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Original Article

Hybrid-capture based genomic profiling identifies BRAF V600 and non-V600 alterations in melanoma samples negative by prior testing

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ABSTRACT

Background: BRAF and MEK inhibitors are approved for BRAF V600-mutated advanced melanoma, with response rates of up to 70%. Responses to targeted therapies have also been observed for diverse non-V600 BRAF alterations. Thus, sensitive, accurate, and broad detection of BRAF alterations is critical to match patients with available targeted therapies.

Methods: Pathology reports were reviewed for 385 consecutive melanoma cases with BRAF mutations or rearrangements identified using a hybrid-capture based next generation sequencing comprehensive genomic profiling (CGP) assay during the course of clinical care.

Results: Records of prior BRAF molecular testing were available for 79 (21%) cases. Of cases with BRAF V600 mutations 11/57 (19%) with available data were negative by prior BRAF testing. Prior negative BRAF results were also identified in 16/20 (80%) cases with non-V600 mutations, two of which harbored multiple BRAF alterations, and in 2/2 (100%) cases with activating BRAF fusions. Clinical outcomes for a subset of patients are presented.

Conclusion: CGP identifies diverse activating BRAF alterations in a significant fraction of cases with prior negative testing. Given the proven clinical benefit of BRAF/MEK inhibitors in BRAF-mutated melanoma, CGP should be considered for patients with metastatic melanoma, particularly if other testing is negative.

Implications for practice:

Published guidelines for melanoma treatment recommend BRAF mutational analysis, but little guidance is provided as to selection criteria for testing methodologies, or as to clinical implications for non-V600 alterations. In this study we report that hybrid-capture based next generation sequencing can detect BRAF alterations in samples from a significant fraction of advanced melanoma patients with prior negative BRAF results. This study highlights the need for oncologists and pathologists to be critically aware of coverage and sensitivity limitations of various assays, particularly regarding non-V600E alterations, of which many are potentially targetable.

Introduction

One of the most compelling examples of clinical utility of targeted therapies is the development of BRAF and MEK inhibitors for the treatment BRAF V600E/K-mutated melanoma in stage IV disease[1,2,3,4], and recently in stage III disease in the adjuvant setting[5]. *BRAF* exon 15 mutations drive proliferation of over 50% of all cutaneous melanomas[6]. Vemurafenib and dabrafenib[7] have shown remarkable clinical activity in patients with BRAF V600E/K-mutated melanoma and received US Food and Drug Administration (FDA) approval for the treatment of metastatic melanoma. Subsequently, combinations of BRAF and MEK inhibitors showed improved efficacy when compared to BRAF inhibitor monotherapy, with responses in approximately 70% of cases and median overall survival exceeding 2 years[8,9,10].

In addition to V600E/K, other substitutions and indels at V600, and many non-V600 BRAF mutations have been found, mostly clustered in the activation segment or in the glycine-rich loop of the kinase domain[11,12]. *BRAF* exon 11 mutations have been associated with responses to diverse multi-kinase inhibitors, such as and sorafenib[13] and dasatinib[14]. In addition to BRAF short variant mutations, constitutively activating fusions retaining the BRAF catalytic domain are also found in melanomas, and enriched in Spitzoid melanomas,[15,16]. Because of the rarity and novelty of these fusions, to date no international clinical trials have been initiated for this subgroup, but MEK inhibitors have shown some clinical efficacy in this context and may constitute a crucial therapeutic option for these patients[15,17].

Current methodology for detecting BRAF alterations in clinical specimens is left to laboratory discretion, and as such multiple assays are used in clinical practice to inform therapy selection[18].

Importantly, limitations and performance characteristics of molecular assays are typically not readily apparent to the treating physician. Given the substantial clinical benefit demonstrated for BRAF and MEK inhibitors in patients with BRAF-V600E mutated melanoma, assessing the limitations of BRAF testing typically used in clinical care is critical. Beyond V600E mutation, other alterations both at V600 and throughout BRAF should be recognized, given early evidence of targetablity.

To this end, a comprehensive review of melanoma cases with BRAF alterations detected using a hybrid capture–based comprehensive genomic profiling (CGP) assay during clinical care was conducted. Both history of prior BRAF testing, as well as available outcomes data were analyzed.

Materials and Methods

A minimum of 50ng of DNA was extracted from 40 µm of formalin-fixed paraffin embedded sections of 385 consecutive melanoma cases submitted during the course of clinical care (March 2016 and March 2017), and CGP was performed on hybridization captured, adaptor ligation–based libraries to a mean coverage depth of >600X for the entire coding sequence of 236 or 315 cancer-related genes plus 19 to 28 introns from genes frequently rearranged in cancer (including all *BRAF* exons and introns 7-10) to identify base pair substitutions, insertions/deletions, copy number alterations, and rearrangements[19]. Testing was performed in a CLIA-certified/CAP-accredited laboratory (Foundation Medicine Inc., Cambridge, MA). Tumor mutational burden (TMB) was characterized as the number of somatic base substitution or indel alterations per megabase (Mb). Prior test results were extracted from provided pathology reports. Approval for

this study, including a waiver of informed consent and a HIPAA waiver of authorization, was obtained from the Western Institutional Review Board (Protocol No. 20152817).

Results

CGP was performed on 385 advanced-stage melanomas during the course of clinical care. The distribution of cases assessed and results of prior BRAF testing are shown in Figure 1. In this set of 385 advanced melanomas, 38 unique BRAF short variant mutations and 5 unique activating rearrangements (fusions or kinase domain duplications) were represented (Figure 2). Prior testing records utilizing diverse methodologies were available for 79/385 (21%) cases (Figure 3). Overall, 29/79 (37%) cases with BRAF alterations detected using CGP were BRAF-negative on prior testing. Of BRAF V600-mutated cases, 11/57 (19%) with available data were negative by prior BRAF testing, including 7/45 (16%) with V600E mutations. In 9/29 cases with prior negative BRAF results, information regarding the prior test methodology was available and suggested that the detected alteration should have been covered (Table 1, Figure 3C). Biopsies with the same surgical pathology ID were tested in 3/9 cases (including the same block or slide in 2 cases). In the remaining cases prior testing was done on a confirmed different sample in 5 cases (including 1 liquid biopsy), and on an unknown sample in 1 case (Table 1, Figure 3C). Of all cases where the BRAF mutation was expected to be covered by the prior assay and the same sample was tested 3/35 (8.6%) appear to have been false negatives (Table 1 patient 5, 6 and 7). For comparison, in cases where the other test methodology covered the detected BRAF mutation the same sample was confirmed to have been tested in 3/9 (33%) prior negative cases and 32/50 (64%) prior positive cases (Figure 3C). Characteristics of cases with BRAF alterations identified by CGP, but not detected by prior BRAF testing, are shown in Figure 2. There was no significant

difference in BRAF mutant allele frequencies (median 35% vs. 40%, $P = 0.25$) or percentage of tumor nuclei (median 50% for both, $P = 0.97$) between BRAF V600-mutated samples with prior negative and prior positive results (Figure 4).

Prior negative results were identified in 16/20 (80%) cases with BRAF non-V600 mutations, two of which harbored multiple BRAF alterations. Specifically, BRAF non-V600 mutations not detected by prior testing included 9 activating mutations: K601E (n=4), G464V, G469V, E586K, L597Q, and A589_T599insT; 4 mutations predicted to result in impaired BRAF kinase activity: D594A/G/N (n=3) and G466V; and 5 uncharacterized mutations: S467L (n=2), L584F (n=2), and, N581I. In addition, 2/2 (100%) cases with activating BRAF fusions (TRIM24-BRAF and SOX5-BRAF) also had prior negative BRAF results (Figure 3A and 3B).

Clinical follow-up was available for 7 patients with BRAF activating alterations identified using CGP following prior negative BRAF testing. One patient (Table 1, patient 2), a 27-year-old male, directly benefited from CGP testing. Initial BRAF testing utilizing melting curve analysis was negative for V600 mutations. The patient received ipilimumab + nivolumab as first-line systemic treatment for stage IV melanoma. After 5 months of immunotherapy, CGP of a second biopsy detected BRAF V600E (68% mutant allele frequency) as well as a tumor mutational burden (TMB) of 18 mutations/Mb. Immediately following this result immunotherapy was discontinued due to symptomatic decline and toxicity, and the patient began dabrafenib + trametinib, resulting in symptom improvement and shrinkage of metastatic lung nodules after 3 months of treatment.

Of the remaining patients, four are currently being treated with immunotherapy with ongoing responses, and may pursue BRAF and/or MEK inhibitors as a next line of therapy. In the sixth case, the patient progressed on immunotherapy and then was too ill to pursue a BRAF/MEK inhibitor after the BRAF V600E positive result was returned. In the final case, a *SOX5* (exons 1-6)-*BRAF* (exons 9-18) fusion was identified. The patient received 6 weeks of cobimetinib with no response and is now on an immunotherapy trial. Shortly after our study, an additional patient with a ERC1-BRAF fusion identified using CGP, with previously negative testing for BRAF, showed a good response to sorafenib after progressing on immunotherapy (Figure 5), and is still under treatment to date.

Discussion

Within the last decade, the use of rationally applied targeted therapy has revolutionized the care of metastatic melanoma, beginning with the identification of BRAF V600 mutations, predominantly V600E, that respond to combined BRAF and MEK inhibitors. For such therapies to be optimally delivered, there is an inherent mandate for specific and sensitive clinical testing to detect BRAF mutations. Two recent studies[20,21] demonstrate that CGP applied in the course of clinical care can identify genomic alterations that guide targeted therapy for advanced non–small cell lung cancer (NSCLC) patients who have been previously tested "negative" by standard-of-care molecular testing.

Despite approved companion diagnostics, significant variability exists in methods used for BRAF testing in the clinical setting. These include BRAF V600E specific clone VE1 immunohistochemistry (IHC)[22,23], polymerase chain reaction (PCR; such as the FDA-approved

COBAS® 4800 test), pyrosequencing, high resolution melting analysis, Sanger sequencing, and next-generation sequencing (NGS). Herein, CGP identified BRAF alterations in 37% of cases with prior negative BRAF results returned using a variety of testing methodologies. For cases with BRAF V600 mutations detected by CGP 19% had prior negative results. For cases with non-V600 alterations were detected by CGP, 80% of cases had prior negative results. No significant differences in mutant allele frequency or tumor fraction were observed in V600-positive cases with prior positive or prior negative results, suggesting that these variables are unlikely to explain the missed detection in most cases. In approximately two-thirds of cases the prior negative result was likely due to limited coverage of the original BRAF testing method. In 2 cases BRAF V600K mutations were not detected when IHC specific for V600E was used, and in 1 case V600R (MAF 49%) was not detected by a PCR assay which indicated that probes were designed for V600E, but that other V600 alterations might be detected with limited sensitivity. Non-V600 alterations (including 4 K601E and 1 BRAF fusion) were not detected in 14 cases in which the mutation present was known to be excluded from coverage by the assay employed. We also acknowledge that among 9 cases with prior negative results where the original assay should have detected the BRAF alteration, the same sample was only confirmed to have been tested in one-third of cases. However, in cases with prior positive results the same sample was only confirmed to have been tested in 64% of cases, and in 30% of cases a different sample was confirmed to have been tested. Further, studies have shown that driver mutations are strongly conserved between primary and metastatic samples[24].

Although dabrafenib and vemurafenib are specifically developed to inhibit BRAF V600 mutations, responses to RAF and/or MEK inhibitors have been reported for many less common

BRAF alterations, including a subset of those observed herein[15,16,25,26,27]. Currently multiple clinical trials are enrolling melanoma patients with BRAF non-V600 alterations (NCT02296112, NCT02465060. This creates an imperative, beyond better BRAF V600 testing, for detection of these diverse kinase activating mutations and fusions.

In addition to high sensitivity for detection of diverse BRAF alterations, CGP determines the TMB of a given sample using an algorithm that, based on the genomic alterations detected on 0.83-1.14Mb of DNA, extrapolates to the genome as a whole[28]. As high TMB may predict responses to immunotherapy in melanoma[29,30], the option to obtain CGP should be accessible to each patient and doctor prior to treatment decision making in the context of metastatic melanoma.

This study highlights the importance of using a sensitive full-coverage assay, such as the HC-based CGP assay employed herein, as opposed to conventional assays that have less sensitivity and/or are limited to small regions of the gene, which may miss less well characterized but still actionable alterations. Published guidelines strongly encourage of the use of BRAF mutational assays, but without explicit mention of performance characteristics, the necessity of rigorous analytic validation, or the potential value of coverage beyond V600. Previously reported responses to targeted therapies in patients with diverse BRAF alterations, as well as clinical cases described herein, highlight the need for consistent accurate detection of these alterations to allow for selection of matched therapies associated with demonstrated clinical efficacy.

Conflict of Interest: AW, JSR, SMA, VAM, and ABS are employed by, and hold equity interest in Foundation Medicine, Inc. LB is a consultant for Leo Pharma.

Acknowledgments: We would like to thank patients and their families.

Figure Legends

Figure 1: Consort diagram of melanoma patients with BRAF alterations identified using comprehensive genomic profiling.

Figure 2. (A) Distribution of BRAF short variant mutations and rearrangements in our set of 385 advanced melanoma cases. SV: short variant; KDD: kinase domain duplication. (B) Median tumor mutational burden (TMB; mutations/Mb) is shown for each subset of cases.

Figure 3: Characteristics of cases with BRAF alterations identified using CGP after prior negative BRAF testing. (A) Characteristics of 29 cases with BRAF alterations identified by hybrid capture-based NGS (HC-NGS) after prior negative BRAF result. MAF: mutant allele frequency; NA: not applicable. *Non-hybrid capture-based NGS assays. #Two samples with activating K601E mutations also has a second activating or uncharacterized BRAF mutation identified. (B) 31 BRAF alterations identified in 29 patient samples by HC-NGS after prior negative result. (C) Fraction of cases in which the detected alteration was expected to be covered by the prior test (100% for prior positive, not shown), as well as the fraction of cases for which the same sample was tested by CGP and the prior BRAF test.

Figure 4: BRAF MAF and tumor fraction of 57 cases with BRAF V600 mutation detected by HC-NGS. Comparison of (A) BRAF V600 mutant allele frequency (MAF), and (B) tumor fraction, in 11 cases with prior negative BRAF results (blue) compared 46 cases with prior positive BRAF results (red).

Figure 5: Photographs of a 43 year-old female patient with stage IV ERC1-BRAF fusion melanoma, taken before (A, C) and after (B, D) 2 months of oral sorafenib 400mg BID. A-B: subcutaneous metastases of the anterior part of the trunk, C-D: subcutaneous metastases of the back, responding to sorafenib, after progressing under immunotherapy.

Figure 1.

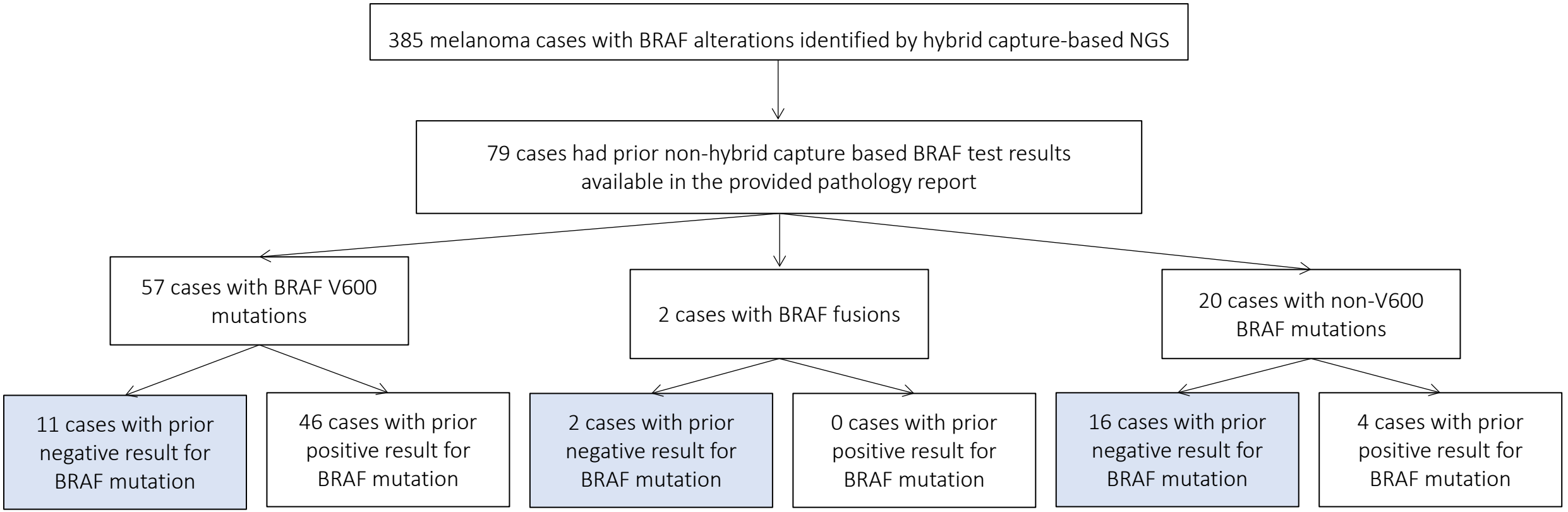

385 melanoma cases with BRAF alterations identified by hybrid capture-based NGS
79 cases had prior non-hybrid capture based BRAF test results available in the provided pathology report
57 cases with BRAF V600 mutations
2 cases with BRAF fusions
20 cases with non-V600 BRAF mutations
11 cases with prior negative result for BRAF mutation
46 cases with prior positive result for BRAF mutation
2 cases with prior negative result for BRAF mutation
0 cases with prior positive result for BRAF mutation
16 cases with prior negative result for BRAF mutation
4 cases with prior positive result for BRAF mutation

Figure 2.

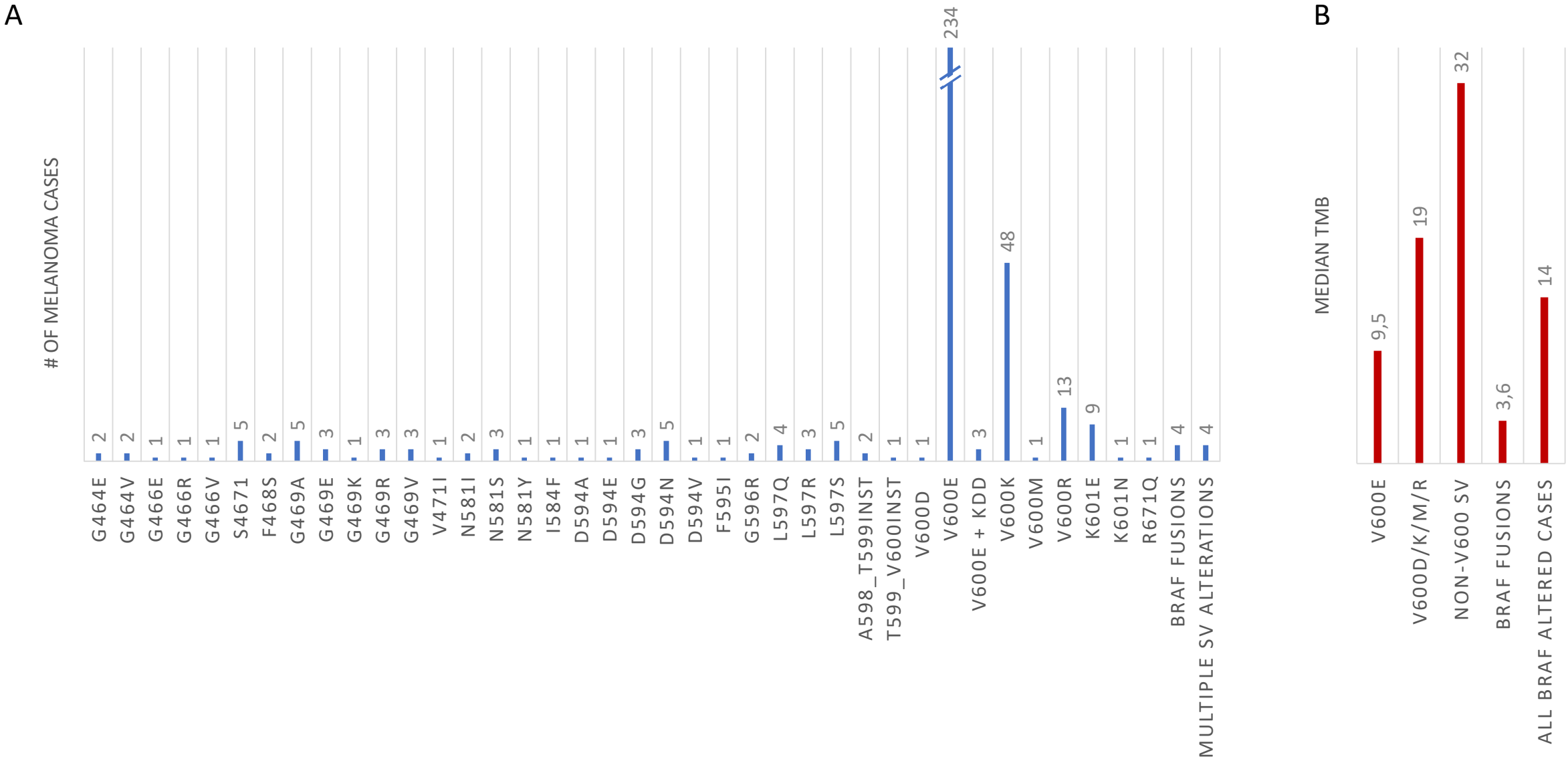

A
OF MELANOMA CASES
G464E 2
G464V 2
G466E 1
G466R 1
G466V 1
S467I 5
F468S 2
G469A 5
G469E 3
G469K 1
G469R 3
G469V 3
V471I 1
N581I 2
N581S 3
N581Y 1
I584F 1
D594A 1
D594E 1
D594G 3
D594N 5
D594V 1
F595I 1
G596R 2
L597Q 4
L597R 3
L597S 5
A598_T599INST 2
T599_V600INST 1
V600D 1
V600E 234
V600E + KDD 3
V600K 48
V600M 1
V600R 13
K601E 9
K601N 1
R671Q 1
BRAF FUSIONS 4
MULTIPLE SV ALTERATIONS 4
B
MEDIAN TMB
V600E 9,5
V600D/K/M/R 19
NON-V600 SV 32
BRAF FUSIONS 3,6
ALL BRAF ALTERED CASES 14

Figure 3.

A

	No. case	Median patient age (range)	Patient gender	Median MAF (range)	Median estimated tumor purity (range)	Prior BRAF testing method	BRAF alterations detected by HC-NGS
All BRAF prior negative cases	29	57 (14-89)	7F: 22M	30.4% (1.2-68%)	60% (20-90%)	IHC (3) NGS* (2) PCR (13) Sanger (5) Other/unspecified (6)	-
BRAF V600 prior negative	11	49 (14-74)	4F: 7M	35.1% (1.2-68%)	50% (20-90%)	IHC (2) NGS* (1) PCR (3) Sanger (2) Other/unspecified (3)	V600E (7) V600K (2) V600M (1) V600R (1)
BRAF non-V600 mutation prior negative	16	59.5 (37-89)	1F: 15M	29.2% (4.0-61.8%)	60% (20-80%)	IHC (1) NGS* (1) PCR (9) Sanger (3) Other/unspecified (2)	Activating# (9) Impaired (4) Uncharacterized (5)
BRAF fusion prior negative	2	34 (30-38)	2F: 0M	NA	60% (30-90%)	PCR (1) Other/unspecified (1)	TRIM24-BRAF SOX5-BRAF

B

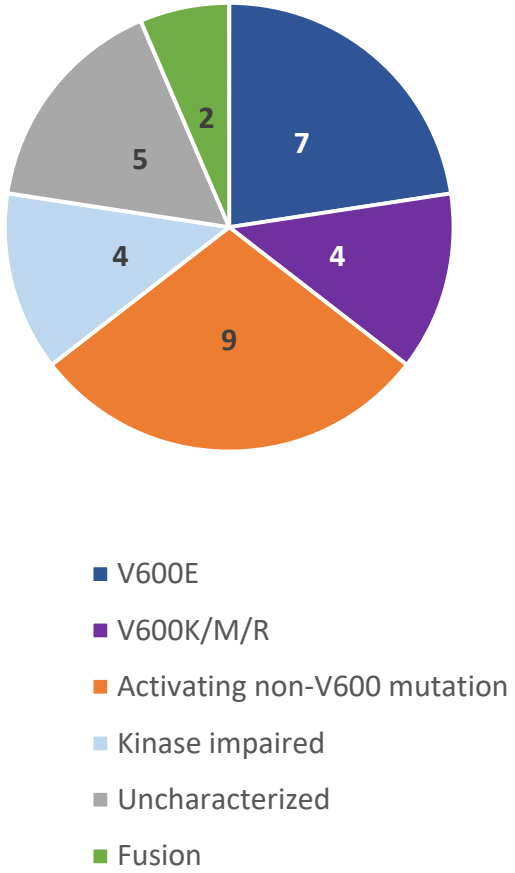


Figure 3.

C

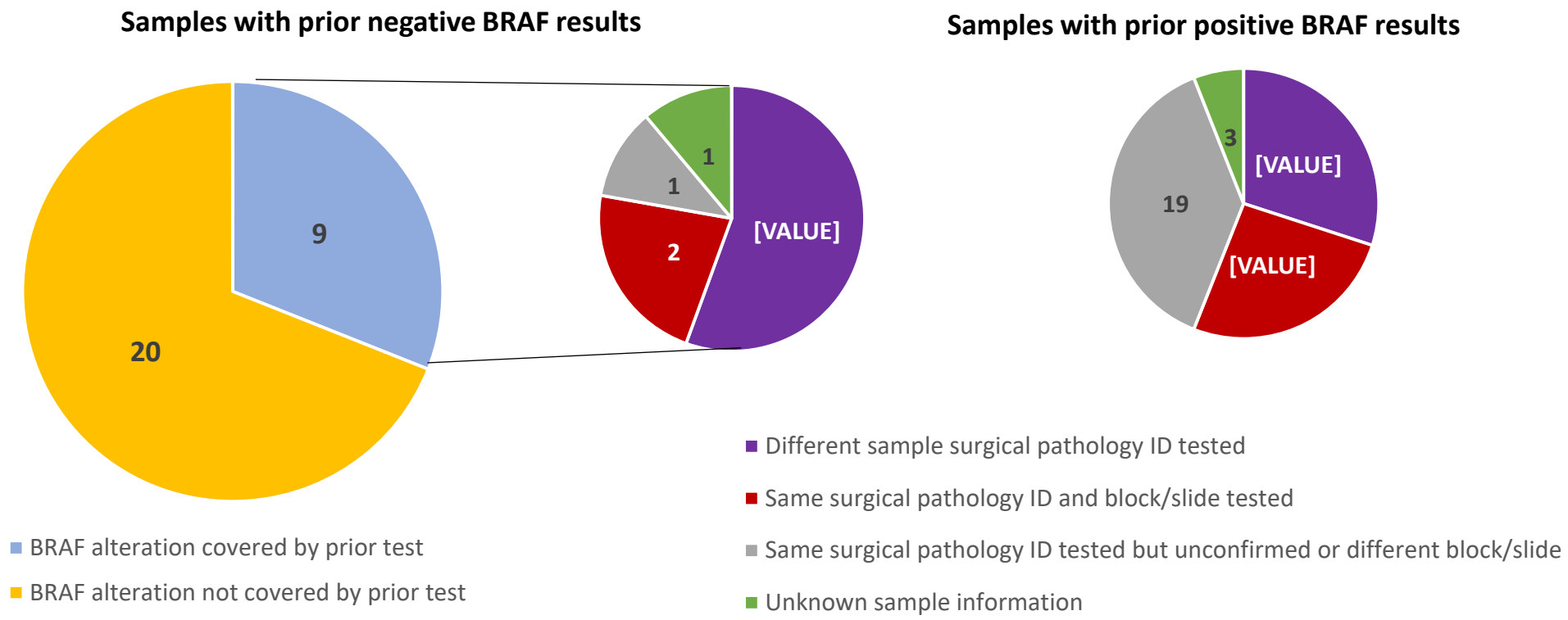
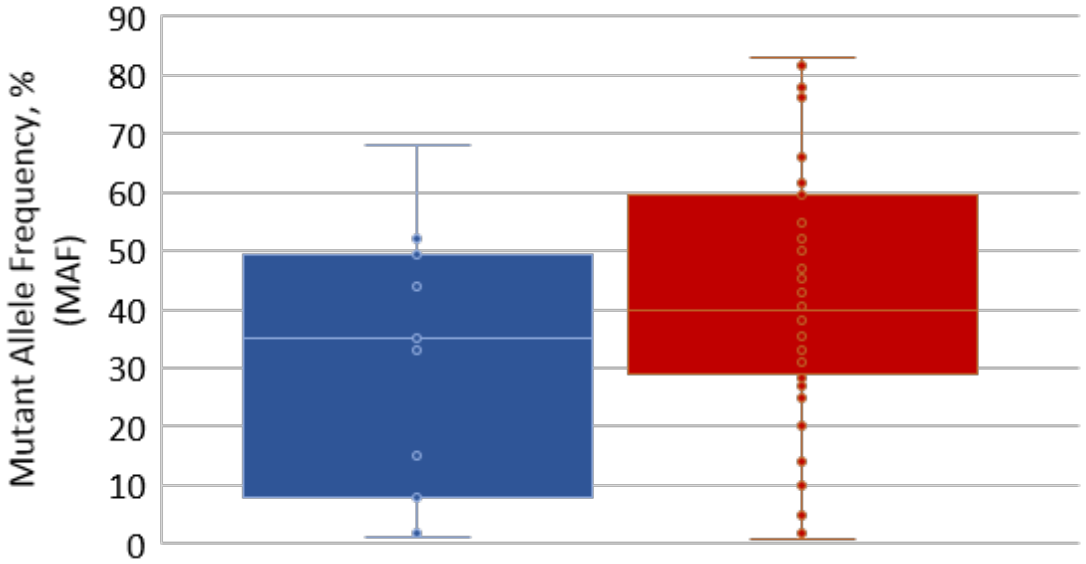

Samples with prior negative BRAF results
9
20
1
1
2
[VALUE]
Samples with prior positive BRAF results
3
19
[VALUE]
[VALUE]
BRAF alteration covered by prior test
BRAF alteration not covered by prior test
Different sample surgical pathology ID tested
Same surgical pathology ID and block/slide tested
Same surgical pathology ID tested but unconfirmed or different block/slide
Unknown sample information

Figure 4.

A

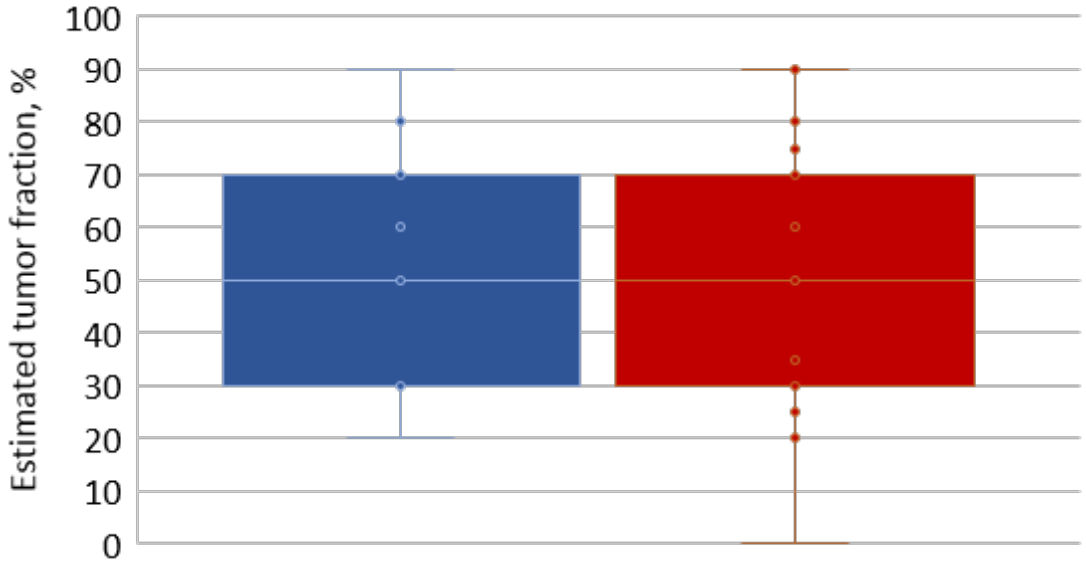


BRAF V600-mutated cases with prior negative results

BRAF V600-mutated cases with prior positive results

Median MAF: 35.1% 39.8% P = 0.25

B



BRAF V600-mutated cases with prior negative results

BRAF V600-mutated cases with prior positive results

Median % tumor: 50% 50% P = 0.97

Figure 5.

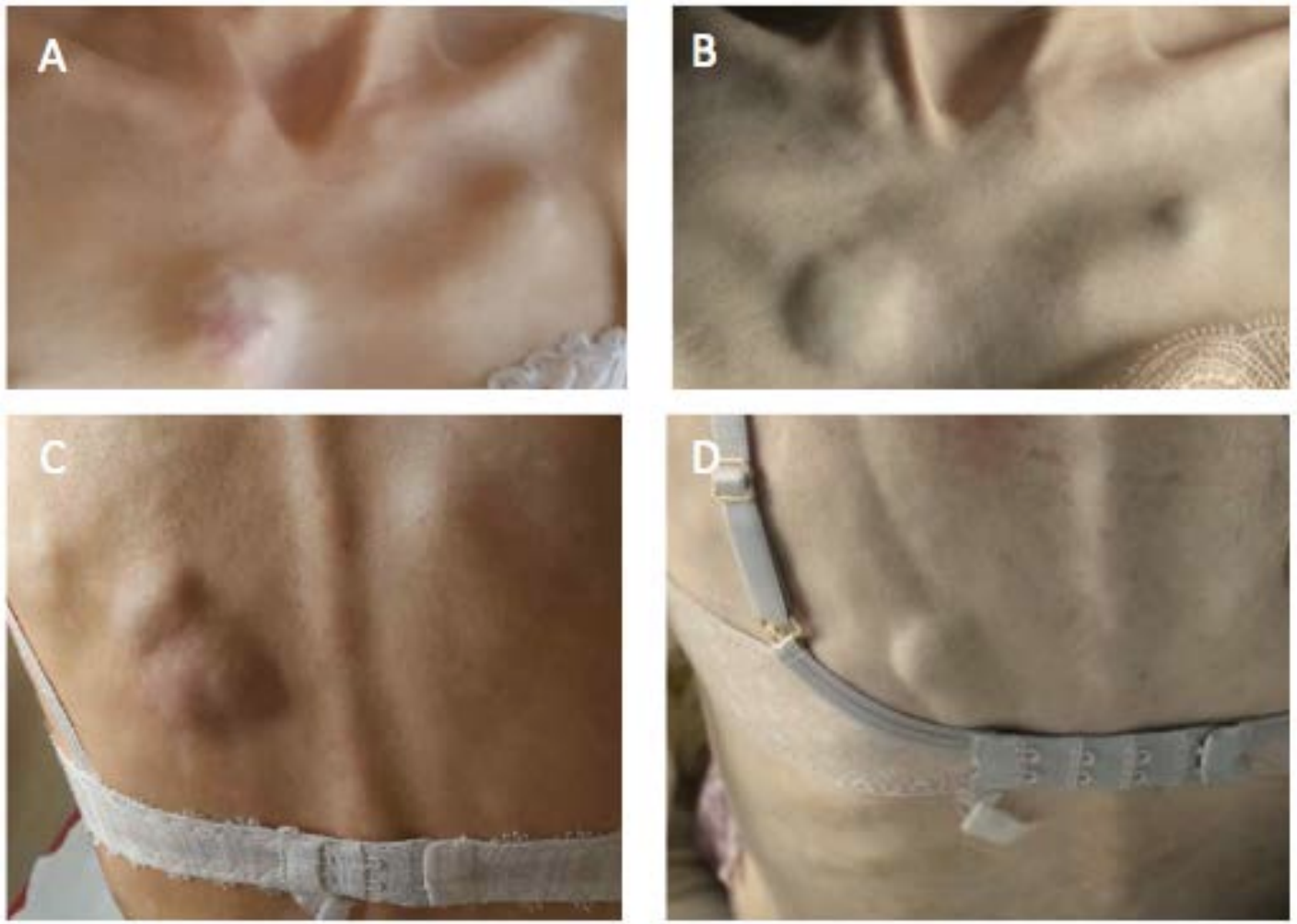


Table 1. Characteristics of 29 melanoma cases positive for BRAF alterations using CGP with prior negative BRAF results.

Patient	BRAF alteration detected by CGP	MAF	Same surgical pathology ID and block/slide tested by other methodology?	Same surgical pathology ID for samples tested by other methodology?	Other testing method	Other testing covers mutation detected?
1	V600E	15	Unknown	Unknown	Unknown	Y[ǂ]
2	V600E	68	N	N	melting curve analysis	Y
3	V600E	8	N	N	Sanger sequencing	Y
4	V600E	44	N	N	Unknown	Y[ǂ]
5	V600E	1.2	Y	Y	PCR	Y
6	V600E	2	Y	Y	Sanger sequencing	Y
7	V600E	35	N[a]	Y	PCR	Y
8	V600K	33	N	N	IHC	N
9	V600K	52	Unknown[b]	Y	IHC	N
10	V600M	44	n/a	n/a[c]	NGS	Y
11	V600R	49	N	Y	PCR	N*
12	K601E	36	N	N	PCR	N
13	K601E	24	N	N	PCR	N[^]
14	K601E + L584F	48	N	N	PCR	N
15	K601E + E586K	32	Y	Y	PCR	N
16	A589_T599insT	11	Y	Y	PCR	N
17	G464V	29	Unknown[d]	Y	IHC	N
18	G469V	9	Unknown	Y	Sanger sequencing	N
19	L597Q	4	n/a	n/a[e]	NGS	N
20	D594A	29	N	N	PCR	N
21	D594G	54	Unknown	Unknown	PCR	N
22	D594N	17	Y	Y	Unknown	Unknown
23	G466V	62	N	N	Sanger sequencing	N
24	S467L	30	N	N	PCR	N
25	S467L	29	N	N	PCR	N
26	N581I	35	Unknown	Unknown	Unknown	Unknown
27	L584F	6.3	N	N	Sanger sequencing	Y[#]
28	SOX5-BRAF fusion	n/a	N[f]	Y	PCR	N

29	TRIM24-BRAF fusion	n/a	Unknown	Unknown	Unknown	Unknown

MAF: mutant allele frequency; CGP: comprehensive genomic profiling.
*Test specifications note probes are designed for V600E but will detect other V600 alterations with limited sensitivity
[^]Test specifications note primers are designed for V600E/K, but may cross react with K601E
[#]Test methodology says BRAF exon 15 is covered, but does not specific individual residues
[ŧ]Prior test detials were not provided; however we make the assumption that any molecular test returning a "BRAF wild-type" result for melanoma would have covered the canonical V600E mutation.
[a]Both samples were from a single mid-back specimen but different slides from the tissue block were tested
[b]Both samples were from a single left-thigh specimen
[c]Liquid biopsy collected 12 months after tissue sample
[d]Both samples were from a right-groin soft tissue mass
[e]Liquid biopsy collected 4.5 months after tissue sample
[f]Both samples were from a single right-thigh specimen but different slides from the tissue block were tested

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