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Short Communication

Detection of BRAF-V600E and V600K in melanoma circulating tumour cells by droplet digital PCR

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ABSTRACT

Objectives: Defining the BRAF mutation status in metastatic melanoma patients is critical to selecting patients for therapeutic treatment with targeted therapies. Circulating tumour cells (CTCs) can provide an alternative source of contemporaneous tumour genetic material. However methodologies to analyse the presence of rare mutations in a background of wild-type DNA requires a detailed assessment. Here we evaluate the sensitivity of two technologies for cancer mutation detection and the suitability of whole genome amplified DNA as a template for the detection of BRAF-V600 mutations.

Design and methods: Serial dilutions of mutant BRAF-V600E DNA in wild-type DNA were tested using both competitive allele-specific PCR (castPCR) and droplet digital PCR (ddPCR), with and without previous whole genome amplification (WGA). Using immunomagnetic beads, we partially enriched CTCs from blood obtained from metastatic melanoma patients with confirmed BRAF mutation positive tumours and extracted RNA and DNA from the CTCs. We used RT-PCR of RNA to confirm the presence of melanoma cells in the CTC fraction then the DNAs of CTC positive fractions were WGA and tested for BRAF V600E or V600K mutations by ddPCRs.

Results: WGA DNA produced lower than expected fractional abundances by castPCR analysis but not by ddPCR. Moreover, ddPCR was found to be 200 times more sensitive than castPCR and in combination with WGA produced the most concordant results, with a limit of detection of 0.0005%. BRAF-V600E or V600K mutated DNA was detected in 77% and 44%, respectively, of enriched CTC fractions from metastatic melanoma patients carrying the corresponding mutations.

Conclusions: Our results demonstrate that using ddPCR in combination with WGA DNA allows the detection with high sensitivity of cancer mutations in partially enriched CTC fractions.

Introduction

In the last few years, substantial advances have been made in the treatment of metastatic melanoma with the advent of therapies, such as vemurafenib and dabrafenib, targeting somatic mutations in position V600 of the v-Raf murine sarcoma viral oncogene homolog B (BRAF) [1,2]. BRAF mutations mediate tumour proliferation and survival via activation of the RAF–MEK–ERK pathway [3]. Around 43–66% of diagnosed melanomas carry BRAF mutations, with the most common being V600E (80%), with other variants found at lower frequencies; V600K (12%), M (4%), R (5%) and D (<5%) [4,5].

Heterogeneity in BRAF mutation within a patient has been described between primary tumours and metastases, and between different metastases [6–8]. Testing BRAF mutation status is usually performed on the most recently resected or biopsy tumour. A recent case study pointed to misclassification using this strategy with the patient missing critical treatment [9]. CTCs provide a sample with which to test for mutations even when the tumour biopsy is too old, not available or difficult to obtain being inaccessible by percutaneous biopsy.

We previously described a method for enrichment of melanoma CTCs from patient blood using immunomagnetic beads [10]. However, after enrichment, CTCs remain in a large background of white blood cells (1000–10,000 cells), which affects our capacity to detect cancer mutations. Two potential technologies to overcome this background and accurately detect the few copies of mutant DNA are competitive allele-specific TaqMan PCR (castPCR) or droplet digital PCR (ddPCR) [11].

Typically only 1–10 CTCs can be found in 4 mL of blood of metastatic melanoma patients [10]. Partitioning of the sample as required for

Abbreviations: BRAF, v-Raf murine sarcoma viral oncogene homolog B; CTCs, circulating tumour cells; ddPCR, droplet digital PCR; castPCR, competitive allele-specific TaqMan; WGA, whole genome amplification.

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droplet digital PCR might miss those rare copies of mutated DNA. Thus, pre-amplification of the input DNA is imperative for sample analysis. However, no previous analysis has been done on the suitability of whole genome amplified (WGA) DNA as a template for castPCR or ddPCR.

In this study we compared the sensitivity of ddPCR to a competitive allele-specific TaqMan (castPCR) assay (Life Technologies) for the detection of BRAF-V600E and V600K mutations using gDNA or WGA DNA as template. Furthermore, we evaluated ddPCR for the detection of BRAF mutations in a CTC enriched fraction from melanoma patients undergoing targeted therapies.

Materials and methods

DNA extraction and serial dilution curve construction

Genomic DNA was isolated from peripheral blood cells of a healthy donor and the melanoma cell line SK-MEL-28 (homozygous for the BRAF-V600E mutation) using an AllPrep DNA/RNA Mini kit (Qiagen).

Patient recruitment

Metastatic melanoma patients were enrolled in the study at Sir Charles Gardner Hospital (SCGH) in Perth, Western Australia, based on having confirmed BRAF-V600E or V600K mutations by molecular analysis of the most recent tumour biopsy. Written informed consent was obtained from all patients. The study was approved by the Human Research Ethics Committees of Edith Cowan University (No. 2932) and Sir Charles Gairdner Hospital (No. 2007-123).

CTC enrichment and nucleic acid extraction

Patient peripheral blood samples were collected in 4 mL EDTA tubes, stored at 4 °C, and processed within 24 h of collection. CTCs were enriched as previously described [10]. In summary, whole blood was treated with red blood cell lysing buffer and remaining cells were incubated with immunomagnetic beads coated with antibodies against MCSP (clone 9.2.27, BD Biosciences), ABCB5 (clone 3C2-1D12, kindly provided by Prof. Markus Frank) [12], MAGEA3 (rabbit polyclonal, Thermo Scientific) or RANK (clone 80704, R&D systems) or a combination of these cell surface antigens to target CTCs. After washing, DNA and RNA were extracted from these CTC fractions using an adapted version of AllPrep DNA/RNA Mini kit, using RNeasy MinElute Spin Columns for RNA extraction (Qiagen). RNA was eluted in 12 μL and DNA is 20 μL of RNAse-free water. Extracted RNA (6 μL) was used for cDNA preparation using the SuperScript® VILO™ cDNA Synthesis Kit (Life Technologies) followed by a TaqMan® PreAmp Master Mix Kit (Life Technologies). The presence of transcripts for melanoma genes MLANA, MAGEA3, Tyrosinase, ABCB5 and PAX3 was determined using TaqMan® probes in a ABI ViiA 7 Real-time instrument (Life Technologies). Detection of 18S was used as a positive control.

Whole genome amplification (WGA) reaction

DNA isolated from enriched CTC preparations as well as mutant/wild-type DNA dilutions were whole genome amplified using the Repli-g Midi Kit (Qiagen). For CTC-DNA, WGA was adapted as follows; 18 μL of DNA was incubated with 2.2 μL of DLB buffer for 3 min at RT, after which 3 μL of stop solution was added. The denatured DNA was then combined with a master mix of 29 μL of reaction buffer and 1 μL of Repli-g DNA polymerase. The reaction was incubated for 16 h at 30 °C followed by 3 min at 65 °C. WGA DNA was stored at −80 °C.

Droplet digital PCR

Either 1 μg genomic DNA or 4 μL of each WGA CTC-DNA sample was digested using 20 U HaeIII (New England Bio) and 1× NE Buffer 4 for 60 min at 37 °C followed by 20 min at 80 °C for enzyme inactivation.

Each droplet of a PCR supermix (Bio-Rad) reaction contained: 1× droplet PCR supermix, 250 nM of each probe, 900 nM primers and 100 ng of digested genomic DNA or 1 uL digested WGA DNA, in a total reaction volume of 20 μL. Samples were analysed for BRAF-V600E or V600K mutations depending on the mutation identified in the patient biopsy. The following probes were used: T1799-VIC WT (VIC-CTAGCT ACAGTGAAATC-MGBNFQ) and A1799-FAM V600E (6FAM-TAGCTACA GAGAAATC-MGBNFQ) or A1799-FAM V600K (6FAM-TAGCTACAAAGA AATC-MGBNFQ). The following primers were used for both assays: 5′-CTACTGTTTTCCTTTACTTACTACTACACCTCAGA-3′ (forward) and 5′-ATCCAGACAACTGTTCAAACTGATG-3′ (reverse). Probes and primers were custom synthesised by Life Technologies. Droplets were generated and analysed using the QX100 system (Bio-Rad). Amplifications were performed using the following conditions: 1 cycle of 95 °C for 10 min, 40 cycles of 94 °C for 30 s and 55 °C for 1 min, and 1 cycle of 98 °C for 10 min. All samples, including no template controls, were run in eight replicates. QuantaSoft analysis software (Bio-Rad) enabled fractional abundance to be calculated for each sample.

CAST PCR analysis

Competitive Allele-Specific TaqMan PCR (castPCR) was performed using TaqMan® Mutation Detection Assays (Life Technologies) for BRAF-V600E. A reference assay is used as a control. Each 20 μL reaction contained: 1× Genotyping master mix, 1× TaqMan BRAF 476 mutant detection assay and 9 μL of template, either 1:20 diluted WGA DNA or 100 ng of genomic DNA. Amplifications were carried out in a ViiA 7 system (Applied Biosystems) under the following cycling conditions: 1 cycle of 95 °C for 10 min, 5 cycles of 92 °C for 15 s and 58 °C for 1 min, then 60 cycles of 92 °C for 10 min and 60 °C for 1 min. Results were analysed using the Mutation Detector software (Life Technologies).

Results

Effect of WGA on the sensitivity of castPCR and ddPCR

Serial dilutions from 5 to 0.005% of mutant BRAF-V600E DNA were prepared in a constant background of homologous wild-type DNA and tested using both ddPCR and castPCR, with and without previous WGA. Wild-type DNA was included as a negative control in each assay. Analysis of BRAF-V600E using gDNA as input template produced comparable results in both castPCR and ddPCR (Fig. 1A). CastPCR overestimated the fractional abundance at 0.005% input frequency. This is consistent with the reported limit of the detection for the assay at 0.1% (www.lifetechnologies.com). Analysis of the results using WGA DNA as input template showed that castPCR yielded consistently lower than expected fractional abundances. This could have been interpreted as a bias amplification of the wild-type gene by the WGA reactions. However, ddPCRs using WGA DNA as template produced the results comparable to gDNA, with highly concordant fractional abundances. Moreover ddPCR was the only analysis which provided accurate fractional abundance estimations at 0.005% of V600E mutant (Fig. 1A). To further evaluate the sensitivity of the ddPCR using WGA DNA as template, a serial dilution curve extended to 0.00005% was evaluated, revealing a limit of sensitivity at 0.0005% (Fig. 1B).

Analysis of BRAF mutation in a CTC enriched fraction from melanoma patients

We next analysed DNA from 30 enriched CTC fractions from 15 metastatic melanoma patients with recorded BRAF-V600E mutated

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