**Grant Ref No: PHD 2015/02 - John Pritchard 3nd Year PhD Progress Report**

**Molecular tracking of muscle invasive bladder cancer by circulating tumour DNA**

Regular monitoring of BC is required due to high relapse rates and disease progression1-2. Patients with late stage BC are often in clinical trials and they benefit from the timely knowledge of tumour status. Circulating tumour DNA (ctDNA)3-based monitoring is known to provide considerable lead-time in detecting recurrence4. The ctDNA levels are shown to be higher in progressive BC than in recurrent disease5. BC has few common mutations. However, the TERT -124 mutation is found in close to 60% of tumours8, and therefore it is an ideal target for a ddPCR assay using liquid biopsy. Futhermore, it has been reported that whole exome sequencing (WES) could be utilised to identify tumour-specific mutations to overcome the issue of uncovering common mutations5. In this study, we aimed to extend the repertoires of tumour-specific mutations that can be assayed by ddPCR to monitor BC in liquid biopsies. The objectives were: 1) to develop an assay for the TERT -124 using ddPCR and monitor disease status of patients using plasma, 2) to evaluate WES as an unbiased approach to identify patient-specific tumour mutations, and 3) to develop a patient-specific assay for CNTNAP4 G727\* identified by WES.

**Methods**

Samples were collected under MI84 ECMC Blood Biomarkers Study. Matched FFPE tumour samples were from the NHS Greater Glasgow & Clyde Biorepository (John Pritchard, Year 1 PhD report). ddPCR assays were developed in-house for mutations in TERT and CNTNAP4. WES of FFPE tumour DNA (pT2G3, n=3) and corresponding patient's germ line DNA (PBMC) was performed using Illumina NextSeq 500 system (Glasgow Polyomics Facility, University of Glasgow) (John Pritchard, Year 2 PhD report).

**Results**

**A ddPCR assay for TERT -124 promoter mutation was optimised using vector DNA.**

To develop a ddPCR assay for TERT -124, annealing temperature, cycle number and primer to probe ratio were optimized, and the specificity and sensitivity of the assay was evaluated. Varying ratios of WT and Mut probes were tested (WT:Mut, 100%:0%, 75%:25%, 50%:50%, 25%:75%, 0%:100%) with a fixed amount of each template. It showed that the Mut probe resulted in false signals on WT template when the probe ratios were WT:Mut 0%:100% and 25%:75%, as well as the WT probe resulting in false signals on the Mut template when the probe ratios were WT:Mut 100%:0% and 75%:25%. However, at the probe ration of Mut:WT 50%:50%, both Mut and WT signals were amplified specifically. In order to identify the level of detection (LOD), 0.07-5% of Mut template DNA was spiked into WT template DNA. LOD of the Mut allele was 0.5% fractional abundance (FA).

**The TERT -124 assay distinguished TERT -124 mutation-positive and negative tumours.**

Patient tumour DNA with known TERT -124 status from SNaPshot assay was subjected to the ddPCR assay optimized above. The ddPCR assay concurred with the SNaPshot assay in 100% of the TERT -124-positive cases (9 out of 9) using as little as 1 ng of template DNA. Eight out of 9 patients (88%), the ddPCR assay correctly identified the tumour as negative with the mutation, agreeing with the SNaPshot assay. One case where SNaPshot was negative, ddPCR called the mutation as positive at 7% FA. The LOD for SNaPshot is reported to be between 5-10%. Therefore, Mut allele is likely to have been below the detection limit of SNaPshot in this case.

**Disease status was monitored in two patients with the TERT -124 mutation**

Case 1: Patient 3 was a 77-year-old male with a pT2-G3 tumour identified as TERT -124 positive by SNaPhot. His tumour was confirmed to be positive for the Mut allele by ddPCR (Fig 1A). The Mut allele was detected in the plasma (Fig 1C) collected at 1 week into chemotherapy, prior to cystectomy. However, Mut allele was not detected in the plasma at 45 months post cystectomy (Fig 1D). This agrees with the clinical information where this patient has been in remission over 5 years.

Case 2: Patient 11 was a 68-year-old male with a pT2-G3 tumour identified as TERT -124 positive by SNaPshot. His tumour was confirmed to be positive for the Mut allele by ddPCR (Fig 1E). Plasma was collected 1-month into chemotherapy prior to cystectomy. Some positive signals were observed, however the FA was below the LOD of this assay (Fig 1F). Further plasma was collected at 2 and 17-months post-cystectomy. In these samples, the Mut FA was above the LOD (Fig 1I). Increase in the FA of Mut allele was observed at 17 months compared to 2 month (Fig 1G-I). Clinically, this patient has had several relapses as well as metastasis. The patient is currently in remission, however, is being carefully monitored by the oncologist.

**Assayable mutations, such as CNTNAP4, was identified by WES.**

In order to evaluate whether patient-specific mutations assayable by ddPCR can be identified by WES, I have developed a system of data filtering. The developed filter identified 54-249 candidate mutations from a total of 19673-39778. The CNTNAP4 G727\* mutation was identified in at least 10% of reads and with a read depth of >50 in one of the patients (Patient 5). A ddPCR assay designed for this mutation was able to distinguish WT and Mut genomic DNA in patient tumour and plasma samples successfully.

**Discussion**

This study has demonstrated the following. Firstly, we have successfully developed an in-house-designed assay for TERT -124 mutation and monitored disease status in two patients. Secondly, WES can be used to identify patient-specific mutations and that this information can lead to development of patient-specific ddPCR assay using CNTNAP4 gene mutation.

Commercially available ddPCR assays are currently limited to the most common cancer mutations. BC is highly heterogeneous and has few common mutations. Therefore, it is important to develop efficient assays for the limited number of assayable mutations. Some common mutations identified are found in the TERT promoter and can be present in up to 85% of BC tumours6-8, TERT -124 mutation is especially prevalent, at 60%. So far, there have been only two publications that had reported ddPCR mutation assays for TERT -124. One study stated it was interrogating the TERT -124 mutation, however the probe appeared to target chr5: 1,295,2259. This differs from our assay and previous literature that state the position of the TERT -124 mutation is chr5: 1,295,2288-9. The second study designed to target two different mutations at once10. The assay uses a single probe to target two mutations and causes a significant shift in amplitude making it difficult to distinguish true positive droplets. Therefore, our assay is the first ddPCR that targets the TERT -124 mutation only.

SNaPshot-based detections of mutations in a panel of 7 genes investigating over 40 well-known mutation sites covered 70% of our cohort (n=21) (Year 1 report). 14% of patients had two mutations identified, 57% had just 1 mutation and 29%, no mutation. Using ddPCR, we were able to successfully monitor 19% of our patient cohort that are undergoing clinical trials.

Possibility of NGS-based mutation detection for development of patient-specific assays has previously been reported5. Our study agrees with the previous report. However, we have recognized a significant workload and time as a challenge. Furthermore, no single common mutation was identified among three tumours subjected to WES, despite the presence of over 19000 mutations. This indicates that identification of any common mutations through NGS sequencing is unlikely. The WES also highlighted that intratumoural heterogeneity could pose a challenge in identifying the trackable mutation after treatment, particularly when clonal pressures are high. The NGS applied directly in liquid biopsies could overcome this challenge in the future.

**The Future Work**

The objectives are: (1) to investigate whether Whole Genome Sequencing (WGS) of patients who are still undergoing treatment would provide any advantage to WES in identifying patient-specific assays in tumour biopsies, (2) to analyse shallow WGS directly applied on plasma samples, as an assay to identify and monitor multiple mutations in n=3 patients. We hope to publish our data as a summary of case studies in successfully monitoring the patient status using PIK3CA, TP53 Y163C, TERT -124 and CNTNAP4 G727\*.

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