Detecting *BRAF* Mutations in Formalin-Fixed Melanoma: Experiences with Two State-of-the-Art Techniques

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Key Words

BRAF mutations · V600E · Mutation detection methods · Melanoma

Abstract

Background: Melanoma is characterized by a high frequency of *BRAF* mutations. It is unknown if the *BRAF* mutation status has any predictive value for therapeutic approaches such as angiogenesis inhibition.
Patients and Methods: We used 2 methods to analyze the *BRAF* mutation status in 52 of 62 melanoma patients. Method 1 (mutation-specific real-time PCR) specifically detects the most frequent *BRAF* mutations, V600E and V600K. Method 2 (denaturing gel gradient electrophoresis and direct sequencing) identifies any mutations affecting exons 11 and 15.
Results: Eighteen *BRAF* mutations and 15 wild-type mutations were identified with both methods. One tumor had a double mutation (GAA) in codon 600. Results of 3 samples were discrepant. Additional mutations (V600M, K601E) were detected using method 2. Sixteen DNA samples were analyzable with either method 1 or method 2. There was a significant association between *BRAF* V600E mutation and survival.
Conclusion: Standardized tissue fixation protocols are needed to optimize *BRAF* mutation analysis in melanoma. For melanoma treatment decisions, the availability of a fast and reliable *BRAF* V600E screening method may be sufficient. If other *BRAF* mutations in exons

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11 and 15 are found to be of predictive value, a combination of the 2 methods would be useful.

Introduction

BRAF gene aberrations became prominent in melanoma in 2002, when Davies et al. [1] published the results of a first genome-wide analysis of melanoma tissue. This study revealed a high frequency of *BRAF* mutations in melanoma patients occurring mainly at codon 600. The V600E mutation occurs in 40–60% and the V600K exchange is observed in up to 20% of *BRAF* mutations [2–4].

Currently, promising therapeutic agents such as vemurafenib (RG 7204; Roche, Basel, Switzerland) are available that specifically target the *BRAF* V600E mutation [5–11] while also exhibiting positive effects in melanoma patients with the V600K variant [7, 12].

Several studies have proven that *BRAF* mutations at V600 lead to reduced overall survival (OS) unless treated with a *BRAF* targeting drug [3, 13]. Therefore, knowing the *BRAF* mutation status in melanoma patients is clinically important. Various detection methods have been applied to detect *BRAF* V600 mutations [1, 3, 5, 13–21].

Here, we describe 2 different *BRAF* mutation detection methods that were utilized in a phase II melanoma trial [13] with regard to their advantages, reliability and limitations. The impact of a *BRAF* mutation on patient outcome was also analyzed.

Patients and Methods

Patient Selection
A total of 62 patients were treated with temozolomide and bevacizumab in this phase II trial; SAKK 50/07. All patients were considered for *BRAF* genotyping. All patients had histologically confirmed melanoma and unresectable stage IV disease. Patient demographics have been described previously [13]. The trial was approved by the local ethics review boards as well as by Swissmedic and was registered at the National Institute of Health (www.clinicaltrial.gov; identifier number: NCT00568048). All patients gave written informed consent before any trial procedure.

Material Preparation and Selection
HE-stained sections of 62 primary melanomas that were fixed in formalin and embedded in paraffin (FFPE) were reviewed by 2 pathologists (D.M.-P., H.M.). The tumor region of interest was marked on each glass slide. DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Basel, Switzerland) and tested for *BRAF* mutations in 2 independent laboratories.

BRAF *Mutation Analysis from Melanoma Tumor Tissues*
Two methods were used for the analysis of *BRAF* mutations. Method 1 was performed according to the method described by Benlloch et al. [14]. This mutation-specific real-time PCR enables the specific detection of the GTG to GAG transversion in codon 600 of *BRAF*, which causes an amino acid exchange from valine to glutamic acid. The sequences of the exon 15-specific primers and the MGB-TaqMan probes are shown in **table 1**. Depending on the genotype, distinct plots for the mutant and wild-type (WT) variants were generated. The threshold between WT and mutated genotypes was adapted to a heterozygous *BRAF* V600E mutated melanoma reference sequence. The reference cell culture was established from 1 melanoma patient. Final evaluation was based on allelic discrimination (**fig. 1**).

In addition to *BRAF* V600E, other less frequent *BRAF* mutations in exons 11 and 15 have been described for melanoma [1]. Method 2 was used to find such mutations. This method is based on a denaturing gel gradient electrophoresis (DGGE) of amplified PCR products of exons 11 and 15, and direct DNA sequencing as previously described [22]. PCR products showing reduced or increased mobility shifts compared to WT *BRAF* were subjected to direct sequencing (**fig. 2**).

Statistical Analysis

Endpoints considered for the statistical analysis were disease stabilization (either complete response, partial response or stable disease) rate at 12 weeks, best overall response, and 3 time-to-event type endpoints: duration of response stabilization, progression free survival (PFS) and OS. PFS was defined as the time from trial registration until either disease progression or death, with patients censored at the time of starting second-line therapy or the last time they were known to be alive without progression.

The *BRAF* mutation status identified by the 2 methods was used as a descriptive secondary endpoint: 3 types of *BRAF* status groups were considered based on either *BRAF* method 1, *BRAF* method 2 or combined *BRAF* methods 1 and 2. All endpoints were evaluated by *BRAF* status by means of the log-rank test for survival-type endpoints and χ^2 or Spearman's rank correlation coefficient allowing for the ordered outcome of tumor response. No adjustment for multiple comparisons was performed. The data was analyzed in SAS (Statistical Analysis Systems, version 9.2).

Results

Detection of BRAF *Mutation Status by Methods 1 and 2*

DNA could be extracted from 52 of 62 (84%) FFPE tumor samples. Due to insufficient or no tumor cells, 10 cases could not be analyzed. The *BRAF* mutation status was analyzable in 36 DNA samples with both methods. Eighteen *BRAF* V600E mutations (50%) and 15 WT mutations (42%) were identified with both methods. The results of 3 samples (8%) were discrepant between the methods. Additional mutations (V600M, K601E) were obtained by using method 2. Notably, 1 of the V600E melanoma results had a GTG to GAA double point mutation. Eight DNA samples were analyzable with method 1, and 8 with method 2. No mutations were seen in *BRAF* exon 11. The results of the 2 *BRAF* mutation analyses are shown in **table 2**. Examples of mutations are shown in **fig. 3**a–e.

BRAF *Mutation Status and Tumor Response*

No statistically significant association was seen between *BRAF* mutation status obtained by method 1, 2 or both and overall best tumor response with the use of angiogenesis inhibition in combination with an alkylating chemotherapy.

BRAF *Mutation Status and Time-to-Event Endpoints*

Among patients with a tumor response and a known *BRAF* mutation status, there was no statistically significant difference in response duration between *BRAF* status groupings. No statistically significant difference in median PFS was observed between *BRAF* status using *BRAF* method 1, 2 or both.

The median OS was 9.6 months (95% CI: 8.0, 11.9). When stratified by method 1, *BRAF* mutation status showed a statistically significant difference (p = 0.0137). Using method 2, *BRAF* mutation status was borderline statistically significant (p = 0.0546) and when combining methods 1 and 2, *BRAF* mutation statuses also resulted in statistical significance (p = 0.0102). The data are shown in **table 3**.

Discussion

Advantages and Limitations of the Used Methods

Method 1 allows the specific detection of the *BRAF* hotspot mutation in melanoma at codon 600. In addition to GAG (V600E) and AAG (V600K) mutations, the DNA sequencing results obtained with method 2 indicate that the TaqMan probe is obviously also able to bind a third variant, namely GAA which also codes glutamic acid. This double mutation at codon 600 is the third most frequent *BRAF* sequence alteration and occurs in about 2% of malignant melanomas [4].

The significant advantage of method 1 lies in the TaqMan approach, which allows the rapid identification of *BRAF* V600E without any additional analysis such as the time-consuming and costly DGGE and DNA sequencing. In contrast, method 2 allows the detection of any possible mutations occurring within exon 15, including other V600 variants. If any other *BRAF* mutations besides that at codon 600 also become therapeutically relevant for melanoma patients, the TaqMan approach would not be sufficient. However, the occurrence of other mutations in melanoma, such as K601E or V600M, is quite low and the long-term relevance has not yet been documented.

Frequency of V600 Mutations

The frequency of the *BRAF* V600E mutation of 50% is comparable to that described in the literature. Recent results show that up to 20% of all *BRAF* mutations lead to a V600K exchange, which is also targetable [23, 24]. We were unable to find this alteration in our melanoma samples. The lack of finding V600K mutations in our patient cohort may be explained due to the relatively low number of analyzable DNA samples. However, our data are in line with the results of other studies in which much lower frequencies of the V600K mutation were found [23, 24]. Based on these data, it is also possible that the frequency of the V600K mutation is overestimated.

Tumor Heterogeneity and Protocol Standardization

An important drawback is the fact that the tumor tissue is often infiltrated and surrounded by non-tumorous cells. Depending on the level of contamination, a *BRAF* mutation may be overlooked, thus producing a false-negative (WT) result. We aimed to circumvent this problem by punching exclusively into areas of the tumor tissue that contained more than 80% tumor cells.

The necessity of standardized protocols for fixation, dehydration and paraffin embedding of tissues is a critical issue, particularly if the specimens to be analyzed derive from different pathology institutes. A reliable *BRAF* mutation analysis was problematic or even impossible for about one-third of the tissue samples, suggesting more or less differing protocols used at the different pathology centers involved in this phase II trial. Standardized and optimal operating procedures are of utmost importance for reliably performing *BRAF* mutation analysis in all tissue samples to guarantee the best benefit for melanoma patients.

Outcome Data

In our trial, statistical differences were between *BRAF* WT and mutant patients regarding OS. Whether the survival difference between *BRAF* WT and mutant patients is only due to the fact that *BRAF* mutational status has prognostic value or if *BRAF*

mutational status may also be predictive for a combination treatment with bevacizumab and temozolomide cannot be concluded definitively from our data set, but should be prospectively studied. If the 2 most frequent *BRAF* mutations, V600E and V600K, are the alterations with predictive value in melanoma patients, techniques identical or similar to method 1 described in this paper may be the detection system of choice.

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Disclosure Statement

Study design, data collection, analysis and interpretation of the data, final approval of the manuscript and the decision to submit the paper for publication were the responsibility of the authors and not the sponsors. R. von Moos: Consultancy/advisory role at Roche, Bristol-Myers-Squibb and Essex AG, and speaking honoraria at Roche. R. Dummer: Had consultancy/advisory role and research funding at Astra-Zeneca and Novartis. S. Gillessen: Advisory role at Novartis. H. Moch: Currently conducting research sponsored by Roche and Novartis. All other authors: no conflict of interest to declare.

Table 1. Primer sequences and MGB-TaqMan probes for *BRAF* exon 15 applied in method 1

Primer	Sequence
BRAF exon 15 forward	5′-CTACTGTTTTCCTTTACTTACTACACCTCAGA-3′
BRAF exon 15 reverse	5′-ATCCAGACAACTGTTCAAACTGATG-3′
BRAF WT probe	5′-VIC-CTAGCTACAG**t**GAAATC-MGB-3′
BRAF V600E probe	5′-FAM-TAGCTACAG**a**GAAATC-MGB-3′

BRAF WT (GtG) and mutated codon 600 (GaG) are indicated in bold lowercase letters.

Table 2. Patient *BRAF* characteristics: a comparison between methods 1 and 2

BRAF analysis results		Frequency, % (n = 52)
method 1	method 2	
V600E	V600E*	18 (34.62)
WT	WT	15 (28.85)
WT	NA	5 (9.52)
NA	WT	6 (11.54)
NA	V600E	1 (1.92)
NA	V600M	1 (1.92)
V600E	**WT**	**1 (1.92)**
WT	**V600E**	**1 (1.92)**
WT	**K601E**	**1 (1.92)**
V600E	NA	3 (5.77)

NA = Not analyzable (no/too little DNA, no PCR product). * One tumor with GAA mutation. Discrepant results are in bold.

Table 3. *BRAF* mutation status and patient outcome

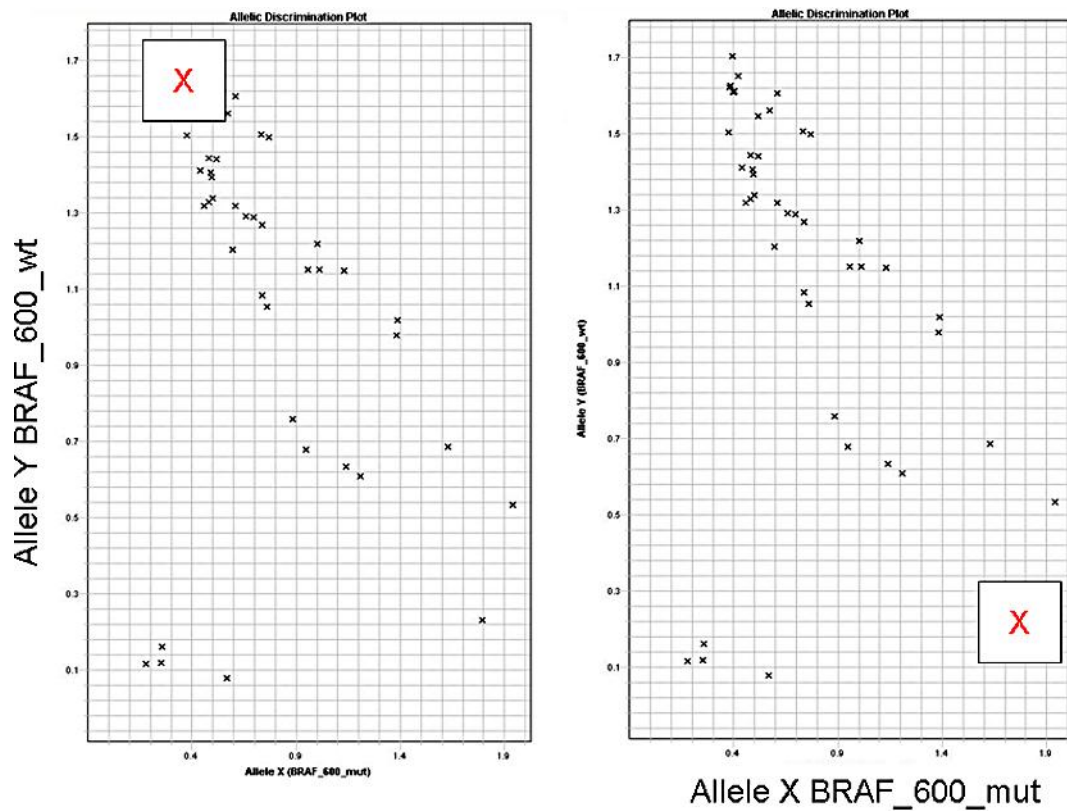| Analysis	Method	*BRAF* status [median event-free survival time, months (95% CI)]		Log-rank p value
		BRAF mutation	*BRAF* WT	
PFS	1	4.0 (1.6, 5.4)	5.3 (2.6, 8.1)	0.0986
	2	4.0 (1.2, 5.5)	5.4 (2.6, 8.1)	0.0571
	1 + 2	4.0 (1.7, 6.6)	5.5 (1.3, 10.9)	0.0647
OS	1	9.2 (6.5, 11.9)	12.0 (7.4, 16.4)	0.0137
	2	9.6 (6.6, 11.9)	12.6 (8.4, 15.6)	0.0546
	1 + 2	9.2 (6.6, 11.8)	12.6 (7.2, 16.4)	0.0102

Fig. 1. Allelic discrimination plot of WT *BRAF* (left: red x) and *BRAF* V600E mutant (right: red x).
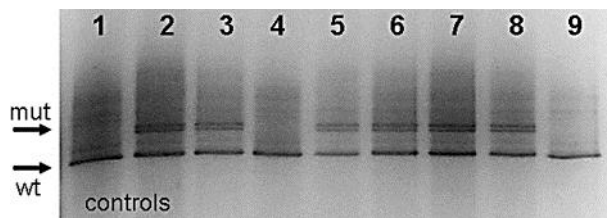

Fig. 2. DGGE of PCR products of *BRAF* exon 15 from 7 melanomas. Lanes 1 + 2: controls for *BRAF* WT and *BRAF* V600E mutation. Lanes 3–9: PCR products of 7 melanomas with *BRAF* WT (wt) and mutation (indicated by arrows).
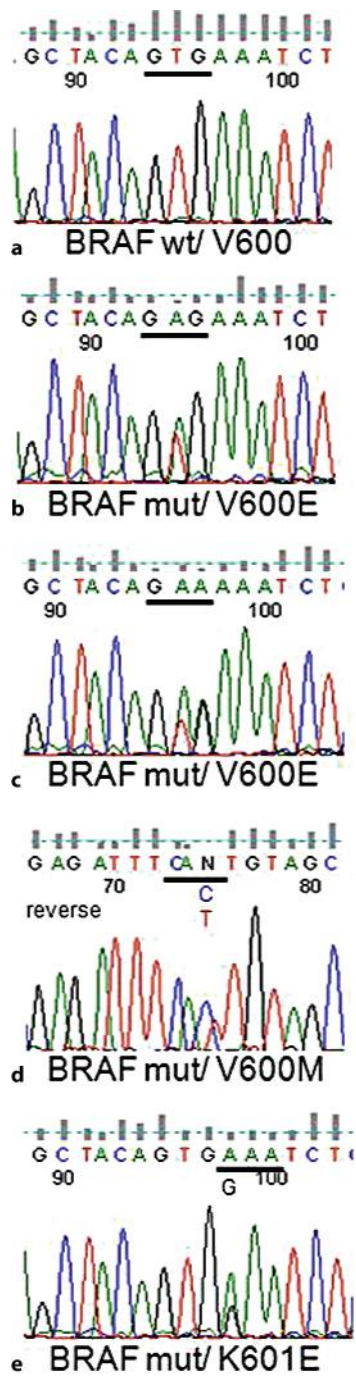
Fig. 3. *BRAF* exon 15 mutation status in 5 melanoma patients. **a** *BRAF* WT. **b**, **c** Single and double mutations in codon 600 leading to V600E. **d** C>T transition (reverse sequence) leading to methionine (ATG) at codon 600. **e** A>G transition at codon 601.

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