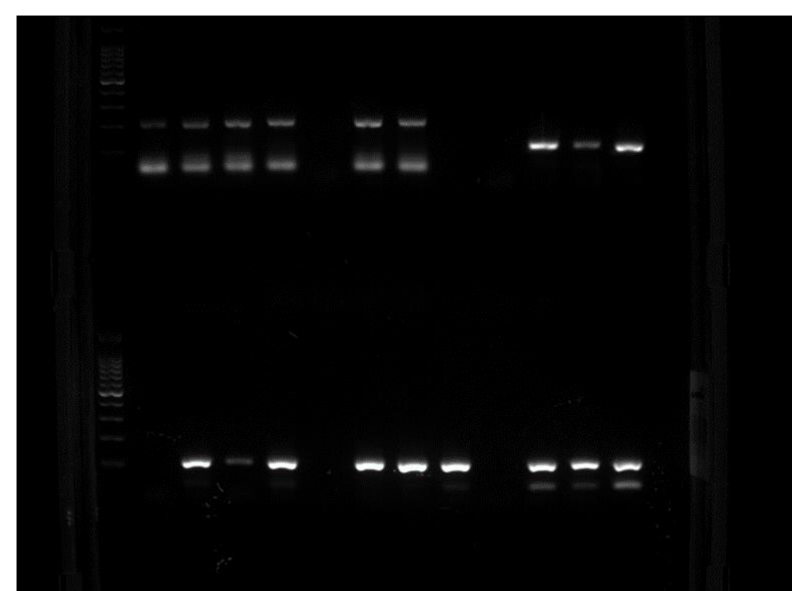
Methylation of DNA is associated with cancer and aging. Promoter associated CpG islands are especially prone to hypermethylation and deactivation during the development of cancer.

Genome wide screening of methylation changes in cancerous cells identified 9 candidate genes functionally relevant to **Acute Lymphoblastic Leukaemia** (ALL). From this pool of 9 genes I was allocated two for investigation; **Wnt7a** and **Them4**.

My objective was to accumulate methylation data for both genes in order to compare levels in 28 adult ALL samples upon diagnosis and relapse. If successful this would lead to further investigation with a larger sample size. This would be used to confirm whether or not the increased levels of methylation at presentation indicate risk of relapse.

Methodology

Stage 1 – Identification of suitable **Polymerase Chain Reaction** (PCR) conditions for primers. This was done by varying the annealing temperature against a $MgCl_2$ concentration in the PCR machine and then running the samples out on a **DNA agarose electrophoresis gel** to see which was most affective



Figue 1: An agarose gel electrophoresis used to test for the presence of amplified DNA and Primer conditions. Electrophoresis works on the principle that larger strands of DNA move more slowly than smaller strands, allowing the length of DNA to be measured by the level of separation using a DNA ladder. By analysing the brightness of the bands the relative level of amplification can be identified. In this case the diagram shows the conditions for Wnt7a proceeding from the seventh top band. The first band at 56°C and 1mmol cannot be seen because the conditions are unsuitable, the brightest bands are all at 61°C.

	Temperature (°C)			
$MgCl_2$ (mmol)	56	58	61	63
1	1	2	3	4
2	5	6	7	8
3	9	10	11	12
4	13	14	15	16

Figure 2: Table showing the combination of temperature and $MgCl_2$ used in combination to establish suitable conditions for PCR. The final conditions for Wnt7a and Them4 were 61C/4mmol $MgCl_2$ and 58°C/3mmol $MgCl_2$ respectively. Variable optimum annealing conditions are caused by different G/C contents of DNA and the length of the sample.

Stage 2 – Testing the accuracy of the primers using DNA of known methylation levels to see if the assays produce methylation data corresponding to the known values. Carried out **bisulphate modification** to replenish stock of methylated DNA.

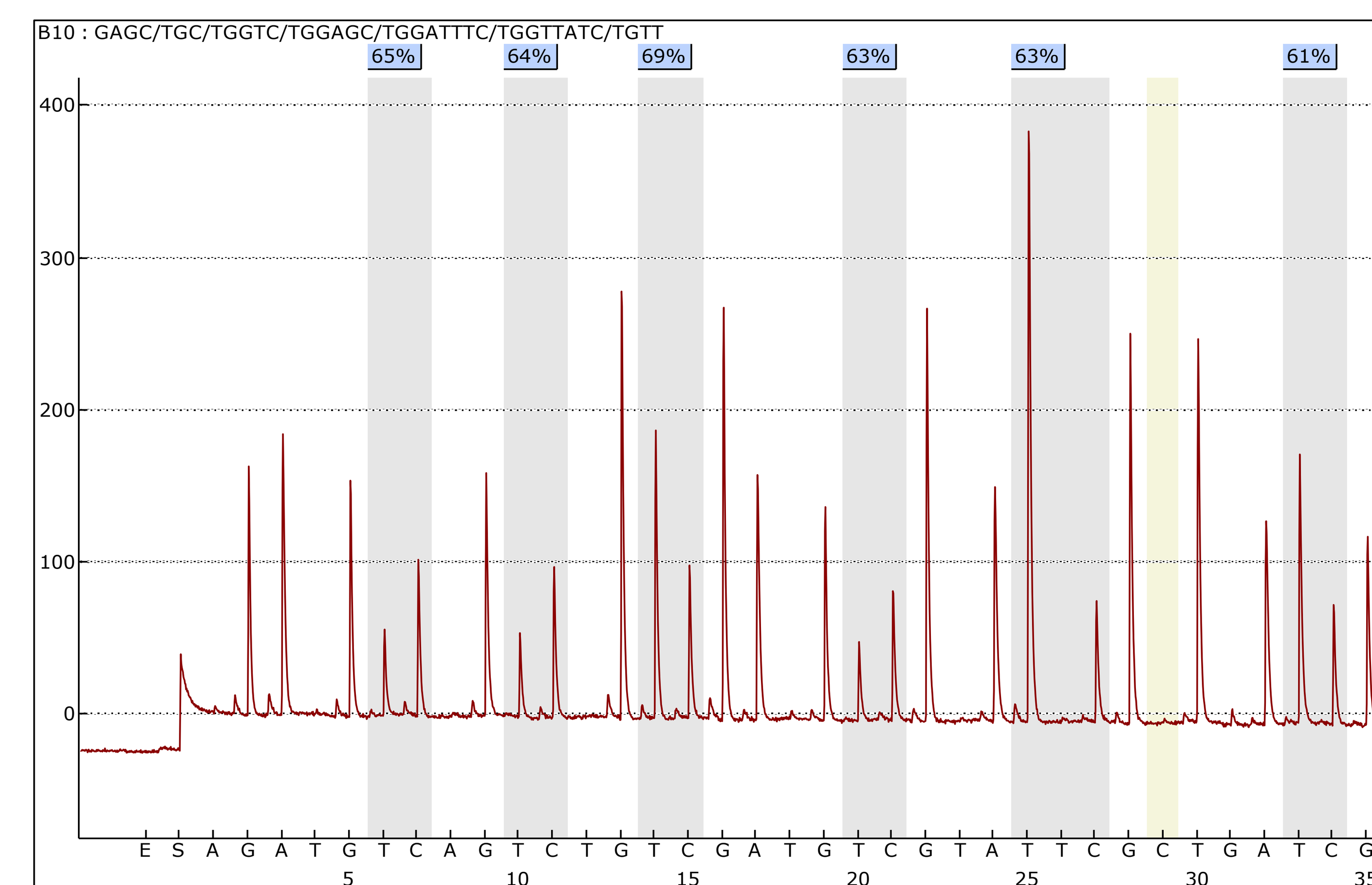


Figure 3: An example of a pyrogram taken during my project. The pyrosequncer distinguishes between methylated and unmethylated cytosine residues due to the bisulphate modification of the samples, which converts the unmethylated cytosine residues to uracil, which is not found in DNA.

Stage 3 – Testing of 28 adult ALL sample sets at diagnosis and relapse by PCR amplification and **pyrosequencing**.

Figure 3: The pyrosequencer housed in the Centre for Aging and Vitality. Pyrosequencing is a form of DNA sequencing which relies on a chemical reaction producing light from with the addition of each correct complimentary base pair to the template sequence. This occurs by a series of reactions beginning with the release of PPi from the DNA strand, which is converted to ATP and then used as a coenzyme to luciferase to produce light from luciferin.

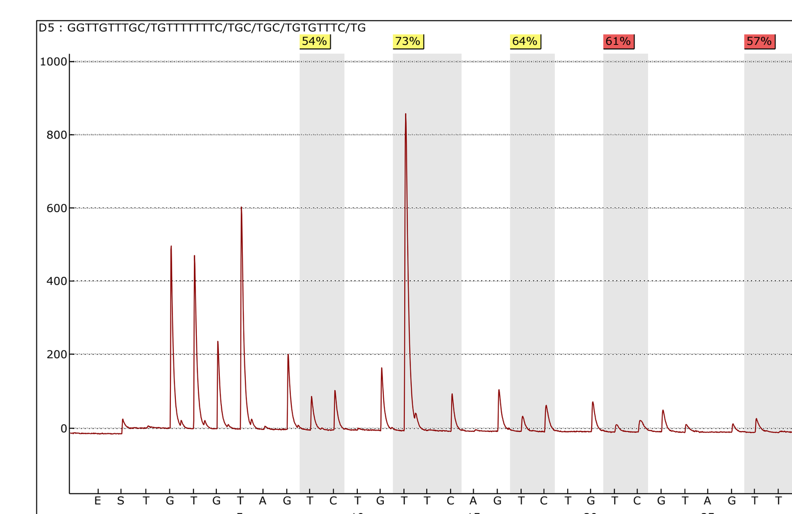


Figure 4: A pyrogram with low peaks, probably due to a flawed bisulphate modification

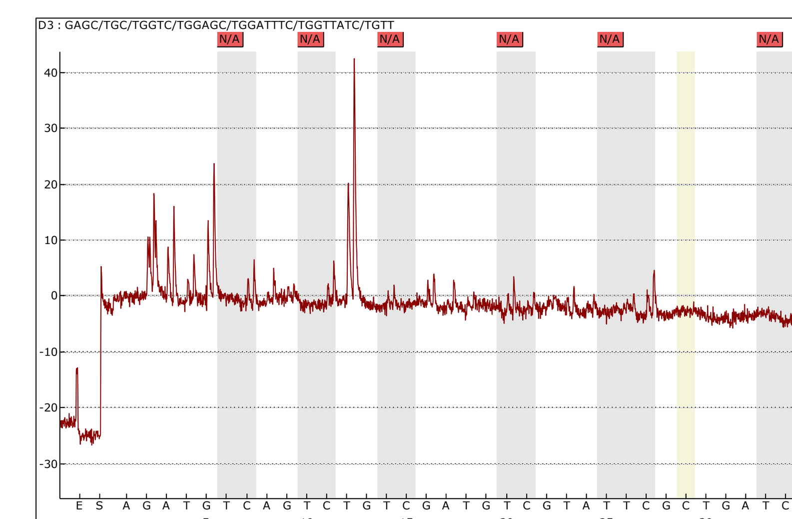


Figure 5: A failed pyrogram due to human error

Results

By taking the mean methylation values of the CpG islands found in the promoter regions of my chosen genes at diagnosis and relapse a clear correlation should have been apparent. This pattern would show high methylation levels upon presentation, and an increased level of methylation upon relapse, however no clear conclusions can be drawn from my results. If my data had shown evidence of this the experiment would have been extended to 150 pairs of patient samples to confirm if high levels at presentation indicate likelihood of relapse in later life. Mainly due to time constraints and technical errors I was unable to take repeat results of my data, and therefore my data is relatively unreliable. In addition to this some of the methylation values were highly unlikely, and therefore probably incorrect. For some of my data the pyrosequencer failed the results because of low peak values, this is most likely due to a problem with the bisulphate modification stage. Due to the small amount of sample available for each patient and limited time I was unable to modify more sample and repeat the process.

		CpG Position Methylation (%)						Statistics calculated over all CpGs
Patient Number		Pos. 1	Pos. 2	Pos. 3	Pos. 4	Pos. 5	Pos. 6	Mean
4	4508	23.7	22.5	33.11	20.97	17.61	24.81	23.78
	4907	33.68	32.96	40.32	31.58	20.89	30.59	31.67
5	827	85.71	77.27	77.4	82.09	61.39	48.3	72.03
	1305	92.22	88.38	94.93	90.45	89.92	86.3	90.37
10	773	48.58	47.9	51.88	46.41	36.96	40.54	45.38
	959	68.17	64.55	71.82	58.5	56.75	56.28	62.68
11	2511	50.76	49.52	56.08	50.57	45.43	47.38	49.96
	2766	80.82	81.69	83.95	68.74	42.19	45.11	67.08
16	3322	83.64	82.22	80.91	81.42	72.39	76.74	79.55
	3718	88.75	78.9	91.98	84.31	73.95	79.26	82.86
20	3684	68.43	64.47	71.73	64.79	66.55	62.2	66.36
	4332	86.48	79.17	86.58	83.12	83.27	96.13	85.79
9	1628	20.04	22.07	11.91	16.82	15.9		17.35
	2202	87.42	92.83	80.9	100	100		92.23
15	7020	48.72	27.65	15.98	13.82	33.67		27.97
	7838	64.35	75.51	54.93	71.43	83.22		69.89
17	938	82.4	93.61	72.57	84.61	100		86.64
	1865	8.35	43.53	25.18	21.27	53.92		30.45

Figure 6: Sample of some of my final results, showing the methylation data for the diagnostic and presentation samples of 9 patients. These statistics were generated from methylation levels of CpG islands across the promotor region measured by the pyrosequencer. Data from patients 4, 5, 10, 11, 16 and 20 are taken from the Them4 gene, and the remaining patients 9, 15 and 17 were taken from the Wnt7a gene.

Setbacks:

- Was unable to find suitable PCR conditions for Them4, new forward and reverse primers were designed to fall in a different position along the promoter sequence.
- The Wnt7a sequencing primer for the pyrosequencer also required redesigning
- Bisulphate modification may have not been effective in some samples, causing low peak values in the pyrograms and resulting in a failed pyrosequence, see figure 4
- Unknown human errors produced a lack of product either at the PCR amplification stage or during pyrosequencing, see figure 5
- DNA contamination of the blank control sample

Conclusions

- No overall trend
- Some non reproducible data, indicating technical flaws

References: Harrington, C. T., et al (2013) 'Fundamentals of Pyrosequencing, *Archives of Pathology and Laboratory Medicine,'* Vol. 137; page 1296
Strathdee, G. R., et al (2014) 'DNA methylation abnormalities at gene promoters are extensive and variable in the elderly and phenocopycancer cells,' *The FASEB Journal,* Vol. 28; page 3261-3272

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