SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
PREDICTIVE MARKERS
TRANSLATIONAL RESEARCH
HIGH-THROUGHPUT SCREENING
MELANOMA

Received
22 February 2013

Accepted
25 March 2013

Published
15 April 2013

Correspondence and requests for materials should be addressed to B.I. (barry.iacopetta@uwa.edu.au)

* These authors contributed equally to this work.

A multisite blinded study for the detection of *BRAF* mutations in formalin-fixed, paraffin-embedded malignant melanoma

Anna Richter[1], Fabienne Grieu[2], Amerigo Carrello[2], Benhur Amanuel[2], Kateh Namdarian[3], Aleksandra Rynska[3], Amanda Lucas[3], Victoria Michael[3], Anthony Bell[3], Stephen B. Fox[4,5], Chelsee A. Hewitt[3], Hongdo Do[3], Grant A. McArthur[4,5], Stephen Q. Wong[3], Alexander Dobrovic[3,4,5]* & Barry Iacopetta[1]*

[1]School of Surgery, University of Western Australia, Nedlands, Australia, [2]Molecular Pathology, PathWest, Sir Charles Gairdner Hospital, Nedlands, Australia, [3]Department of Pathology, Peter MacCallum Cancer Centre, Melbourne, Australia, [4]Department of Pathology, University of Melbourne, Australia, [5]Sir Peter MacCallum Department of Oncology, University of Melbourne, Australia.

Melanoma patients with *BRAF* mutations respond to treatment with vemurafenib, thus creating a need for accurate testing of *BRAF* mutation status. We carried out a blinded study to evaluate various *BRAF* mutation testing methodologies in the clinical setting. Formalin-fixed, paraffin-embedded melanoma samples were macrodissected before screening for mutations using Sanger sequencing, single-strand conformation analysis (SSCA), high resolution melting analysis (HRM) and competitive allele-specific TaqMan® PCR (CAST-PCR). Concordance of 100% was observed between the Sanger sequencing, SSCA and HRM techniques. CAST-PCR gave rapid and accurate results for the common V600E and V600K mutations, however additional assays are required to detect rarer *BRAF* mutation types found in 3–4% of melanomas. HRM and SSCA followed by Sanger sequencing are effective two-step strategies for the detection of *BRAF* mutations in the clinical setting. CAST-PCR was useful for samples with low tumour purity and may also be a cost-effective and robust method for routine diagnostics.

Mutations in the *BRAF* oncogene are found in approximately 50% of all sun-exposed melanomas[1–5]. The majority of mutations (70–80%) comprise a single base substitution in codon 600, identified as c.1799 T > A p.Val600Glu and commonly referred to as V600E. Of the remaining *BRAF* mutations in melanoma, by far the most common involves the mutation of two adjacent nucleotides and is identified as c.1798_1799delinsAA p.Val600Lys, or V600K[3,5–8]. Rarer mutations include c.1798_1799delinsAG p.Val600Arg (V600R), c.1801A > G p.Lys601Glu (K601E) and c.1799_1800delinsAA p.Val600Glu (V600E2).

BRAF mutation results in hyperactivation of the MAPK signalling pathway, causing deregulation of cell proliferation and oncogenesis without the requirement for Ras activation[1,9]. Braf is the most potent of the Raf proteins to activate the downstream signalling cascade, hence mutant Braf was identified as a novel target for kinase inhibitors such as vemurafenib (PLX4032/RG7204) [6,10,11] and dabrafenib[12].

In a recent phase 3 trial, treatment with vemurafenib was associated with improved survival of metastatic melanoma patients with the *BRAF* 600E mutation[13]. As a result of these clinical findings, vemurafenib was approved by the US Food and Drug Administration (FDA) for the treatment of advanced stage melanoma harbouring the V600E mutation. Promising data has also been reported with the Braf inhibitor dabrafenib[12,14]. Accurate determination of the *BRAF* status of melanomas is therefore crucial in deciding upon the use of Braf inhibitors in individual patients. For this purpose a companion diagnostic kit, the Cobas 4800 BRAF V600 mutation test (Roche Diagnostics) was also approved by the FDA. Although this test screens for the V600E mutation, it also shows some cross reactivity for the V600K mutation[15]. There is some evidence to suggest that patients with the V600K mutation and other rarer codon 600 and 601 mutations also respond to Braf or MEK inhibitors[13,16–19]. Hence the identification of these and other non-V600E mutant cases is critical to allow stratification of patients for possible treatment.

Table 1 | *BRAF* mutation detection in melanoma samples using four different methods

Sample	HRM	SEQ	SSCA	CAST	Mutation[1]	AA change
P01	○	○	○	○	wildtype	
P04	●	●	●	●	c.1798_1799delinsAA, p.Val600Lys	V600K
P05	○	○	○	○	wildtype	
P06	●	●	●	●	c.1798_1799delinsAA, p.Val600Lys	V600K
P08	○	○	○	●	c.1799T>A, p.Val600Glu	V600E
P09	○	○	○	○	wildtype	
P11	●	●	●	●	c.1798_1799delinsAA, p.Val600Lys	V600K
P12	●	●	●	●	c.1799T>A, p.Val600Glu	V600E
P13	●	●	●	●	c.1799T>A, p.Val600Glu	V600E
P14	○	○	○	●	c.1799T>A, p.Val600Glu	V600E
P15	○	○	○	○	wildtype	
P17	●	●	●	○	c.1801A>G, p.Lys601Glu	K601E
P19	○	○	○	○	wildtype	
P20	●	●	●	●	c.1799T>A, p.Val600Glu	V600E
P21	●	●	●	○	c.1799_1800delinsAA, p.Val600Glu	V600E2
P22	●	●	●	●	c.1798_1799delinsAA, p.Val600Lys	V600K
P23	●	●	●	○	c.1799_1800delinsAA, p.Val600Glu	V600E2
P24	●	●	●	●	c.1798_1799delinsAA, p.Val600Lys	V600K
P25	○	○	○	○	wildtype	
P26	●	●	●	●	c.1798_1799delinsAA, p.Val600Lys	V600K
P27	●	●	●	●	c.1799T>A, p.Val600Glu	V600E
P28	○	○	○	○	wildtype	
P29	●	●	●	●	c.1799T>A, p.Val600Glu	V600E
P30	●	●	●	●	c.1799T>A, p.Val600Glu	V600E
P31	○	○	○	○	wildtype	
P32	○	○	○	○	wildtype	
P33	●	●	●	●	c.1799T>A, p.Val600Glu	V600E
P34	●	●	●	●	c.1798_1799delinsAA, p.Val600Lys	V600K
P35	●	●	●	○	c.1801A>G, p.Lys601Glu	K601E
P37	●	●	●	●	c.1798_1799delinsAA, p.Val600Lys	V600K
P38	○	○	○	○	wildtype	
P39	●	●	●	●	c.1798_1799delinsAA, p.Val600Lys	V600K
P40	●	●	●	●	c.1798_1799delinsAA, p.Val600Lys	V600K
P41	●	●	●	●	c.1798_1799delinsAA, p.Val600Lys	V600K
P42	●	●	●	●	c.1799T>A, p.Val600Glu	V600E
P43	●	●	●	●	c.1798_1799delinsAA, p.Val600Lys	V600K
P44	●	●	●	●	c.1799T>A, p.Val600Glu	V600E
P45	●	●	●	●	c.1799T>A, p.Val600Glu	V600E
P46	●	●	●	●	c.1799_1801delinsAGG, p.Val600_Lys601delinsGluGlu	V600E K601E
P47	●	●	●	○	c.1801A>G, p.Lys601Glu	K601E
P48	●	●	●	●	c.1798_1799delinsAA, p.Val600Lys	V600K
P49	●	●	●	●	c.1799T>A, p.Val600Glu	V600E
P50	●	●	●	●	c.1798_1799delinsAA, p.Val600Lys	V600K
M01	○	○	○	○	wildtype	
M02	○	○	○	○	wildtype	
M03	●	●	●	○	c.1798_1799delinsAG, p.Val600Arg	V600R
M04	○	○	○	○	wildtype	
M05	○	NA	○	○	wildtype	
M06	○	○	○	○	wildtype	
M07	○	○	○	○	wildtype	
M08	○	○	○	○	wildtype	
M09	●	●	●	●	c.1799T>A, p.Val600Glu	V600E

Table 1 | Continued

Sample	HRM	SEQ	SSCA	CAST	Mutation[1]	AA change
M10	●	●	●	●	c.1799T>A, p.Val600Glu	V600E
M11	●	●	●	●	c.1799T>A, p.Val600Glu	V600E
M12	○	○	○	○	wildtype	
M13	●	●	NA	NA	c.1798_1799delinsAA, p.Val600Lys	V600K
M14	○	○	○	○	wildtype	
M15	○	○	○	○	wildtype	
M16	○	○	○	○	wildtype	
M17	●	●	●	●	c.1799T>A, p.Val600Glu	V600E
M18	○	○	○	○	wildtype	
M19	○	○	○	○	wildtype	
M20	○	○	○	○	wildtype	
M21	●	●	●	●	c.1798_1799delinsAA, p.Val600Lys	V600K
M22	●	●	●	●	c.1799T>A, p.Val600Glu	V600E
M23	○	○	○	○	wildtype	
M24	○	○	○	○	wildtype	
M25	○	○	○	○	wildtype	
M26	○	○	○	○	wildtype	
M27	○	○	○	○	wildtype	
M28	○	○	○	○	wildtype	
M29	●	●	●	●	c.1799T>A, p.Val600Glu	V600E
M30	●	●	●	●	wildtype	
M31	●	●	●	●	wildtype	
M32	●	●	●	○	c.1801A>G, p.Lys601Glu	K601E
M33	●	●	●	●	c.1799T>A, p.Val600Glu	V600E
M34	○	○	○	○	wildtype	
M35	●	●	●	●	c.1799T>A, p.Val600Glu	V600E
M36	○	○	○	○	wildtype	
M37	●	●	●	●	c.1799T>A, p.Val600Glu	V600E
M38	○	○	○	○	wildtype	
M39	○	○	○	○	wildtype	
M40	●	●	●	●	c.1798_1799delinsAA, p.Val600Lys	V600K
M41	●	●	●	●	c.1799T>A, p.Val600Glu	V600E
M42	○	○	○	○	wildtype	
M43	●	●	●	●	c.1799T>A, p.Val600Glu	V600E
M44	○	○	○	○	wildtype	
M45	○	○	○	○	wildtype	
M46	○	○	○	○	wildtype	
M47	○	○	○	○	wildtype	
M48	○	○	○	○	wildtype	
M49	○	○	○	○	wildtype	
M50	●	●	●	●	c.1798_1799delinsAA, p.Val600Lys	V600K

[1]The mutant sequence was determined in all cases by bidirectional Sanger sequencing. HRM, high resolution melt analysis; SEQ, Sanger sequencing; SSCA, single strand conformation analysis; CAST, CAST-PCR; AA, amino acid; open circle represents wild type *BRAF*; closed circle represents mutant *BRAF*.

We and others have reported the frequency of the V600K mutation may be as high as one third of all *BRAF* mutations in melanoma[3,5,6,16]. Indeed, our earlier study of 183 consecutive cases of metastatic melanoma found the ratio of V600K : V600E mutation was almost 1 : 2 [5]. Because some of the assays commonly used in the clinical setting may underestimate the frequency of V600K mutation, the aim of the current study was to evaluate four different platforms for the detection of *BRAF* mutations and to assess the sensitivity and specificity of each platform. We report here the results of a two institute, blinded study on 93 melanoma samples using the mutation detection methods of Sanger dideoxy sequencing, single strand conformation analysis (SSCA), high resolution melting analysis (HRM) and competitive allele-specific TaqMan (CAST)-PCR (Life Technologies).

Results

The results of mutation testing are summarized in Table 1. Of 93 samples tested, 91 gave results using all four platforms. One sample (M05) could not be adequately sequenced and another (M13) failed analysis with both SSCA and CAST-PCR. Representative examples of data output using the sequencing, SSCA, HRM and CAST-PCR methods are shown in Figure 1 for wildtype *BRAF* and for the V600E, V600K and K601E mutations. A total of 24 V600E, 18 V600K, 4 K601E, 2 V600E2 and one V600R mutation were detected. An exchange of both amino acids at codons 600 (Valine) and 601 (Lysine) to Glutamine (c.1799_1801delinsAGG, p.Val600_Lys601delinsGluGlu, V600E/K601E) was observed in one sample (P46, Figure 2A).

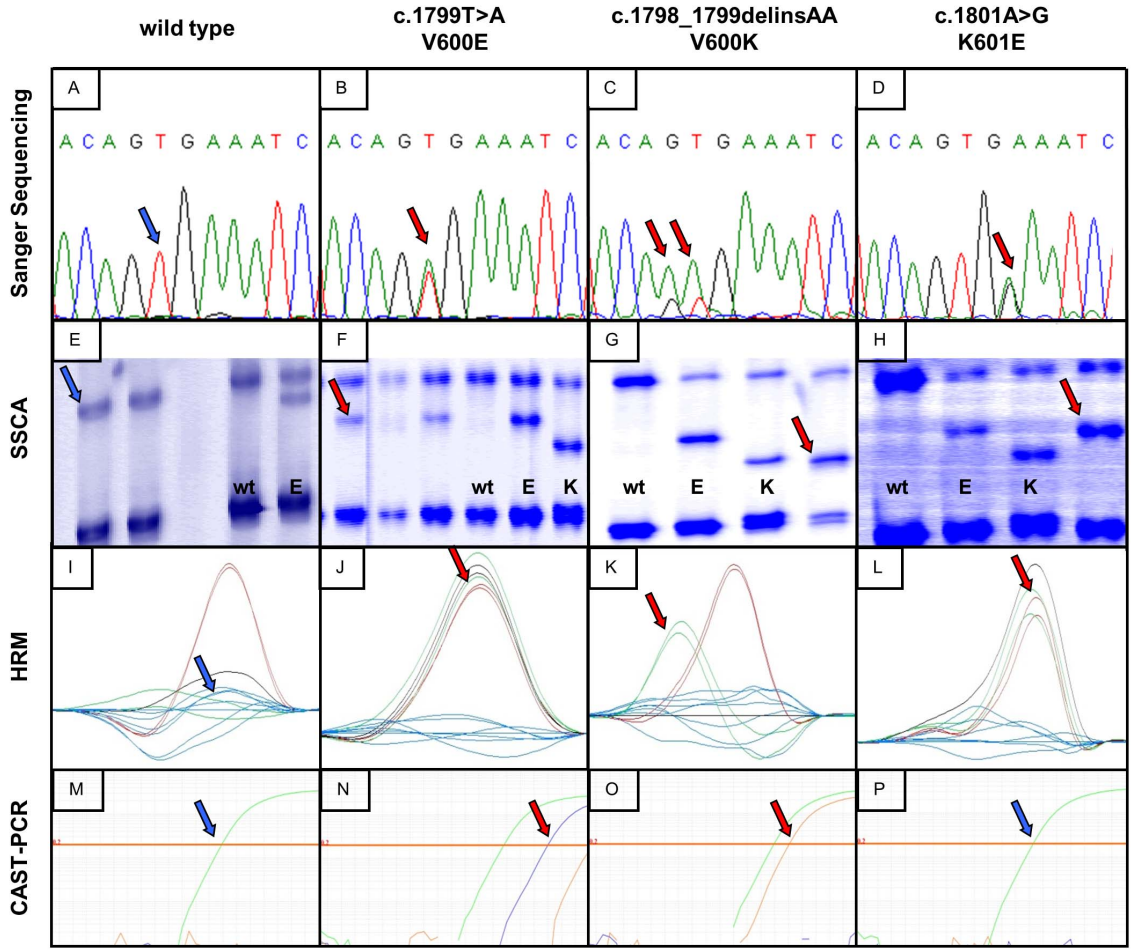

Figure 1 | Representative results for *BRAF* mutation screening using Sanger sequencing (A–D), single strand conformation analysis (SSCA; E–H), high resolution melting analysis (HRM; I–L) and competitive allele-specific TaqMan (CAST-PCR; M-P). The first column is an example of wildtype *BRAF*, the second column of V600E, the third column of V600K and the fourth of K601E. Blue arrows indicate the wildtype profile, while red arrows indicate mutant profiles. For SSCA, E represents the V600E mutation and K the V600K mutation. In HRM graphs (difference plots), red lines indicate positive controls, blue lines negative controls, green lines the sample and black lines a sample/wildtype mix. In CAST PCR, green lines indicate the reference assay, blue lines the V600E assay and orange lines the V600K assay. Slight cross reactivity of V600E positive samples with the V600K assay (orange line) can be seen (N), but not the reverse (O). CAST-PCR using probes for V600E and V600K did not detect the K601E mutation (P).

Concordance between HRM, SSCA and sequencing for the detection of mutations was 100%, although only the sequencing method was capable of identifying the exact mutation. CAST-PCR detected all V600E and V600K mutations, but failed to detect the K601E, V600E2 and V600R cases because probes specific for these mutations were not used in the analysis. Interestingly, the double V600E/K601E mutation (P46) was detected by CAST-PCR (Figure 2D) with the V600E probe. Some cross reactivity was observed by CAST-PCR between the V600K probe and V600E mutations (Figure 1N and 3D), but not vice versa (Figure 1O). In two cases (P33, M33) the threshold dCt value was reached with both probes, with dCt values of 2.77 and 9.45, and 3.52 and 8.98 for the V600E and V600K probes, respectively (Figure 3D). Sequencing, SSCA and HRM results confirmed that both cases were V600E (P33 shown in Figure 3 A–C).

For two samples (P08, P14), CAST-PCR detected a V600E mutation that was not found by sequencing, SSCA or HRM (P08 shown in Figure 4). The amount of mutant allele for these samples was estimated using the Mutation Detector™ software (Life Technologies, USA) to be just 1.7% and 2.2%, respectively. The dCt values for both samples were confirmed to be < 9.96 in two separate runs (Figures 4D and 4E), thus signalling the presence of a mutation. The CAST-PCR results were validated using limited copy number (LCN)-HRM which identifies low frequency mutations by limiting dilution and enables identification of the mutation by Sanger sequencing[20–22]. Analysis of sample P14 using LCN-HRM revealed the presence of a V600E mutation at an estimated allele frequency of less than 5% (Figure 5).

Discussion

This blinded study to evaluate *BRAF* mutation screening methods was carried out by two laboratories with extensive experience in HRM, SSCA and Sanger sequencing. A concordance of 100% for mutation detection was found with all 3 methods for 91 melanoma samples (Table 1). The only discordance was the identification of one sample (P22) as V600E by HRM but as V600K by CAST-PCR and the SSCA banding pattern. Sanger sequencing confirmed this sample was V600K. In this instance, CAST-PCR was very useful in confirming the exact mutation and it also demonstrated an ability to detect low level mutations which can be difficult to read from Sanger sequencing. Similar to previous results with *KRAS* mutation testing[23], the present study demonstrated a high concordance for *BRAF* mutation detection when screening was carried out by experienced laboratories working in a clinical setting.

Although HRM and SSCA are rapid, inexpensive and sensitive screening techniques, the drawback is that they do not identify the exact mutant sequence. CAST-PCR also proved in the present study to be a rapid and sensitive technique for *BRAF* mutation screening.

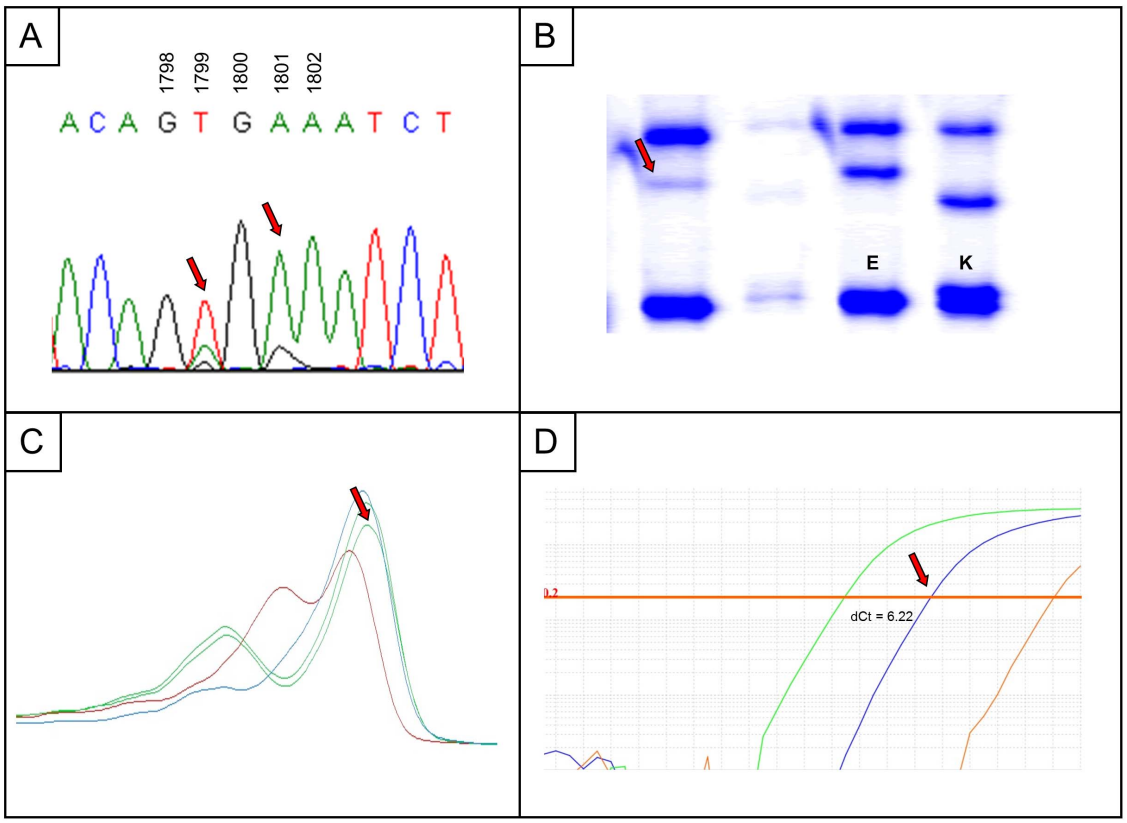

Figure 2 | Results for sample P46 containing the double mutation c.1799_1801delinsAGG (p.Val600_Lys601delinsGluGlu). This mutation was detected by CAST-PCR with the V600E probe (D), as well as by SSCA (B) and HRM (C).

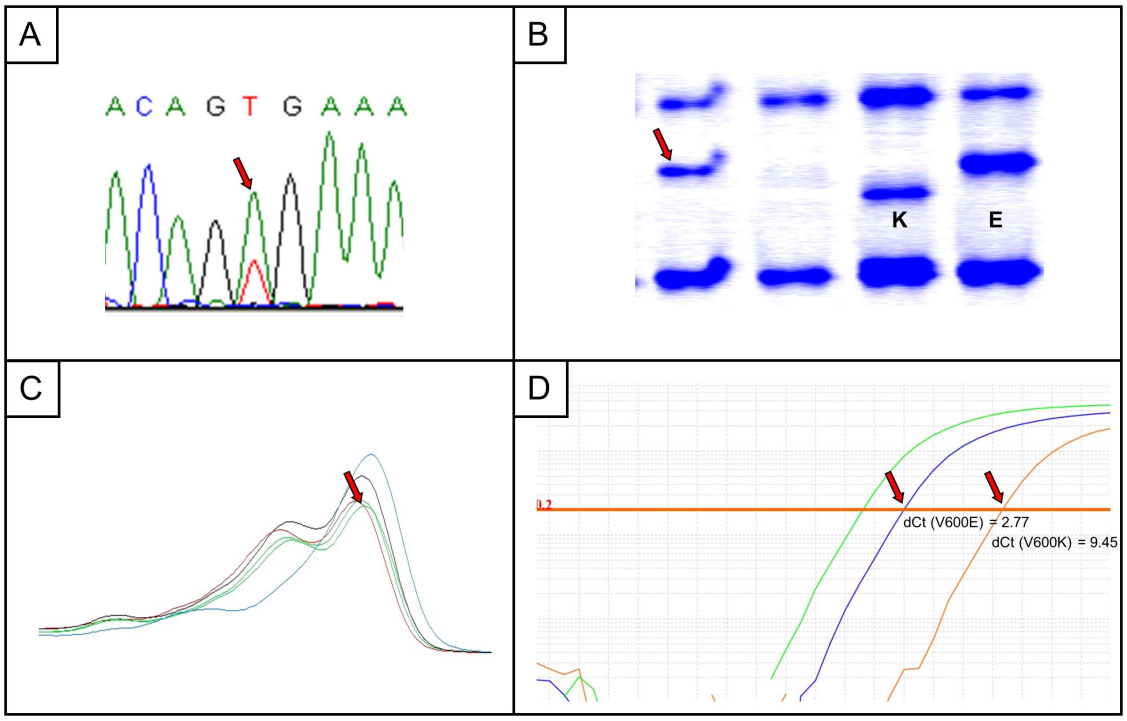

Figure 3 | Results for sample P33 showing cross reactivity between the V600K probe (orange line) and a V600E mutation (blue) in CAST-PCR (D). In this case the dCt value for the V600K assay was lower than the threshold value of 9.96, however Sanger sequencing (A) clearly showed this to be a V600E mutation. The results for SSCA (B) and HRM (C) also suggest a single V600E mutation.

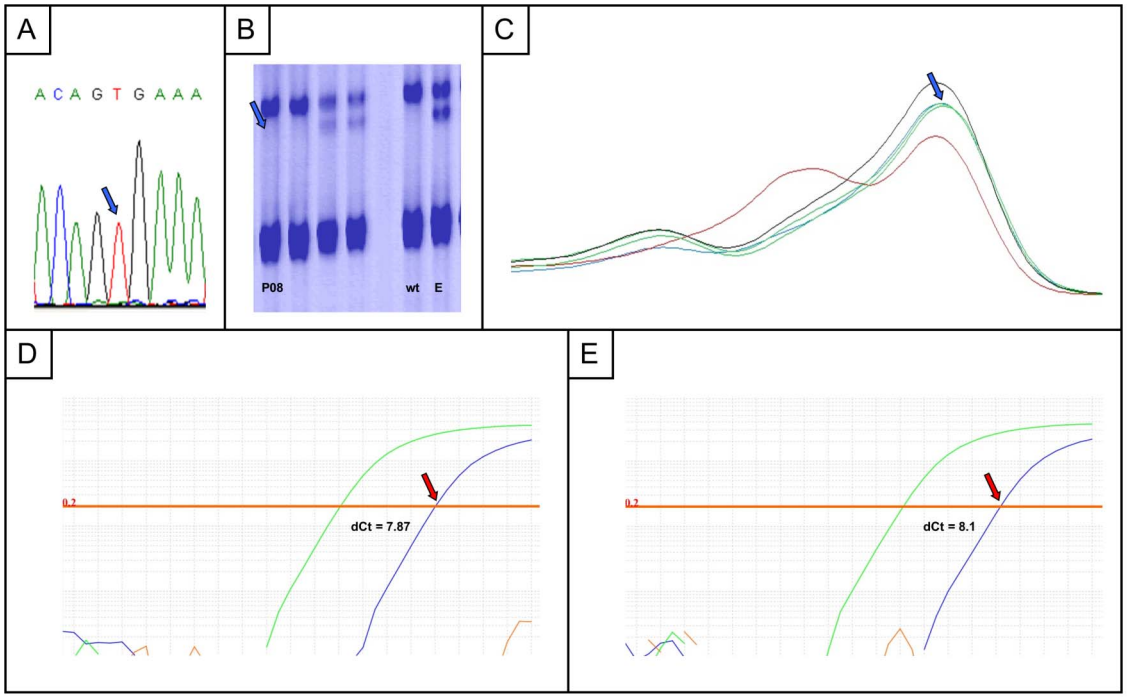

Figure 4 | Results for sample P08 showing wildtype profiles (blue arrows) by sequencing (A), SSCA (B) and HRM (C). Two separate runs of CAST-PCR (D, E) revealed a V600E mutation for this sample, with dCt values in each case of <9.96.

This method was able to identify samples containing as little as 2% mutant allele (Figure 4), confirming recent findings by Didelot *et al.* on the high sensitivity of this method[24]. CAST-PCR is therefore ideal for samples with low tumour purity. However, the clinical relevance of very low mutant allele content for the response of melanoma to BRAF inhibitors is currently unknown. Treatment of wild type *BRAF* melanoma cells with inhibitors of the MAPK pathway may in some circumstances stimulate the growth of these cells[25–27]. Genotypic heterogeneity within tumours and between primary and metastatic tumours may also give rise to low mutant allele content[28–30]. Further studies are required to determine whether the treatment of patients with apparently low percentages of mutant allele has an overall positive or negative clinical impact.

The present work on CAST-PCR used assays for the detection of V600E and V600K mutations only, meaning that rarer mutations such as V600E2, V600R and K601E were undetected (Table 1). In a recent study of 1,112 unselected cases, non-V600E and non-V600K mutations were estimated to occur in approximately 3–4% of melanomas[8]. Probes for the rare *BRAF* mutations are commercially available for CAST-PCR but would add to the cost of screening. The assay cost to screen both the V600E and V600K mutations is approximately $US25 per sample. Widening the screen to include the rarer K601E, V600E2 and V600R mutations would add another $US30 per sample, although this could be carried out as a second step only for the 50–60% of samples with apparently wild type *BRAF*. A possible limitation of the CAST-PCR method in some circumstances is the requirement for relatively large amounts of DNA given that multiple assays are required. Using the manufacturer's recommendation of 20 ng of DNA per assay, at least 120 ng of DNA would be required to screen for the V600E, V600K, V600E2, V600R and K601E mutations. However, in the current study we were able to use as little as 5 ng of DNA per reaction, suggesting this may not be a serious drawback for the vast majority of samples.

This study also confirms the use of LCN-HRM for identifying low allele frequency mutations in a sequence agnostic fashion[20–22]. In turn, this has further validated the CAST-PCR approach for low frequency mutations.

Sanger sequencing is considered by many to be the gold standard for mutation detection and has the advantage over many of the rapid screening methods in being able to identify the exact mutation

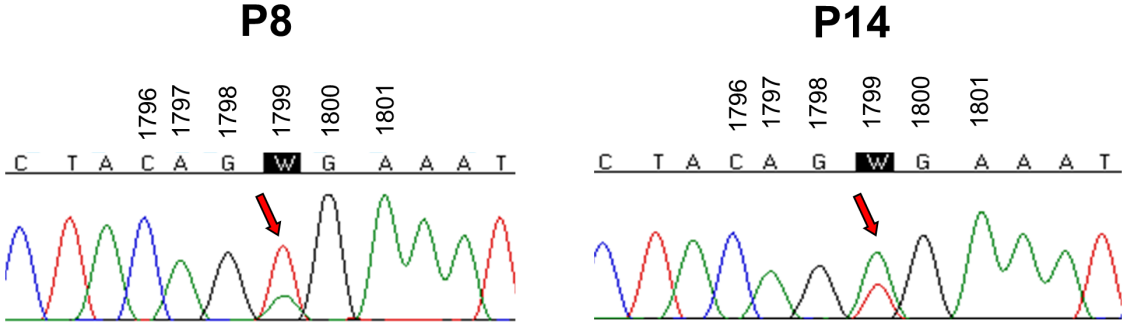

Figure 5 | Two samples (P8 and P14) were positive for the *BRAF* V600E mutation using CAST-PCR but negative using SSCA, HRM and Sanger sequencing. Shown are representative sequencing traces from one of the positive replicates for each of P8 and P14 using LCN-HRM. Red arrows indicate the *BRAF* V600E mutation.

sequence. However in the routine clinical setting it suffers from two major limitations. Firstly, macrodissection of the specimen by a pathologist is required to ensure the tumour cell content is at least 25%. Secondly, the time taken to obtain the sequence readout can be up to several days depending on the turnaround times of individual and service laboratories. The perfect concordance observed in the present study between Sanger sequencing and the HRM and SSCA methods is likely due to the routine enrichment of tissue samples with tumour following careful evaluation of H&E sections by the pathologist.

Several other methods not tested here have been used for *BRAF* mutation screening, including pyrosequencing[31–33], competitive amplification of differentially melting amplicons (CADMA)[30], matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) [8], allele-specific PCR (Taqman)[34] and quantitative allele-specific PCR using a heterozygous plasmid containing wild-type and mutant sequences as a calibrator[35]. Pyrosequencing is a robust methodology but in Australia at least this platform is limited by restricted distribution of the instrumentation. Next generation sequencing platforms including Illumina, Helicos and 454 pyrosequencing have also been used to detect *BRAF* V600E mutations in melanoma[36], but these are inefficient unless multiple loci are screened. Immunohistochemistry with the VE1 antibody (Ventana) was recently shown to be highly specific for the V600E mutation and whilst data remains limited, no cross-reactivity with other *BRAF* mutation has been reported. If used in isolation, immunohistochemistry with VE1 antibody will miss significant numbers of non-V600E *BRAF* mutations and at times weak staining and heavy melanin pigment makes interpretation difficult requiring confirmation by molecular methods[37,38]. A two step screening procedure involving immunohistochemistry followed by pyrosequencing for VE1-negative or uninterpretable cases was proposed by Colomba *et al*[38].

The FDA approved Cobas® 4800 BRAF V600 Mutation Test is based upon the use of allele-specific Taqman probes directed at the V600E mutation (c.1799T > A)[15,39,40]. This test was reported to have a very high sensitivity when the V600E mutant allele content was greater than 10%[30,39]. However, a limitation of the Cobas test is its ability to detect non-V600E mutations, estimated to account for one quarter of all *BRAF* mutations in a study of 1,112 cases of melanoma[8]. Anderson *et al*. reported the Cobas BRAF V600 assay was able to detect 70% of V600K mutant samples through cross-reaction with the TaqMan V600E probe[15]. Further studies are needed to determine whether this result can be reproduced in independent laboratories where the Cobas test is run in parallel with other mutation detection methods. It also remains to be determined whether the frequency of cross-reaction with V600K is influenced by the mutant allele content. This is especially important in light of clinical findings that suggest V600K mutant tumours respond to vemurafenib[13,16,19,41].

The demonstration of clinical activity for Braf inhibitors has created the need for accurate, robust, rapid and cost-effective *BRAF* mutation screening assays. The present work has shown that HRM and SSCA are able to meet these criteria in the clinical setting and are thus useful as a first screen prior to confirmation and identification of the mutation by Sanger sequencing. The two major limitations of Sanger sequencing are the need to enrich for tumour cell content using macrodissection and the long turnaround time. CAST-PCR was shown here to be a sensitive, rapid and robust method for *BRAF* mutation screening and can be cost effective through the use of a two step screening procedure. This would involve an initial screening for the V600E and V600K mutations, followed by screening for the rarer mutations only in those samples showing apparently wildtype status. Although HRM, SSCA or allele-specific real-time PCR followed by Sanger sequencing are used routinely in many laboratories, several other strategies for achieving complete *BRAF* mutation screening are also likely to be effective and could include two step CAST-PCR, pyrosequencing alone, or immunohistochemistry for V600E followed by Sanger sequencing for non-V600E cases. Ultimately, the choice of method used is also dependent on the clinical importance in identifying the 3–4% of melanoma cases known to harbour non-V600E/non-V600K mutations[3,5,8]. As more data emerges on the activity of Braf inhibitors in such patients, it is expected that diagnostic methods capable of detecting these mutations will become standard.

Methods

Melanoma samples. A total of 43 formalin-fixed and paraffin embedded (FFPE) melanoma samples were obtained from the PathWest laboratory, Sir Charles Gairdner Hospital, Perth. These comprised a selected subset from a consecutive series that had previously been investigated for *BRAF* mutation by bidirectional Sanger sequencing and in some cases SSCA[5]. In addition, all samples were evaluated by CAST-PCR. A further 50 FFPE melanoma samples were obtained from the Melbourne Melanoma Project (http://melbournemelanomaproject.com/). Approval for the study was obtained from the human research ethics committees at the Peter MaCallum Cancer Centre and the Sir Charles Gairdner Hospital. Cases from both sites were enriched for tumour cell content by macrodissection following evaluation of the H&E section by a specialist pathologist. Approximately 1–3 cm^2 of tissue sections (5 μm thickness) was used to extract DNA from each sample. DNA from the Perth samples was sent to Melbourne for analysis using HRM and sequencing, while samples from Melbourne were sent to Perth for analysis by sequencing, SSCA and CAST-PCR. All testing was carried out blinded to the result obtained from the other laboratory. In the Perth laboratory, testing with SSCA, sequencing and CAST-PCR was carried out blinded to results from the other tests. In the Melbourne laboratory, sequencing was carried out only on samples showing a positive or equivocal result with HRM. Controls were conducted for all assays and included the absence of DNA and samples with known wild type or mutant status.

Single strand conformation analysis (SSCA). The primers used for fluorescent SSCA were identical to those used for dideoxy sequencing, but with additional fluorescent HEX dye labelling at the 5′ end (GeneWorks, Adelaide, Australia). PCR conditions were the same as for sequencing. Three μl of fluorescent PCR product was mixed with 6 μl deionized formamide loading buffer and heated to 95°C for 5 minutes. Two μl of this mix was then loaded onto a 10% polyacrylamide/2% glycerol gel (100 μm thick, 18 cm long) and run in a DNA fragment analyser (GS-2000, Corbett Life Sciences, Australia). After pulsing for 20 sec at 1400 V the wells were rinsed and the gel was run at 1400 V for 90 min in 0.8× TBE buffer at a constant gel temperature of 24°C. ONE-Dscan 1.3 software (Scanalytics, Billerica, MA) was used to analyse the gel run. The mutation status of the samples was determined by comparison of the sample bands to those of wild type and mutant controls run in parallel.

CAST-PCR. Samples were analysed by CAST-PCR using the BRAF_476_mu and BRAF_473_mu probes for the detection of V600E and V600K mutations, respectively (Life Technologies, USA). Mastermixes were prepared as recommended by the manufacturer and distributed in a 96-well plate. DNA for Perth (20 ng) and Melbourne (5 ng) samples was added to each well and the reaction carried out in a ViiA™ 7 Real-Time PCR system. A lower amount of DNA was used for the Melbourne samples due to the limited availability of this resource. The PCR conditions comprised an initial denaturation step of 10 minutes at 95°C followed by 5 cycles of 15 sec denaturation at 95°C and one minute extension at 58°C. This was followed by 40 cycles of 15 sec denaturation at 95°C and one minute extension at 60°C. Real time data was collected during the last 40 cycles of amplification and analysed using the Mutation Detector™ software v.2.0 (Life Technologies, USA). Samples with a delta(d)Ct of less than 9.96 were considered positive for mutation, where dCt = Ct mut – Ct ref.

High resolution melt (HRM) analysis. PCR and HRM were performed using the LightCycler 480 (Roche Diagnostics). The primer sequences used were 5′-CCTCACAGTAAAAATAGGTGATTTTGG-3′ and 5′-GGATCCAGACAACTGTTCAAACTGA-3′, giving an amplicon size of 88 bp. The reaction mixture included 1× PCR buffer, 2.5 μM $MgCl_2$, 200 nM of each primer, 200 μM of dNTPs, 5 μM of SYTO 9 (Invitrogen, Carlsbad, CA, USA), 0.5U of HotStarTaq polymerase (Qiagen), 10 ng DNA and PCR grade water in a total volume of 10 μl. PCR conditions included an activation step of 15 min at 95°C followed by 55 cycles of 95°C for 10 sec, annealing for 10 sec comprising 10 cycles of a touchdown from 65°C to 55°C at 1°C/cycle followed by 35 cycles at 55°C, and extension at 72°C for 30 sec.

Limited copy number – high resolution melt (LCN-HRM) analysis. To examine whether a low frequency of *BRAF* V600E mutation was present in two samples (P8, P14) that were positive by CAST-PCR but negative by HRM, SSCA and Sanger sequencing, limited copy number – high resolution melting (LCN-HRM) was performed as previously described[20]. In brief, DNA from these samples was diluted to ensure that only a few copies of template were added to LCN-HRM reactions. PCR cycling and HRM were performed on the RotorGene Q (Qiagen) and positive LCN-HRM reactions showing aberrant melting profiles were chosen for Sanger sequencing. The LCN-HRM reaction mixture in a total volume of 20 μL was prepared

as follows for an estimated 2–4 copies of template: 1× PCR buffer, 2.5 μM $MgCl_2$, 400 nM of each primer, 200 μM of dNTPs, 5 μM of SYTO 9 and 0.5 U of HotStarTaq polymerase. PCR conditions included an activation step of 15 min at 95°C followed by 60 cycles of 95°C for 10 sec, annealing for 20 sec comprising 10 cycles of a touchdown from 65°C to 60°C at 0.5°C/cycle followed by 50 cycles at 60°C, and extension at 72°C for 30 sec. Each sample was tested in 66 LCN-HRM replicates. LCN-HRM products showing aberrant melting profiles were used as templates for Sanger sequencing.

Sanger sequencing (Perth laboratory). Bidirectional Sanger sequencing was performed as described earlier[5]. Briefly, PCR was carried out in a final volume of 25 μL using the forward primer sequence 5′- TCATAATGCTTGCTCTGATAGGA-3′ and the reverse primer sequence 5′-GGCCAAAAATTTAATCAGTGGA-3′ to give an amplicon size of 224 base pairs. PCR reactions contained 10–50 ng of DNA, 0.5 μM of each primer, 200 μM of dNTPs, 3 μM $MgCl_2$ and 0.4 units of Taq polymerase (Qiagen, Australia). The PCR conditions were an initial denaturation period of 5 minutes at 94°C, followed by 40 cycles of denaturation at 94°C for 40 sec, annealing at 60°C for 40 sec and extension at 72°C for 40 sec. The program was terminated by a final step of 7 min at 72°C. PCR products were purified using the QIAquick PCR purification Kit (Qiagen, Australia). Two nanograms of PCR product was then used as template for sequencing using the BigDye Terminator version 3.1 Cycle. Controls without added DNA were included with each PCR and run on a 2% agarose gel to demonstrate the absence of contamination. Sequence analysis was carried out using Sequence Scanner v1.0 (Applied Biosystems) and Assign™ ATF 1.5 (Conexio Genomics Pty. Ltd.) software.

Sanger sequencing (Melbourne laboratory). Samples showing aberrant melting curves by HRM were sequenced following amplification of genomic DNA. The reaction mixture included 1× PCR buffer, 2.5 μM $MgCl_2$, 400 nM of each primer, 200 μM of dNTPs, 0.5 U of HotStarTaq polymerase (Qiagen), 10 ng DNA and PCR grade water in a total volume of 20 μl. The primer sequences used were 5′- caggaaacagctatgaccCATGAAGACCTCACAGTAAAAATAGGT-3′ and 5′- tgtaaaacgacggccagtCATCCACAAAATGGATCCAGACAAC-3′, giving an amplicon size of 143 bp. M13 tags are shown in lower case and *BRAF*-specific sequences in uppercase. PCR was run on a Veriti 96-well Thermal Cycler (Applied Biosystems, Life Technologies, Foster City, CA) under the following conditions: an activation step of 15 min at 95°C followed by 11 cycles of 95°C for 10 sec, annealing for 10 sec at 65°C and extension at 72°C for 20 sec. This was followed 40 cycles of 95°C for 10 sec, annealing for 10 sec at 55°C, extension at 72°C for 20 sec and a final extension at 72°C for 10 mins. After PCR amplicon clean-up using ExoSAP-IT® (Affymetrix) or Agencourt® AMPure magnetic beads (Beckman Coulter), forward and reverse sequencing reactions were performed using M13 forward and reverse primers. Excess dideoxy terminators were removed from the sequencing reaction with the Agencourt CleanSEQ reagent (Beckman Coulter) and the reactions analysed by capillary electrophoresis on an ABI3730 sequencer (Applied Biosystems). In some cases where additional genomic DNA was unavailable, M13 tags were added to the HRM product and directly sequenced.

1. Davies, H. *et al.* Mutations of the BRAF gene in human cancer. *Nature* **417**, 949–954 (2002).
2. Cohen, Y. *et al.* Exon 15 BRAF mutations are uncommon in melanomas arising in non-sun-exposed sites. *Clin Cancer Res* **10**, 3444–3447 (2004).
3. Long, G. V. *et al.* Prognostic and clinicopathologic associations of oncogenic BRAF in metastatic melanoma. *J Clin Oncol* **29**, 1239–1246 (2011).
4. Ellerhorst, J. A. *et al.* Clinical correlates of NRAS and BRAF mutations in primary human melanoma. *Clin Cancer Res* **17**, 229–235 (2011).
5. Amanuel, B., Grieu, F., Kular, J., Millward, M. & Iacopetta, B. Incidence of BRAF p.Val600Glu and p.Val600Lys mutations in a consecutive series of 183 metastatic melanoma patients from a high incidence region. *Pathology* **44**, 357–359 (2012).
6. Halaban, R. *et al.* PLX4032, a selective BRAF(V600E) kinase inhibitor, activates the ERK pathway and enhances cell migration and proliferation of BRAF melanoma cells. *Pigment Cell Melanoma Res* **23**, 190–200 (2010).
7. El-Osta, H. *et al.* BRAF mutations in advanced cancers: clinical characteristics and outcomes. *PLoS One* **6**, e25806 (2011).
8. Greaves, W. O. *et al.* Frequency and spectrum of BRAF mutations in a retrospective, single-institution study of 1,112 cases of melanoma. *J Mol Diagn* **15**, 220–226 (2012).
9. Deichmann, M., Thome, M., Benner, A. & Näher, H. B-raf exon 15 mutations are common in primary melanoma resection specimens but not associated with clinical outcome. *Oncology* **66**, 411–419 (2004).
10. Solit, D. B. *et al.* BRAF mutation predicts sensitivity to MEK inhibition. *Nature* **439**, 358–362 (2006).
11. Yang, H. *et al.* RG7204 (PLX4032), a selective BRAFV600E inhibitor, displays potent antitumour activity in preclinical melanoma models. *Cancer Res* **70**, 5518–5527 (2010).
12. Falchook, G. S. *et al.* Dabrafenib in patients with melanoma, untreated brain metastases, and other solid tumours: a phase 1 dose-escalation trial. *Lancet* **379**, 1893–1901 (2012).
13. Chapman, P. B. *et al.* Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med* **364**, 2507–2516 (2011).
14. Hauschild, A. *et al.* Dabrafenib in BRAF-mutated metastatic melanoma: a multicentre, open-label, phase 3 randomised controlled trial. *Lancet* **380**, 358–365 (2012).
15. Anderson, S. *et al.* Multisite analytic performance studies of a real-time Polymerase Chain Reaction assay for the detection of BRAF V600E mutations in formalin-fixed, paraffin-embedded tissue specimens of malignant melanoma. *Arch Pathol Lab Med* **136**, 1385–1391 (2012).
16. Rubinstein, J. C. *et al.* Incidence of the V600K mutation among melanoma patients with BRAF mutations, and potential therapeutic response to the specific BRAF inhibitor PLX4032. *J Transl Med* **8**, 67 (2010).
17. Klein, O. *et al.* BRAF inhibitor activity in V600R metastatic melanoma. *Eur J Cancer* **49**, 1073–1079 (2013).
18. Dahlman, K. B. *et al.* BRAF (L597) mutations in melanoma are associated with sensitivity to MEK inhibitors. *Cancer Discovery* **2**, 791–797 (2012).
19. McArthur, G. *et al.* Efficacy of vemurafenib in BRAF V600K mutation positive melanoma disease – results from the phase 3 clinical study BRIM3. *Pigment Cell & Melanoma Res* **25**, 871 (2012).
20. Do, H. & Dobrovic, A. Limited copy number-high resolution melting (LCN-HRM) enables the detection and identification by sequencing of low level mutations in cancer biopsies. *Mol Cancer* **8**, 82 (2009).
21. Do, H. & Dobrovic, A. Dramatic reduction of sequence artefacts from DNA isolated from formalin-fixed cancer biopsies by treatment with uracil-DNA glycosylase. *Oncotarget* **3**, 546–558 (2012).
22. Tan, A. Y. *et al.* Rapid detection of FLT3 exon 20 tyrosine kinase domain mutations in patients with acute myeloid leukemia by high-resolution melting analysis. *Leuk Lymphoma* **53**, 1225–1229 (2012).
23. Whitehall, V. *et al.* A multicenter blinded study to evaluate KRAS mutation testing methodologies in the clinical setting. *J Mol Diagn* **11**, 543–552 (2009).
24. Didelot, A. *et al.* Competitive allele specific TaqMan PCR for KRAS, BRAF and EGFR mutation detection in clinical formalin fixed paraffin embedded samples. *Exp Mol Pathol* **92**, 275–280 (2012).
25. Hatzivassiliou, G. *et al.* RAF inhibitors prime wild-type RAF to activate the MAPK pathway and enhance growth. *Nature* **464**, 431–435 (2010).
26. Poulikakos, P. I., Zhang, C., Bollag, G., Shokat, K. M. & Rosen, N. RAF inhibitors transactivate RAF dimers and ERK signalling in cells with wild-type BRAF. *Nature* **464**, 427–430 (2010).
27. Heidorn, S. J. *et al.* Kinase-dead BRAF and oncogenic RAS cooperate to drive tumour progression through CRAF. *Cell* **140**, 209–221 (2010).
28. Yancovitz, M. *et al.* Intra- and inter-tumour heterogeneity of BRAF(V600E) mutations in primary and metastatic melanoma. *PLoS One* **7**, e29336 (2012).
29. Wilmott, J. S. *et al.* Intratumoural molecular heterogeneity in a BRAF-Mutant, BRAF inhibitor-resistant melanoma: a case illustrating the challenges for personalized medicine. *Mol Cancer Ther* **11**, 2704–2708 (2012).
30. Lade-Keller, J. *et al.* Evaluation of BRAF mutation testing methodologies in formalin-fixed paraffin-embedded cutaneous melanomas. *J Mol Diagn* **15**, 70–80 (2013).
31. Tan, Y. H. *et al.* Detection of BRAF V600E mutation by pyrosequencing. *Pathology* **40**, 295–298 (2008).
32. Edlundh-Rose, E. *et al.* NRAS and BRAF mutations in melanoma tumours in relation to clinical characteristics: a study based on mutation screening by pyrosequencing. *Melanoma Res* **16**, 471–478 (2006).
33. Spittle, C. *et al.* Application of a BRAF pyrosequencing assay for mutation detection and copy number analysis in malignant melanoma. *J Mol Diagn* **9**, 464–471 (2007).
34. Lang, A. H. *et al.* Optimized allele-specific real-time PCR assays for the detection of common mutations in KRAS and BRAF. *J Mol Diagn* **13**, 23–28 (2011).
35. Szankasi, P. *et al.* A quantitative allele-specific PCR test for the BRAF V600E mutation using a single heterozygous control plasmid for quantitation: a model for qPCR testing without standard curves. *J Mol Diagn* **15**, 248–254 (2013).
36. Curry, J. L., Torres-Cabala, C. A., Tetzlaff, M. T., Bowman, C. & Prieto, V. G. Molecular platforms utilized to detect BRAF V600E mutation in melanoma. *Semin Cutan Med Surg* **31**, 267–273 (2012).
37. Capper, D. *et al.* Immunohistochemical testing of BRAF V600E status in 1,120 tumour tissue samples of patients with brain metastases. *Acta Neuropathol* **123**, 223–233 (2012).
38. Colomba, E. *et al.* Detection of BRAF p.V600E mutations in melanomas: comparison of four methods argues for sequential use of immunohistochemistry and pyrosequencing. *J Mol Diagn* **15**, 94–100 (2013).
39. Halait, H. *et al.* Analytical performance of a real-time PCR-based assay for V600 mutations in the BRAF gene, used as the companion diagnostic test for the novel BRAF inhibitor vemurafenib in metastatic melanoma. *Diagn Mol Pathol* **21**, 1–8 (2012).
40. Lopez-Rios, F. *et al.* Comparison of testing methods for the detection of BRAF V600E mutations in malignant melanoma: pre-approval validation study of the companion diagnostic test for Vemurafenib. *PLoS One* **8**, e53733 (2013).
41. Sosman, J. A. *et al.* Survival in BRAF V600-mutant advanced melanoma treated with vemurafenib. *N Engl J Med* **366**, 707–714 (2012).

Author contributions

The experimental work for this project was carried out by A.R., F.G., A.C., K.N., A.R., A.L., V.M., A.B., C.H., H.D. and S.W. Identification of suitable samples was carried out by S.F.

and B.A. G.M., A.D. and B.I. wrote the manuscript text. All authors approved the final manuscript.

Additional information

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Richter, A. *et al.* A multisite blinded study for the detection of *BRAF* mutations in formalin-fixed, paraffin-embedded malignant melanoma. *Sci. Rep.* **3**, 1659; DOI:10.1038/srep01659 (2013).

Author/s:
Richter, A; Grieu, F; Carrello, A; Amanuel, B; Namdarian, K; Rynska, A; Lucas, A; Michael, V; Bell, A; Fox, SB; Hewitt, CA; Do, H; McArthur, GA; Wong, SQ; Dobrovic, A; Iacopetta, B

Title:
A multisite blinded study for the detection of BRAF mutations in formalin-fixed, paraffin-embedded malignant melanoma

Date:
2013-04-15

Citation:
Richter, A., Grieu, F., Carrello, A., Amanuel, B., Namdarian, K., Rynska, A., Lucas, A., Michael, V., Bell, A., Fox, S. B., Hewitt, C. A., Do, H., McArthur, G. A., Wong, S. Q., Dobrovic, A. & Iacopetta, B. (2013). A multisite blinded study for the detection of BRAF mutations in formalin-fixed, paraffin-embedded malignant melanoma. SCIENTIFIC REPORTS, 3 (1), https://doi.org/10.1038/srep01659.

Persistent Link:
http://hdl.handle.net/11343/265290

File Description:
Published version