**Final PhD Pathsoc report: Investigating radiation responsiveness in rectal cancer**

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**Background and aims: Initial aims of the project:** 1)Establish radiation-sensitive and radiation-resistant primary cell lines from rectal tumours and test them with radiosensitising chemotherapy that inhibit either glutathione or thioredoxin proteins. 2)Investigate the redox-homeostasis proteome and common rectal cancer oncoproteins as a predictive marker in biopsy specimens and primary cell lines. 3)Evaluate the utility of cell-free DNA (cfDNA) as a marker of tumour response to neoadjuvant CRT.

 **Background:** In the first PathSoc report, full-COLD PCR (Milbury et al, 2011) was used to determine mutations in corresponding FFPE tissue and cell-free DNA. It was deemed an unreliable and ineffective method which lead to the development of our own mutation detection system to complete the planned research. At the time we were not able to find another system which was publishing reliable data on cell-free DNA. Cell-free DNA research is still currently lacking in an effective patient-specific genome-wide mutation detection method that is rapid, reliable, cheap and sensitive. The research conducted in this PhD has aimed to solve the issue in order to broaden the effectiveness of cell-free DNA analysis in the future for all cancer’s and treatment modalities. Since the second-year report, great progress has been made in advancing and validating the highly optimised annealing temperature PCR (HOT\_PCR) liquid biopsy pipeline. The umbrella name is now referred to as HOT\_MAMAS PCR which includes 3 different methods sharing the same modification with different advantages: HOT\_PCR, HOT\_PI (probe-inhibited) PCR and HOT\_ARMS (amplification refractory mutation system) PCR. HOT\_PCR is a rapid multiplex protocol which is combined with high-resolution melting analysis and Sanger sequencing for FFPE sample mutation screening. HOT\_ARMS PCR is an ultra-sensitive real-time PCR based single nucleotide mutation detection system specifically designed for cell-free DNA which can scale across the whole genome with detection as low as 1 mutant copy. HOT\_ARMS PCR allows for the generation of personalised mutation detection panels for cancer treatment-response monitoring based on prior mutation analysis of FFPE biopsy or resection specimens. HOT\_PI PCR is a wild-type blocking mutation enrichment system designed to improve the limit of detection of existing mutation detection technologies for cell-free DNA analysis. HOT\_PI PCR is capable of up to 60x fold mutation enrichment on up to 10 nucleotides across a 50 base pair region whilst remaining consistently reliable. This allows for a reduction in the number of tests required for personalised HOT\_ARMS panels where the mutation of interest is located in areas of the genome with cancer-specific mutation hotspot clusters or in the future may allow for early cancer detection on cell-free DNA by mutation screening of a panel of frequently mutated genes without prior knowledge of mutations from FFPE samples.

 Due to a crisis in the Biobank, blood samples could not be collected from rectal cancer patients. Instead, plasma collected in year 1 of the PhD from 22 colon cancer patients undergoing surgery was used as a source of cell-free DNA for testing of the PCR pipeline. The pipeline is intended for treatment-response monitoring of all cancer’s; making results translational to rectal cancer patients.

 The Biobank crisis further impacted the primary tissue culture segment of the PhD plan as only 1 tissue sample was received. However, the culture was successful using the protocol from Ashley et al (2014) but 1 sample was not sufficient for the intended experimental plan and the work had to be halted.

 A total of 50 rectal biopsies with Mandard score were collected to search for predictive biomarkers of radiation response. RNA was extracted from all 50 samples and after strict quality control; 30 samples remained. Half of the samples were Mandard 1 and 2 which were classed as responders and the other half were Mandard 3-5 which were classed as non-responders. A total of 36 assays were designed and validated; including 33 target genes and 3 housekeeping genes. All target genes were either previously reported as predictive or gene’s responsible/ closely related to the thioredoxin or glutathione pathway.

**Results**: Further development of HOT\_ARMS includes: rapid BRAF V600E testing in under 30 minutes for outpatient’s department cfDNA testing; validation and development of multiplexing to cut down testing time; validation of the dynamic range on FFPE and cfDNA to prove the method can cope with extremely low DNA input; creation of an in-house master mix for improved reliability; successful blind testing of cfDNA samples (provided by Qiagen) against Qiagen’s new Therascreen test where it achieved 100% sensitivity and specificity; and successful blind testing of cross-reactivity using cfDNA samples from Qiagen where HOT\_ARMS did not produce false positives from similar sequence changes on the same nucleotide. HOT\_ARMS primers have also been updated to become more effective and 3 new assays have been generated for EGFR T790M, ESR1 E380Q and ESR1 D538G which are critical for treatment response monitoring in lung cancer and breast cancer. With a limited amount of plasma and cfDNA left from patient’s undergoing surgery in year 1, only HOT\_ARMS was carried out for mutation detection on cfDNA rather than validation with both HOT\_ARMS and HOT\_PI. Results are shown in table 1 where HOT\_ARMS was able to detect mutations in pre-surgery cfDNA samples for 6 out of the 9 total patients that harboured mutations in their FFPE tumour block. Tumour blocks were tested for hotspot mutations in PIK3CA, BRAF and KRAS. 8 of the 9 patients showed no presence of cfDNA mutations 1 day after surgery which was concordant with the follow-up data (an example is shown in figure 1). The single patient with a persistent mutation signal in both pre- and post-surgery did not present a recurrence or metastases up to February 2019. 1 day after surgery may not be sufficient for all patients to clear residual tumour DNA resulting in a false-positive signal.

 HOT\_PI PCR mutation enrichment caused dramatic gains in the sensitivity of Sanger sequencing and high-resolution melting analysis. Improving Sanger sequencing from a poor limit of detection of 20% mutant allele frequency to detection of 1% (figure 2) and high-resolution melting analysis from 6% to a reliable 0.2%. HOT\_PI PCR was tested using mutations in KRAS exon 2, codons 12 and 13. Equal mutation enrichment was achieved across all 3 commonly mutated bases with 7 sequence changes tested in total. By inference it can be assumed that other sequencing technologies such as pyrosequencing (5% LOD) and NGS (1-3% LOD) could undergo HOT\_PI mutation enrichment to improve sensitivity.

 9 out of 33 targets screened by RT-qPCR had very low expression in rectal cancer biopsies which was not subject to analysis. From the remaining targets, only 1 out of 22 targets was found to be significantly predictive whilst 1 other marker presented interesting data. COX-2 was found to be significantly predictive of response to chemoradiotherapy and was upregulated in non-responders compared to responders (median=0.025, n=13 vs median=0.09609, n=17); P=0.0349). TXNIP presented interesting data where a large sub-group of non-responders presented lower expression compared to non-responders. However, with a limited amount of statistical power the null hypothesis was favoured.

**Conclusions:** Since beginning in 2016 with no useable cell-free DNA from extraction and no reliable mutation detection method for analysis; a significant advancement in cell-free DNA analysis has been made. The HOT\_MAMAS liquid biopsy pipeline is ultrasensitive, cheap, reliable and scalable unlike anything else currently available and will allow cell-free DNA as a biomarker to be fully explored without worries of a bias in analysis due to limitations in methods used. The pilot study of liquid biopsies from colorectal cancer patients undergoing surgery has shown that surgical clearance can be determined in real-time on stage 1 and 2 tumours. These results are translational to rectal cancer patients undergoing chemoradiotherapy. Patient’s undergoing chemoradiotherapy have later stage tumours which are known to be easier to detect due to larger and more aggressive tumours which may release DNA into the blood more frequently.

 COX-2 has been found to be potentially predictive of radiation response previously (Min et al, 2008; Kobayashi et al, 2006; Spolverato et al, 2011). This research adds further validation to the predictive value of COX-2 overexpression presenting unfavourable response to radiotherapy for rectal cancer patients. The use of aspirin to downregulate COX-2 should be more widely investigated to potentially enhance rectal cancer tumour downstaging. Moreover, the study further confirms that TP53, BAX, EGFR and P21 which also required further validation from previously published work are not predictive of response as well as many thioredoxin and glutathione genes. COX-2 overexpression in non-responders proves that this data set is reliable and gives a platform for further investigation of predictive markers/novel targets for improving response.

**How Closely Have the Original Aims been Met:** The original aims considering the Biobank crisis have been met as well as they could have been given time and costs. All work which did not require the Biobank for completion was carried out.

**Outputs (including meeting abstracts, oral presentations, original papers, review articles) from the study in which the Pathological Society has been acknowledged:** Oral presentation on HOT\_ARMS PCR given at Leeds Pathsoc 2019

**References: 1)** Ashley, N., Jones, M., Ouaret, D., Wilding, J. and Bodmer, W. (2014). Rapidly derived colorectal cancer cultures recapitulate parental cancer characteristics and enable personalized therapeutic assays. Journal of Pathology, Jul; 234: 34-35. Doi: 10.1002/path. 4371. **2)** Milbury, C., Li, J., Liu, P. and Makrigiorgos. (2011). COLD-PCR: improving the sensitivity of molecular diagnostic assays. Expert review of molecular diagnostics, Mar; 11(2): 159-169. Doi: 10.1586/erm. 10.115/. **3)** Byung Soh Min, MD; Yoon Jung Choi, MD; Hong Ryull Pyo, MD; Hogeun Kim, MD; JinSil Seong, MD; Hyun Cheol Chung, MD; Sun Young Rha, MD; Nam Kyu Kim, MD, PhD. (2008). Cyclooxygenase-2 expression in pretreatment biopsy as a predictor of tumour responses after preoperative chemoradiation in rectal cancer. The journal of the American medical association, Arch Surg. 2008; 143(11):1091-1097. doi: 10.1001/archsurg.143.11.1091. **4)** H. Kobayashi , Y. Hashiguchi , H. Ueno , E. Shinto , Y. Kajiwara , H. LenzK. D. Danenberg , P. V. Danenberg , H. Mochizuki. (2006). Pretreatment COX-2 protein expression is a predictor of tumor regression in rectal cancer treated with preoperative short-term chemoradiotherapy. Journal of clinical Oncology, DOI: 10.1200/jco.2006.24.18\_suppl.3610 Journal of Clinical Oncology 24, no. 18\_suppl (June 20, 2006) 3610-3610. **5)** Spolverato, G., Pucciarelli, S., Bertorelle, R., De Rossi, A., & Nitti, D. (2011). Predictive factors of the response of rectal cancer to neoadjuvant radiochemotherapy. Cancers, 3(2), 2176–2194. doi:10.3390/cancers3022176.

**Tables/figures**

**Table 1: cell-free DNA testing by HOT\_ARMS on liquid biopsies obtained from colorectal cancer patients undergoing surgery to determine surgical clearance.**

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| **Sample** | **FFPE sample mutation** | **Pre-surgery mutation status HOT\_ARMS** | **Post-surgery mutation status HOT\_ARMS** | **Dukes/Metastasis/LN/V/****max tumour diameter (mm)/G/T** | **Metastases or recurrence occurred?** |
| **3** | KRAS c.35G>T | Wild | Wild | C1/MX/N1/V0/26/G2/pT3 | None – December 2018 |
| **4** | KRAS c.34G>T | Mutant | Wild | B/MX/N0/V0/32/G2/pT4 | None – January 2019 |
| **5** | KRAS c.34G>T | Mutant | Wild | C1/MX/N1/V1/55/G2/pT3 | No follow up data |
| **11** | KRAS c.38 G>A | Wild | Wild | A/MX/N0/V0/15/G2/pT1 | None – February 2019 |
| **12** | KRAS c.35G>A | Mutant | Mutant (1 day after surgery) | B/MX/N0/V0/35/G2/pT2 | None – February 2019 |
| **14** | KRAS c.35G>A | Mutant | Wild | A/MX/N0/V0/25/G2/pT1 | No follow up data |
| **18** | PIK3CA E545K | Mutant | Wild | B/MX/N0/V1/23/G2/pT3 | None – October 2018 |
| **21** | KRAS c.35G>T | Mutant | Wild | B/MX/N0/V1/50/G2/pT4 | None – May 2018 |
| **22** | KRAS c.35G>A | Wild | Wild | A/MX/N0/V0/35/G2/pT1 | None – June 2018 |

Figure 2. Sanger sequencing of a 95 base pair amplicon for KRAS exon 2 (codon 12 and 13 shown) which has undergone mutation enrichment by the highly optimised annealing temperature probe inhibited PCR system. Image A represents wild-type sequence from the HEK293T cell line; Image B shows a homozygous G>T mutation found in the colon cancer cell line SW480 (c.35G>T) on the second DNA base in the image; Image C shows SW480 spiked into HEK293T (wild-type) at 1% mutant allele frequency (MAF); Image D shows SW480 spiked into HEK293T (wild-type) at 5% MAF; Image E shows SW480 spiked into HEK293T (wild-type) at 10% MAF; Image F shows SW480 spiked into HEK293T (wild-type) at 20% MAF. As the limit of detection for Sanger sequencing is 10-20%, image C (1% MAF), D (5% MAF) and E (10% MAF) should be completely wild and image F (20% MAF) should show a small peak for Thymine. However, the mutation enrichment is so strong that the 1% MAF shown in image C becomes easily detectable and Thymine becomes the dominant peak in image D (5% MAF), E (10% MAF) and F (20% MAF).

