**Consultant’s pump-priming small grants scheme - Grant report**

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*Methylation-specific markers for circulating tumour DNA (ctDNA) in patients with chordoma*

**Introduction and purpose of investigation**

The clinical outcome for the majority of patients with sarcomas has not improved over the last 30 years, and patients would benefit from a non-invasive surveillance assay, which could be employed to assess treatment response to current therapy, and for assessment of suitability for recruitment into clinical trials.

We have developed a comprehensive investigation of the DNA methylation profiles from more than 10,000 cases of tumour and normal tissues, including 800 sarcoma cases, and identified CpG sites capable of separating chordomas from other cancers and blood samples. The aim of this project was to verify that these chordoma-specific CpG sites were indeed chordoma-specific and to demonstrate that these possess a level of sensitivity and specificity which could potentially allow them to be employed for early detection of clinical relapse by measuring ctDNA in plasma samples from patients with chordoma.

**Results**

The methylation status of tumour and germline DNA at the chromosomal locations of the chordoma-specific CpG sites was validated by Sanger sequencing: tumour-derived DNA was as expected methylated (n=12) and germline DNA was unmethylated (n=12).

Next, we designed digital droplet PCR assays for the four assays, and tested them both as single assays and in duplex. The temperature gradient for two of the biomarkers showed similar performance using methylated and unmethylated control DNA in both single and duplex, however the singleplex reactions for two of the biomarkers performed more sensitively and robustly than the duplex reaction.

To stringently assess the performance of the assays, the limit of blank (LOB) was determined. The LOB is defined as the highest apparent analyte concentration expected to be found when replicates of a blank sample containing no analyte are tested essentially determining the number of false positives. Given the intended purpose of these assays for clinical use, the LOB was established using germline DNA which would be expected not to contain methylated analyte to ensure a highly conservative measure.

At this stage the four assays, in singleplex and duplex, were unable to discriminate between tumour and blood-derived DNA with sufficient specificity to be taken forward for testing on plasma samples. A significant difference between the measured tumour and germline methylation fractions (p<0.00001 and p=0.0002) was detected however, the non-specific binding of all assays to unmethylated DNA and low methylation fraction in tumour-derived DNA rendered these assays of insufficient specificity and sensitivity to be developed further for clinical use.

Other steps were taken in an attempt to optimise technical parameters such as reverting to singleplex reactions, however there was insufficient improvement in the specificity. Moreover, to ensure that the signal detected in the blood-derived DNA was not indicative of contamination by ctDNA in a patient with recurrence for example, the assays were tested on 10 germline samples from patients with osteosarcoma. In a similar fashion to the chordoma patients ≥3 droplets were detected in 7 out of 10 osteosarcoma samples which indicates methylated status suggesting that the lack of specificity is a technical issue rather than a biological phenomenon associated with chordoma. Finally, the probes for one of the assays was also redesigned to target the neighbouring nucleotides and to include a further CpG locus to see if an increase in specificity could be achieved by altering the microenvironment targeted by the assay. This modification did not result in a significant improvement.

*Circulating-free DNA*

For a number of other cancers, including breast, colon, lung and prostate, a robust association between clinical outcome and level of cell free DNA (cfDNA) has been reported such that the highest levels of cfDNA are predictive of poorer clinical outcome (Fernandez-Garcia, 2019; Tissot, 2015; Mehra, 2018). This represents a cost-effective alternative approach to personalised genetic assays. cfDNA has not been investigated in chordoma patients and we therefore utilised the internal control assays from our previous study to measure cfDNA prior to treatment and determine if this correlates with clinical outcome in chordoma as seen in other cancers. More specifically, we asked if cfDNA predicts outcome (overall survival and relapse free survival) in patients with chordoma? To resolve this, we have identified and run an initial cohort of 34 patients for whom there is survival data available and have sufficient follow up if they have not recurred or progressed (~7 years from diagnosis). We measured the level of cfDNA in these samples and compared it to clinical outcome. Unfortunately, we did not see a correlation between the cfDNA levels and outcome in chordoma.

**Conclusions**

* DNA methylation profiles can distinguish chordoma samples from other cancer and normal samples
* Chordoma-specific assays are highly sensitive for chordoma tumours, but are not sufficient specific to be applied in a clinical setting.