Introduction

Chronic Lymphocytic Leukaemia (CLL) is the most common form of Leukaemia in the UK. It is derived from mature B lymphocytes and usually occurs in adults over the age of 60 when mononuclear B cells with a specific immunophenotype accumulate in areas such as the bone marrow and lymph nodes \[1\]. It has a slow progression, with patients often monitored for long periods of time before starting treatment. Treatment relapse occurs frequently and is often associated with drug resistance, the molecular cause of which is usually unknown.

It has previously been found that methylation levels of KCNMA1 (potassium calcium-activated channel subfamily M alpha 1) and HOXA4 (homeobox A4) measured before initiation of treatment is an indicator of post-treatment survival. When DNA is methylated, a methyl (CH$_3$) group is added to the cytosine bases of the DNA sequence. If this occurs in the promoter regions of a gene, it usually acts to reduce gene transcription and thus expression. Increased methylation of KCNMA1 and HOXA4 genes is associated with increased risk of death due to treatment resistance \[2\]. Most molecular markers in CLL are predictive of time until first treatment but are only weakly associated with survival duration after therapy initiation. Thus they do not predict if patients will respond well to treatment or predict which treatments would be optimal for a particular patient. If the combined KCNMA1/HOXA4 epigenetic marker can be confirmed as a predictor of survival duration after therapy initiation, it would be a valuable addition to the currently used molecular markers.

The aim of my project was to perform methylation analysis on the KCNMA1 gene, allowing me to assess the utility of the combined KCNMA1/HOXA4 marker for chemotherapy resistance in CLL.

Methods

Methylation was assessed in 143 CLL patient samples using the COBRA (combined bisulphite restriction analysis) method. This involved:

1. **Bisulphite DNA modification** – Introducing methyltransferase-dependent sequence differences into the DNA by converting unmethylated cytosine bases to uracil (methylated cytosines are unaffected).

2. **Polymerase Chain Reaction (PCR) amplification**, producing many copies of the target DNA sequence.

3. **Enzymatic restriction digest**, in which sites that were initially methylated are cleaved into specific fragments.

4. **Polyacrylamide gel electrophoresis** – This allows visualisation of digested fragments and subsequent analysis of methylation levels.

Five controls were also present on each COBRA assay – 0%, 33%, 66% and 100% methylated, as well as a negative (water) control (figure 1). These allowed calculation of the percentage methylation levels in each sample and visualisation of any contamination. Six primer sets were tested to determine the optimal one to use, and the one performing optimally (seq6) was chosen for further use. This required the use of enzymes BisEl and TaqI.

Results

The size of the bands present on the imaged gel depended on the size of the fragments produced by the restriction digest. The band size was assessed using a 100bp DNA ladder. The brightness of the bands corresponded to the levels of methylation present. By looking at the controls, the percentage methylation levels present in each sample could be calculated.

![Figure 1: A diagram visualizing an ideal set of controls on an COBRA assay (Source: Dr Gordon Strathdee)](image)

![Figure 2: An example of an imaged COBRA assay showing a DNA ladder, 10 samples plus 5 controls; 0%, 33%, 66% and 100% methylation, and a water (negative) control.](image)

![Figure 3: A table showcasing KCNMA1 methylation results alongside other relevant data, such as HOXA4 methylation and the patient’s age, sex, and survival.](image)

![Figure 4: A graph comparing the methylation levels of HOXA4 and KCNMA1 genes. The r value is equal to -0.36, meaning that there is a negative correlation, however as the p value is 0.154 (3SF) and greater than 0.05 these data are not statistically significant.](image)

Discussion

- Slight negative correlation seen between HOXA4 and KCNMA1 methylation ($r = -0.36$) (figure 4). However $p > 0.05$ therefore these data are not statistically significant.

- There was also no statistically significant correlation between KCNMA1 methylation and other variables, including patient age ($p=0.332$), overall survival ($p=0.308$) and gender (data displayed in figure 3). $P > 0.05$ for all of these variables.

- Issues with contamination and problems with primer sequences caused the restriction digest or PCR to often fail.

- Therefore a lack of viable results was produced, and KCNMA1 methylation was only calculated for 10 our of the original 143 DNA samples - a very small sample size.

- 0% methylation control on COBRA in figure 2 did not work, so I had to compare the samples to a 0% methylation control on another gel, possibly affecting the accuracy of my results.

- Background bands are also present on the COBRA, making it difficult to identify the relevant bands and thus their methylation levels.

- If the methylation analysis was expanded to include all 143 samples then significant correlation between the data may be seen.

Conclusion

- Still uncertain whether KCNMA1 methylation can act as a marker for chemotherapy resistance in CLL.

- Data suggests some correlation between KCNMA1 and HOXA4 methylation, but it is not statistically significant.

- Further testing is required to find the components that produce the best results. The study could then be run again with a larger sample size, clarifying any possible correlation.

- Further research is required to expand the KCNMA1 methylation analysis and to assess ability of the combined KCNMA1/HOXA4 epigenetic marker before a full conclusion can be drawn.

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References
