Assessing Melanoma BRAF status through ddPCR of cfDNA

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Background

Treatment of recurrent and metastatic melanoma has been revolutionised by the advent of targeted therapy and immunotherapy. The BRAF gene is known to be mutated in ~60% of melanomas[1]. Inhibitors of mutant BRAF are a systemic treatment offered for patients with stage III/IV melanoma who are known to carry a mutation in BRAF[2]. Currently, patients' BRAF mutation status is assessed through molecular analysis of tissue specimens.

Early detection of relapse allows for the most benefit to be derived from targeted therapies. Liquid biopsy of circulating free DNA (cfDNA) provides a sample of circulating tumour DNA (ctDNA), the levels of which can be used to non-invasively detect active disease and predict survival in melanoma[3].

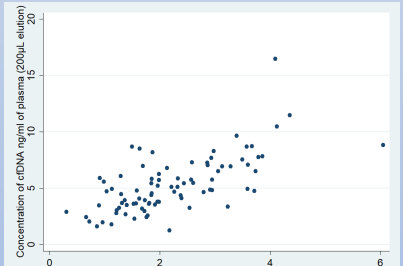
cfDNA provides a non-invasive method for assessing BRAF mutation status in patients with melanoma. This is useful in determining initial treatment, and in monitoring treatment response[4]. This project aimed to ascertain BRAF mutation status in cfDNA through digital droplet PCR (ddPCR) from patients with melanoma. Further work will include longitudinal analysis of BRAF status.

Materials & Methods

Plasma samples were collected according to an SOP optimised for cfDNA[3] by Dr. Silva as part of the Markers of Relapse in melanoma (MRM) study. Patients had either active unresected disease, or recent resection of cutaneous melanoma, at the time of recruitment. Baseline plasma samples were stored at -80°C.

Plasma was thawed and centrifuged at 1600g. Circulating DNA was extracted from 1-2ml of plasma using the QIAamp circulating nucleic acid kit (QIAGEN®) following manufacturer protocol and eluting cfDNA into 100µL. Circulating DNA was quantified with SYBR green quantitative real-time PCR (Life Technologies), based on an 87bp GAPDH gene amplicon.

ddPCR™ was performed at Sheffield Genetics Diagnostics Service using the Bio-Rad QX200 Droplet Generator™ and Droplet Reader as per manufacturer protocol. Human mutant BRAF assays with HEX and FAM fluorophores were used. Results were analysed with Bio-Rad QuantaSoft.



Graph 2: Scatter graph comparing cfDNA concentrations achieved with 100µL (x axis) and 200 µL elution volumes (y axis.) Pearson's r = 0.6687 (p<0.0005)

References:
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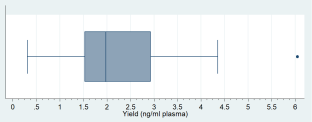
Results

Circulating free DNA extraction

Of 100 cases recruited to the MRM study, cfDNA was extracted from 85 baseline plasma samples. Of the remaining 15, 5 had insufficient samples for extraction. 4 cases had been withdrawn from the study. 7 samples have previously been tested for BRAF and NRAS by ddPCR by Dr Silva, with one of these being repeated in this project.

A median yield of 1.97ng/ml (range 0.31ng/ml - 6.05ng/ml) was achieved when cfDNA was eluted into 100µL. The distribution of yields is demonstrated in **Graph 1**.



Graph 1: Boxplot of cfDNA yield extracted from 85 cases. Min: 0.31ng/ml, median 1.97ng/ml, max: 6.05ng/ml. SD: 1.02.

ddPCR of cfDNA

74 samples yielded >10,000 droplets and were included for analysis. 12 samples contained BRAF mutant positive droplets. Concordance rate between tissue BRAF status and the presence/absence of cfDNA BRAF mutant droplets was 74%.

7/18 tissue BRAF mutant samples were cfDNA BRAF mutant positive, in contrast to 2/32 tissue BRAF wild-type samples. (**Figure 1**) The presence of BRAF mutant positive droplets in cfDNA was significantly different between tissue BRAF mutant and tissue BRAF wild-type groups (p = 0.004).

The presence of BRAF mutant droplets did not confer any significant difference in rate of relapse (p=0.758) or mortality rate (p=0.654).

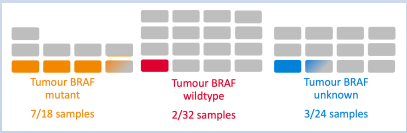


Figure 1: Concordance between tumour BRAF status and cfDNA BRAF status. Each square represents 2 patient samples. Coloured squares = BRAF mutant droplets detected by ddPCR for those samples.

A limitation of cfDNA is the low yield extracted from plasma, which is demonstrated in our results. The use of a 100µL elution volume differs from that used in the previous MRM study (**Graph 2**), and is known to reduce DNA yield during extraction.

Digital droplet PCR (ddPCR) permits detection of ctDNA mutations at low allele fractions. ctDNA exists at low fractions due to tumour heterogeneity and dilution within physiological circulating cell-free DNA. PCR-based approaches using cfDNA in melanoma have been used to detect BRAF mutations, and to monitor response to response to BRAF inhibitors[5].

Next generation sequencing has shown that longitudinal BRAF monitoring predicts patient responses to targeted therapy and immunotherapy[6]. Increasing presence of mutant BRAF can predict progression of disease, and accompanying analysis of NRAS can predict response to immunotherapy and elucidate mechanisms of treatment resistance[4].