**RCPA Foundation Grant Final Report – Dr Stephanie Richards**

Haemophagocytic lymphohistiocytosis (HLH) is a potentially fatal, multisystem clinical syndrome that is characterised by an excessive and ineffective immune response to various stimuli, with subsequent development of a hyperinflammatory state. There are well described molecular defects that can predispose patients to the development of HLH, including X-linked inhibitor of apoptosis (XIAP) deficiency, caused by mutations in the *BIRC4* gene. This molecular defect was first described in male patients with X-linked lymphoproliferative syndrome type 2 (XLP2). Typically considered to affect only males, more recently symptomatic female carriers with skewed X chromosome inactivation have also been described. A rapid flow cytometry screening test for measurement of intracellular XIAP expression has been described and is used in the evaluation of patients with clinical features suggestive of XIAP-deficiency. In 2014, the diagnostic immunology laboratory at the Royal Children's Hospital, Melbourne was involved in the evaluation of a male patient with clinical HLH on a background of severe inflammatory bowel disease from early childhood. Flow cytometry identified absent intracellular XIAP protein expression, which was confirmed on a duplicate sample. The patient, visiting from the UK, was treated and stabilised, and returned to the UK where he underwent a haematopoietic stem cell transplant. The absence of intracellular XIAP expression was confirmed in the immunology laboratory at Great Ormond Street Hospital in London, however no *BIRC4* mutation was identified by Sanger sequencing of this gene in two separate laboratories in Australia and the UK.

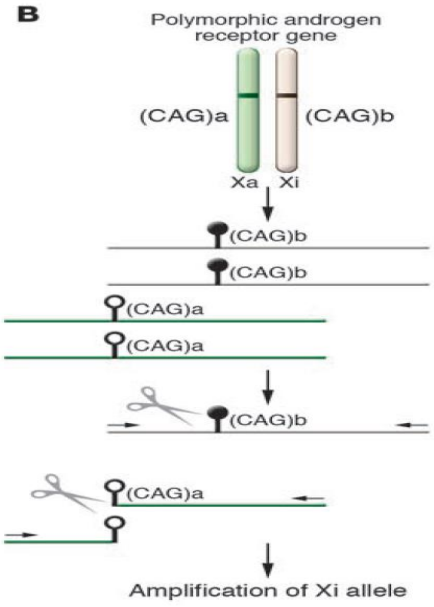
The aim of this project was to investigate the possibility of altered X chromosome gene expression in a male patient with clinical manifestations of HLH and absent intracellular X-linked inhibitor of apoptosis (XIAP) expression, with no identifiable gene variant in the *BIRC4* gene. The laboratory work involved in this project was based in the molecular genetics laboratory at Cincinnati Children's Hospital Medical Centre (CCHMC) under the supervision of Dr Kejian Zhang. This laboratory has a particular interest in the gene defects associated with HLH, and also first described skewed X chromosome inactivation resulting in XIAP deficiency in a female patient.

X chromosome inactivation (XCI) was first described by Lyon in 1966 and is the process by which one of the two copies of the X chromosome present in female somatic cells is inactivated in order to correct the dosage imbalance of X-linked genes between males and females. This process is random and occurs early in development, with the expected ratio of expression from maternally and paternally inherited chromosomes of 50:50 in normal females. However, abnormal patterns of X chromosome silencing can occur, and skewed X chromosome inactivation can result in symptoms of disease in carriers of X-linked conditions. Determination of maternal and paternal X chromosome activation status is useful in the diagnostic analysis of non-random, or skewed X chromosome inactivation patterns. Several different genes have been used to assess X chromosome inactivation however the majority of published studies make use of the human androgen receptor (HUMARA) gene. Allen *et al* identified that the HUMARA gene contains a highly polymorphic trinucleotide repeat in the first exon, and that methylation of restriction enzyme sites 100bp from this short tandem repeat (STR) correlates with X chromosome inactivation. This enabled the development of a PCR assay to determine the methylation status of maternal and paternal alleles, thereby identifying the pattern of X chromosome

inactivation. Using a methylation sensitive restriction enzyme (*HpaII)*, an unmethylated gene on the active X chromosome will be completely digested, so that only digestion-resistant HUMARA genes on an inactivated X chromosome remain, allowing amplification of these sequences by PCR. Following PCR, DNA fragmentation analysis can identify the X chromosome activation pattern for maternal and paternal alleles (Figure 1).

The first phase of this project involved the establishment of the HUMARA assay in the diagnostic molecular genetics laboratory at CCHMC, as this assay had previously only been performed in a research laboratory. This initially involved primer design for the HUMARA gene and was then followed by optimisation of the assay using control DNA samples from 10 male and female patients from the CCHMC laboratory. The assay itself involved four steps – (1) DNA digestion using restriction enzymes, (2) DNA precipitation, (3) PCR and (4) DNA fragment analysis by capillary electrophoresis, with the assay taking a total of two days to perform. Optimisation of the assay included shortening the digestion reaction time, identifying the optimal reaction volume to enable utilisation of the smallest amount of patient DNA, optimising PCR cycle times and lengthening the extension time of the PCR to identify the ideal conditions for this assay. Following assay optimisation, the second phase of this project involved analysis of the patient DNA in triplicate (separate assays) alongside three control samples; a normal male, normal female and a skewed female XCI DNA sample. The results indicated that there was normal HUMARA gene expression in our patient, with no obvious alteration in X chromosome methylation which may have explained the patient's lack of XIAP expression despite no genetic variant being identified in *BIRC4* (Figure 2). Due to time limitations in establishing the assay and analysis of the patient DNA sample using the HUMARA assay, it was not possible to perform further genetic testing on the patient DNA. However, I was fortunate to be able to spend time with the bioinformatics team at CCHMC during this project, and this enabled identification of several areas of interest in the intronic regions of the *BIRC4* gene that may be relevant for further investigation as a potential location for a genetic variant that may explain this patient's clinical and immunological phenotype. It is anticipated that as a result of this project, there will be ongoing collaboration between the diagnostic immunology laboratory at RCH, the molecular genetics laboratory at CCHMC and the patient's clinical team in the UK, which will continue to enhance international relationships in the field of primary immunodeficiency genetics, and ultimately improve patient diagnostics and care.

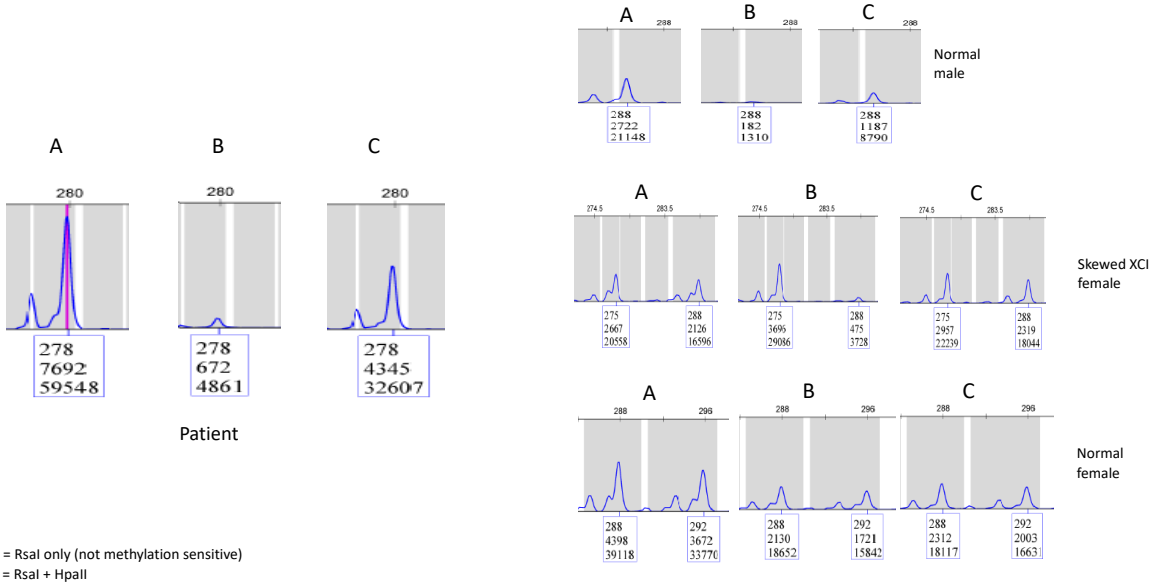
# HUMARA assay principle




- Highly polymorphic CAG repeat (n = 9 to 36) present in exon 1 of the androgen receptor gene on the X chromosome
- Differential methylation of the active and inactive chromosome
- Cleavage sites for methylation-sensitive restriction enzymes in close proximity to the CAG repeat
  - Hypermethylated on Xi
  - Unmethylated on Xa
- Methylation-sensitive restriction enzymes are only able to digest DNA on Xa (unmethylated)
- Used to distinguish between active and inactive X chromosomes (and maternal/paternal X chromosomes)
- PCR of the digested DNA
  - Amplification of the *AR* gene from the inactive X chromosome only (methylated)

Minks J, Robinson W and Brown C. J Clin Invest 2008;118(1):20-23

**Figure 1:** HUMARA assay summary




A = RsaI only (not methylation sensitive)
B = RsaI + HpaII
C = No enzyme control

**Figure 2:** Representative patient results of HUMARA assay