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ORIGINAL ARTICLE

BRAF mutation testing in melanoma: results from a German observational multicenter study

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Abstract

Quality control of *BRAF* mutation testing methods used in routine practice is crucial for optimal treatment selection. In this prospective study, we assessed the impact of patient/sample characteristics on *BRAF* mutation testing results in patients with melanoma, during clinical practice. Data were collected on routine testing practices and documented mutation status in patients with melanoma stages IIIB, IIIC, or IV across 28 diagnostic pathology centers in Germany. Patient/sample data collected included: patient age, location of primary melanoma and metastases, origin of sample, melanoma subtype, and quality of tissue. Statistical influence of patient/sample characteristics on *BRAF* mutation rate was assessed using multiple logistic regression analyses and statistical models developed to predict the probability of *BRAF* mutations for individual patient cohorts. Data/samples from 642 patients with melanoma were analyzed. *BRAF* mutations were documented in 241/642 patients (37.5%). The primary statistical model to predict *BRAF* mutation rates included: age (continuous), origin of sample, method of mutation analysis, and quality of tissue. Analyses of post hoc collected data identified major deviations between documented mutation rates included in this study vs. routinely recorded mutation rates for three centers. When samples from these centers were excluded, the influence of testing method was no longer statistically significant. The final model included patient age, origin of sample (including metastasis location), and quality of tissue. Once validated in an independent population, this type of model could allow pathology centers to compare the performance of their testing methods with what would be expected based on patient, tumor, and sample characteristics.

Keywords *BRAF* mutation · Melanoma · Quality control · Mutational analysis · Multiple logistic regression

Introduction

BRAF plays a key role in the mitogen-activated protein kinase (MAPK) signaling pathway, which is responsible for coordinating responses to extracellular signals and regulating cell proliferation, differentiation, senescence, and apoptosis [1]. Activating mutations of the *BRAF* gene are detected in 40–50% of patients with melanoma [2–6], and targeted therapy with BRAF kinase inhibitors such as vemurafenib (Zelboraf®, F. Hoffmann-La Roche Ltd., Basel, Switzerland), dabrafenib (Tafinlar®, Novartis, Basel, Switzerland), and their combination with MEK inhibitors such as cobimetinib (Cotellic®, F. Hoffmann-La Roche Ltd., Basel, Switzerland) and trametinib (Mekinist®, Novartis, Basel, Switzerland), have become a crucial part of the treatment of advanced metastatic melanoma [7]. To date, the clinical efficacy and safety of these drugs has only been demonstrated in patients with tumors that harbor mutations in codon

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600 of the *BRAF* gene (specifically *V600K* and *V600E*; clinical data for other V600 mutations are lacking due to their rarity) [8, 9]. In the EU, BRAF inhibitors are therefore only indicated for use in patients with $BRAF^{V600}$ mutation–positive tumor status, as confirmed by a validated test [10, 11].

Mutational analysis is required to identify patients who are eligible to receive BRAF inhibitor therapy, and for treatment selection in patients with wild-type *BRAF* who may benefit from alternative therapies, e.g., cancer immunotherapy [7]. Quality control of the diagnostic methods used for mutational analysis in routine clinical practice is therefore crucial to ensure optimal treatment for the individual patient. In this prospective study of $BRAF^{V600}$ mutation status, we assessed the routine testing practices and recorded mutation status in patients with melanoma across 28 diagnostic molecular pathology centers in Germany. The primary aim of this study was to evaluate the impact of multiple parameters on routine $BRAF^{V600}$ mutation testing.

Methods

Study design

This was a non-interventional, prospective study of routine testing methods used to determine $BRAF^{V600}$ mutation status in patients with melanoma stages IIIB, IIIC, or IV at 28 diagnostic molecular pathology centers across Germany. Ethics approval was obtained at the University of Erlangen-Nürnberg (approval-no. 14_13 B dated Feb 19th 2013). Since patient data were acquired fully anonymized from routine testing, no informed consent was required.

Data collection

$BRAF^{V600}$ mutation testing was performed on the primary tumor or metastatic tissue from patients with melanoma during routine clinical testing. The study required each center to submit data on a maximum of 30 samples for inclusion in this analysis (two centers provided more than 30 samples). There were no standard selection criteria for the samples provided and centers were not required to submit consecutive samples. However, the study allowed only samples with sufficient availability of tissue to be included. The physician's choice of diagnostic methods, patient therapy, or the frequency of medical examination during and after treatment was not influenced by this non-interventional study. Routine methods for *BRAF* mutation analysis used within participating centers included Sanger sequencing, the cobas® 4800 BRAF V600 Mutation Test (Roche Diagnostics GmbH, Mannheim, Germany), pyrosequencing, reverse transcription polymerase chain reaction (PCR)–based assays, BRAF StripAssay® (ViennaLab Diagnostics GmbH, Vienna, Austria), high-resolution melting assay, and next-generation sequencing (NGS) (see Table 1).

Data were systematically collected on the following clinical, histological, and mutational findings: routine *BRAF* testing methods used; documented *BRAF* mutation rate in total and per center (relative frequency of mutation based on the mutation status of individual samples submitted by each center); routine *BRAF* mutation rate per center (relative frequency of mutation based on the mutation status of individual samples submitted by each center post hoc); patient and tumor characteristics (age, sex, American Joint Committee on Cancer [AJCC] stage, tumor, node, and metastasis [TNM] classification, melanoma subtype); origin of sample (primary tumor or metastasis, plus location from which the sample was taken).

Statistical methods

Data were analyzed descriptively with summary statistics, 95% Wilson score–based confidence intervals (CIs) and graphical methods (as appropriate). The combined impact of multiple parameters on routine $BRAF^{V600}$ mutation testing was evaluated through multiple logistic regression analysis. We developed a statistical model to predict the probability of *BRAF* mutations for individual patient cohorts and centers.

Univariate analyses were used to identify factors potentially associated with mutation status. The following variables were assessed: patient age (continuous or grouped), sex, origin of tumor sample (primary vs. metastasis), location of metastasis, location of the primary melanoma, AJCC tumor stage, TNM status, melanoma subtype, method of $BRAF^{V600}$ mutation analysis, quality of tissue (self-reported per criteria used at each center), and quality of DNA (self-reported by centers; determined by agarose gel electrophoresis or PCR control). We further assessed the combined statistical influence of these variables on $BRAF^{V600}$ mutation status using multiple logistic regression analyses. The variable "origin of

Table 1 Distribution of *BRAF* testing methods across centers

First *BRAF* analysis method	Centers, *n*	Samples tested, *n*
Next-generation sequencing	2	45
High-resolution melting assay	3	62
Cobas® 4800 BRAF V600 Mutation Test	5	72
Sanger sequencing	11	148
Pyrosequencing	8	154
RT-PCR-based assay	4	61
BRAF strip assay	2	63
Other assay	3	37

Centers may have used more than one method and be counted multiple times

PCR, polymerase chain reaction; *RT-PCR*, reverse transcription PCR

sample" was re-grouped including the variable "location of sample" (e.g., liver, lung) into one new variable for logistic regression analysis. Missing values were categorized as "NA" (not available) and modeled as a separate level for each independent variable.

The best-fitting statistical model for predicting *BRAF* mutation rates was developed through step-wise backward elimination of variables from a comprehensive model that initially included all covariate candidates. The covariates of the best-fitting statistical model were further reviewed for clinical and scientific appropriateness. The relative importance of covariates for the *BRAF* mutation rate was assessed using the model's p value as a measure of statistical significance and by estimating the amount of contribution of each covariate to the variation of the predicted probability of mutation. We used the area under the receiver operating characteristic curve (ROC AUC) to investigate sensitivity, specificity, and predictive strength of the model. A prediction profiler was applied to visualize the relationship between mutation status and the adjusted influence of covariates.

We further conducted an analysis comparing the documented mutation rate (= relative frequency of mutated samples) and mean predicted probability of mutation of each center with its routine mutation rate (routine mutation rate data collected post hoc).

The sample size estimate was based on the precision of the overall mutation rate estimate. Assuming an overall $BRAF^{V600}$ mutation rate of 37%, a two-sided tolerable error of 5%, and a score-based confidence interval of 99%, a sample size of 613 evaluable tumor samples was needed.

Additional exploratory analyses were performed as required. Statistical analyses were performed using SAS JMP V12.2.0 (SAS Institute, Cary, NC, USA).

Results

Final analysis set

Tumor samples were evaluated between April 2013 and April 2015, based on the availability of clinical data. Patient/sample characteristics from 642 patients with melanoma (642 samples respectively) at 28 centers across Germany were analyzed; 398/642 patients (62.0%) were male, and the mean age was 65.6 years (range 16–91 years). The median number of samples provided per center was 25 (range 5–54). *BRAF* mutations were detected in 241/642 patients (37.5%); mutation status was confirmed by retesting at each individual center in 116/642 samples (18.1%).

The majority of $BRAF^{V600}$ mutations identified (188/241; 78.0%) were $BRAF^{V600E}$. Other *BRAF* mutations identified were V600K (23/241; 9.5%), V600R (3/241; 1.2%), and other non-V600 mutations (12/241; 5.0%); 15 samples exhibited *BRAF* mutations but the exact mutation type could not be identified. The distribution of the origin of samples included in this analysis is shown in Fig. 1. *BRAF* mutation frequencies by individual variables are shown in Table 2. Approximately, 80% of samples with available data had tumor cell content of 20–50% or 50–80% before microdissection (50–80% or > 80% after microdissection) and, of these, the majority were classified as "high" or "very high" tissue quality (Online Resources 2 and 3).

Statistical influence of independent variables on BRAF mutation rate

In the primary multiple logistic regression model ($n = 642$), age (years), origin of sample (primary tumor, unknown origin, or metastasis [split by location]), *BRAF* mutation testing method and quality of tissue had a statistically significant, or borderline significant influence on the detected *BRAF* mutation rate (Table 3; primary model). Origin of sample had the highest covariate importance in predicting the *BRAF* mutation rate (total effect = 0.379). The continuous covariate "age" had the lowest p value in the model ($p < 0.001$) and ranked third for covariate importance in predicting *BRAF* mutation. We further tested for interactions of variables, but no indication of an interaction was found ($p > 0.25$).

Statistical model for predicting BRAF mutation probabilities

The primary statistical model used to calculate predicted *BRAF* mutation probabilities included the following covariates: patient age (continuous), origin of sample (location of primary tumor or metastasis [split by location]), method of mutation analysis, and quality of tissue. All predicted mutation probabilities were within the 95% confidence limits of the documented mutation rates at each center (Online Resource 4), indicating no statistically significant deviations from the predicted rates. It should be noted, however, that the confidence intervals were quite wide for some centers with low sample size. In these cases, the deviation was assessed by the difference between the mutation rates predicted by the model and the documented mutation rates, taking into account the uncertainty indicated by the size of the confidence interval. Furthermore, in some centers, we observed deviations between the documented *BRAF* mutation rates included in this study and the rates routinely reported at the respective centers.

Comparison of documented and routine BRAF mutation rates in diagnostic testing by center

Routine *BRAF* mutation rates were collected post hoc from each center and compared with the data collected within this study. Documented *BRAF* mutation rates at centers 2, 12, and

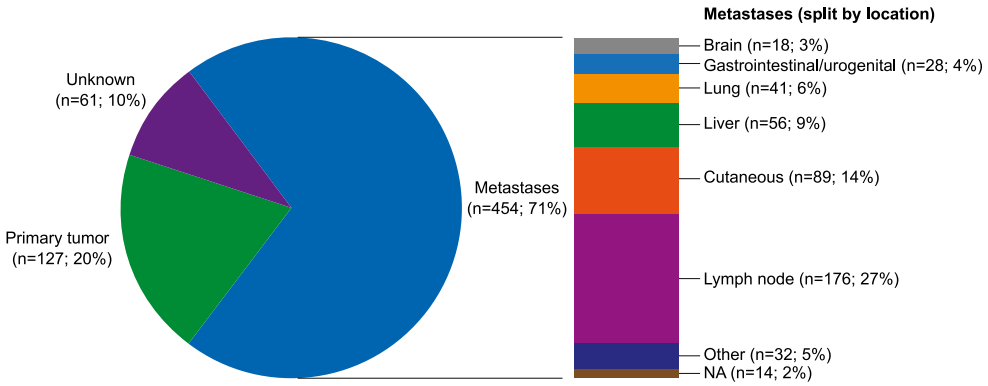

Fig. 1 Distribution of the origin of samples included in this analysis (primary tumor or metastasis [split by location]). NA, not available

18 showed major deviations from their routinely reported rates: samples from centers 12 and 18 had documented mutation rates that were substantially lower than the routine mutation rate collected post hoc from these centers; center 2 had a higher documented mutation rate, compared with its routinely reported rate (Online Resource 5).

Due to the discrepancy between documented and routine mutation rates, the primary model was rebuilt excluding the deviating centers 2, 12, and 18 (Table 3; reduced model). After the exclusion of the data from these centers, the sample size for the reduced model was 597 evaluable samples. In this second analysis, the testing method was no longer associated with the *BRAF* mutation rate ($p = 0.392$); therefore, the method of mutation analysis was excluded from the reduced model. In addition, the p value for the "quality of tissue" variable increased from $p = 0.075$ to $p = 0.223$. The ROC AUC was 0.648, indicating a moderate model fit.

Prediction of BRAF mutation rate based on covariates included in the primary and reduced statistical models

The influence of each individual covariate on the predicted *BRAF* mutation rate is illustrated by its prediction profiler trace (Fig. 2), which is adjusted for the influence of all other covariates at a level indicated by the vertical dashed red lines in each compartment.

In the prediction profiler based on the primary model (Fig. 2 [top panel]), the predicted probability of *BRAF* mutation decreased with increasing age; brain metastases had the highest predicted probability of *BRAF* mutation (~ 60%) and NGS had the lowest predicted *BRAF* mutation probability. However, data for NGS were derived from centers 18 and 26, one of which (center 18) reported major deviations from its routine rate as noted previously, indicating a potentially biased estimate (see Discussion).

The prediction profiler based on the reduced statistical model, excluding centers with major deviations, is shown in Fig. 2 (bottom panel). As with the primary model, the predicted probability of *BRAF* mutation decreased with increasing age; brain metastases had the highest predicted *BRAF* mutation rate and liver metastases the lowest predicted rate. However, no influence of the testing method was presented, as this variable was excluded from the reduced model. The pattern of dependency of the probability of *BRAF* mutation for the other three covariates was remarkably similar in the two models.

When applying the primary model to a hypothetical patient (age 65 years; primary tumor sample origin; method of mutation analysis—pyrosequencing mutation analysis; high tissue quality), the model predicted a probability of carrying a *BRAF* mutation of 38.8% (95% CI 28.4–50.3%) for this combination of covariates. The same covariates applied to the reduced model resulted in a 39.9% (95% CI 31.0–49.5%) predicted probability of *BRAF* mutation.

Discussion

This study assessed the combined impact of multiple parameters on *BRAF* mutation testing results in clinical practice. These data were then used to develop a statistical model to predict the expected frequency of *BRAF* mutations for individual patient cohorts. Essentially, the proposed model has to be validated in an independent study population; if validated, such a model could potentially enable pathology institutes to compare the performance of their testing methods with what would be expected based on patient, tumor, and sample characteristics.

The overall *BRAF* mutation rate for melanoma in this study was 37.5%. This was lower than the 43–62% mutation rates reported in other large studies [5, 12–16], but higher than that reported in a 2016 study in Taiwan (24.6%) [17], and within the broad range of 27–70% noted by Garnett and Marais [1]. The relatively high age of the patients included in our study—the median age of our study population was 68.0 years, compared with the lower median ages (range 51.9–66.0 years) reported in previous studies [5, 14–16]—may be one possible explanation for the low reported rate, given that *BRAF* mutation rate decreased significantly with age. Moreover, many of

Table 2 Documented *BRAF*^{V600} mutation status by individual covariates

	Samples tested, *n*	Mutation, *n* (%)	No mutation, *n* (%)
All samples	642	241 (37.5)	401 (62.5)
Patient age			
< 35 years	11	6 (54.6)	5 (45.5)
35–44 years	31	13 (41.9)	18 (58.1)
45–54 years	96	47 (49.0)	49 (51.0)
55–64 years	132	50 (37.9)	82 (62.1)
65–74 years	184	66 (35.9)	118 (64.1)
75–84 years	156	51 (32.7)	105 (67.3)
≥ 85 years	32	8 (25.0)	24 (75.0)
Origin of sample			
Primary tumor	127	46 (36.2)	81 (63.8)
Unknown	61	27 (44.3)	34 (55.7)
Metastasis (split by location)			
Liver	56	9 (16.1)	47 (83.9)
Lung	41	11 (26.8)	30 (73.2)
Gastrointestinal/urogenital	28	8 (28.6)	20 (71.4)
Lymph node	176	72 (40.9)	104 (59.1)
Cutaneous	89	39 (43.8)	50 (56.2)
Brain	18	9 (50.0)	9 (50.0)
Other	32	15 (46.9)	17 (53.1)
NA	14	5 (35.7)	9 (64.3)
Method of mutation analysis[a]			
Cobas® 4800 BRAF V600 Mutation Test	72	22 (30.6)	50 (69.4)
High-resolution melting assay	62	25 (40.3)	37 (59.7)
Next-generation sequencing	45	6 (13.3)	39 (86.7)
Pyrosequencing	154	57 (37.0)	97 (63.0)
Sanger sequencing	148	58 (39.2)	90 (60.8)
RT-PCR based assay	61	25 (41.0)	36 (59.0)
BRAF strip assay	63	30 (47.6)	33 (52.4)
Other assay	37	18 (48.6)	19 (51.4)
Quality of tissue[b]			
Very high	168	60 (35.7)	108 (64.3)
High	353	150 (42.5)	203 (57.5)
Low	16	3 (18.8)	13 (81.3)
NA	105	28 (26.7)	77 (73.3)
Quality of DNA			
Very high	181	67 (37.0)	114 (63.0)
High	387	148 (38.2)	239 (61.8)
Low	9	1 (11.1)	8 (88.9)
NA	65	25 (38.5)	40 (61.5)
Histological subtype			
Acral lentiginous melanoma	21	4 (19.0)	17 (81.0)
Lentigo maligna melanoma	4	1 (25.0)	3 (75.0)
Nodular melanoma	94	41 (43.6)	53 (56.4)
Superficial spreading melanoma	47	14 (29.8)	33 (70.2)
Other	71	23 (32.4)	48 (67.6)
NA	405	158 (39.0)	247 (61.0)

NA, not available; *PCR*, polymerase chain reaction; *RT-PCR*, reverse-transcription PCR

[a] RT-PCR-based assay, *BRAF* strip assay, and other PCR-based methods were included as a single combined variable for univariate analysis

[b] Quality of samples as self-reported by individual centers per internal criteria

the above-cited references reported on *BRAF* mutation rates in the USA, or high-incidence regions such as Australia. Comparisons between regions are difficult due to the underlying differences between populations and exposure to ultraviolet radiation.

Four covariates were initially identified as having a significant, or at least borderline significant, effect on the probability of *BRAF* mutation: age, origin of sample, quality of tissue, and *BRAF* mutation testing method. These covariates were used to generate the primary statistical model for predicting *BRAF* mutation rates. However, subsequent analyses showed that the significance of the influence of testing method on *BRAF* mutation probability was being driven by documented mutation rates from centers that showed major deviations from the centers' routinely reported mutation rates (rates for the samples in this study were substantially different from the routine rates reported at the respective centers). A reduced model was therefore developed that included only patient age, origin of sample, and quality of tissue. Potential reasons for the observed center effect are discussed below.

Patient age and origin of sample showed clear influences on the documented *BRAF* mutation rate in this study, with higher mutation rates in younger versus older patients, the lowest mutation rates observed in liver metastases and the highest rates in brain metastases. The association of younger age with *BRAF* mutations has been reported previously [5, 14, 15]. A prior study comparing the frequencies of *BRAF* mutations between metastatic sites reported similar distributions between sites (e.g., 48% *BRAF* mutation rate for brain metastases and 45% for liver metastases) [18]. However, an additional retrospective review reported that patients with *BRAF* mutations were more likely to have CNS involvement at the time of metastasis [5], in line with the higher frequency observed in our study. It should be noted that this study included samples from melanomas known to have low and high frequencies of *BRAF* mutation detection, e.g., uveal melanomas with very low frequency of *BRAF* mutations were included [19]. The quality of tissue showed borderline significance in terms of influence on *BRAF* mutation status in the primary model ($p = 0.075$), with poor-quality tissue samples associated with lower *BRAF* mutation rates. The influence of tissue quality became non-significant in the reduced model (deviating centers excluded). However, it may still be appropriate to consider reporting a negative *BRAF* mutation result only in cases where the quality of tissue is considered sufficient. In cases where the quality of tissue is poor, a repeated *BRAF* mutation analysis on an additional sample should be considered.

This study was limited by low overall sample size, low sample sizes at some centers, unbalanced distribution of methods used at the different centers, and high rates of missing values of some variables (including for melanoma subtype). Where samples numbers were low for a specific type of melanoma, these were pooled into an "other" group for analysis and not analyzed separately. Further limitations were potential variation in the assessment of tissue quality introduced by the self-reporting of tissue quality according to criteria used in

Table 3 Effect of the independent variables in each model on *BRAF* mutation rate: likelihood ratio chi-square test results of covariates and their relative importance in predicting the *BRAF* mutation rate in the primary model and the reduced model (excluding deviating centers)

	Primary model			Reduced model		
Variable	*p* value[a]	Main effect[b]	Total effect[b]	*p* value[a]	Main effect[b]	Total effect[b]
Age (years)	*< 0.001*	0.172	0.221	*0.002*	0.282	0.298
Origin of sample[c]	*0.005*	0.324	0.379	*0.035*	0.554	0.571
$BRAF^{V600}$ testing method	*0.008*	0.248	0.304	–	–	–
Quality of tissue	0.075	0.074	0.113	0.223	0.121	0.135

DF, degrees of freedom

[a] All *p* values are adjusted for the influence of all other independent variables

[b] The relative contribution of each factor alone is shown by the main effect; the total effect comprises the relative contribution of that factor alone and in combination with the other factors

[c] Origin of sample is divided into primary tumor, unknown origin or metastasis; metastasis was further split by location

Degrees of freedom were taken into consideration in statistical testing analyses as usual but were not considered in the estimation of the relative contribution of factors to the variation of predicted mutation probability, resulting in two different views of variable importance. Italics text denotes $p < 0.05$

each laboratory; an example of the criteria used at one participating center is provided in Online Resource 6.

Finally, there may have been potential patient and/or sample selection bias as consecutive samples were provided by some centers but not others. In particular, at centers recruiting patients into interventional clinical trials of BRAF inhibitors, it cannot be excluded that *BRAF* mutation–positive patients were preferentially enrolled into interventional trials, which may have led to a greater proportion of *BRAF* mutation–negative patients being included in the present study. The above mentioned limitations may explain, at least in part, the deviations observed at some centers, which were addressed in a second model excluding samples from centers judged to have the highest likelihood of bias according to the analysis of post

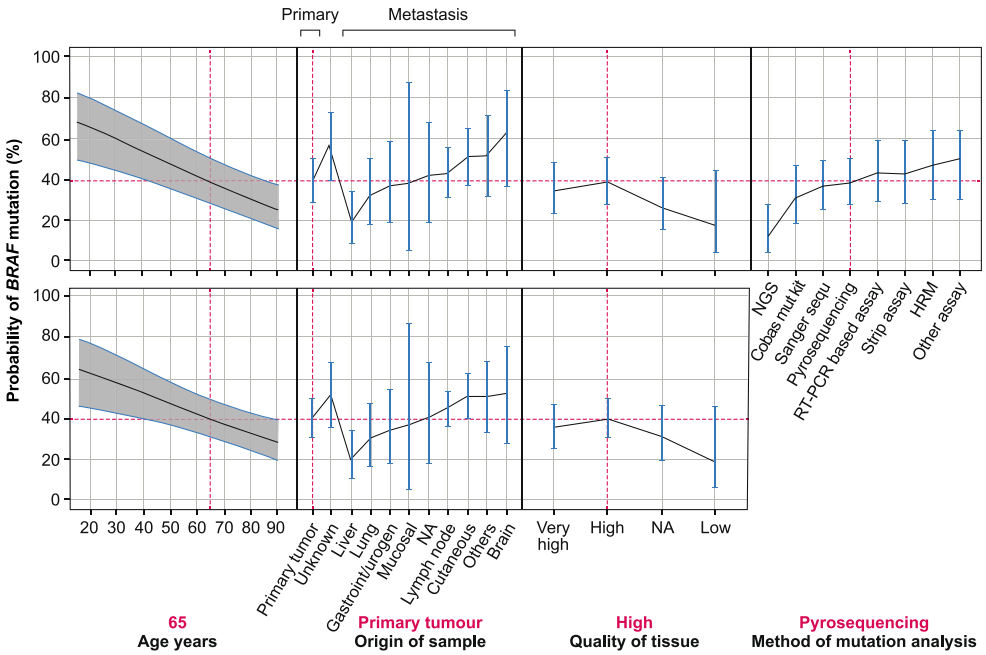

Fig. 2 Predicted probabilities of *BRAF* mutation according to the primary statistical model (top panel) and the reduced statistical model[a] (bottom panel). Data on the y-axis are predicted *BRAF* mutation probabilities. 95% confidence intervals are shown as gray-shaded areas (age) or error bars (origin of sample, method of mutation analysis, and quality of tissue). Age is displayed as a continuous variable. Origin of sample is divided into primary tumor, unknown, or metastasis; metastases are reported by location. The characteristics of the example patient are indicated as dashed red vertical lines. Each compartment shows the functional dependence of the probability of mutation from the levels of one covariate adjusted for the influence of all other covariates at the vertically dashed red lines. Cobas mut kit cobas® 4800 BRAF V600 Mutation Test (Roche Diagnostics GmbH, Mannheim, Germany); Gastroint/urogen, gastrointestinal/urogenital; HRM, high-resolution melting; NA, not available; NGS, next-generation sequencing; PCR, polymerase chain reaction; RT-PCR, reverse-transcription PCR; Sanger sequ, Sanger sequencing. [a]Reduced statistical model excluded all variables with $p > 0.2$ once deviating centers were excluded

hoc collected data. Some of the above limitations relate to the underlying aim of the study: to prepare a model based on a snapshot of the real-world testing practices in Germany at the time of the study, without imposing external criteria/processes. In cases such as the imbalance in testing methods, these limitations may potentially be addressed within a future validation study.

Conclusions

Valid analytical methods to identify *BRAF* mutations with high sensitivity and specificity are mandatory for accurate therapy selection. To our knowledge, this study is the first to establish a statistical model for predicting the $BRAF^{V600}$ mutation state in individual melanoma patients. The model is not intended to replace mutation testing by pathology institutes; instead, it is hoped that the model may one day be adapted for use as a reference tool to support standardization and quality control of testing methods, by enabling centers to compare the mutation rates they detect with the predicted rates typically associated with an individual's patient- and tumor-related characteristics. As with any statistical model, independent validation using a separate population is required before the model can become a tool for use in routine practice. The current work has highlighted the key points that need to be addressed within a future validation exercise, based on a more robust study design, and prospectively collected data are required to confirm the reproducibility and applicability of such a statistical model. However, even without further validation, our findings reflect an association between key variables, such as age, origin of sample, and *BRAF* mutation status, in line with current literature.

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Author contribution statement A Hartmann contributed to study design and study protocol development; A Hartmann, P Schirmacher, W Sterlacci, and C Garbe contributed to data acquisition; P Schirmacher, W Sterlacci, W Koch, DB Liesenfeld, and B Schif were involved in data analysis and A Hartmann, P Schirmacher, W Sterlacci, W Koch, DB Liesenfeld, B Schif, and C Garbe in data interpretation; W Koch, DB Liesenfeld, and B Schif were responsible for figure development. All authors were involved in writing the paper and had final approval of the submitted and published versions.

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Compliance with ethical standards

Conflict of interest A Hartmann has received research funding from Sysmex, BioNTech, Nanostring, and Novartis, and has received honoraria from Roche, AstraZeneca, BMS, and MSD, and payment for consultancy or advisory roles from Medoc, Roche, AstraZeneca, BMS, and MSD. P Schirmacher has received research funding from Roche, AstraZeneca, Novartis, Chugai, Thermo Fisher, and Sanofi Aventis, honoraria from Novartis, AstraZeneca, and Roche, and payment for consultancy or advisory roles from Roche, AstraZeneca, Novartis, BMS, MSD, Merck, and Amgen. W Sterlacci has received payment for consultancy or advisory roles from Roche. W Koch is an employee of BDS Koch, which receives payment for statistical services from Roche. B Schif and DB Liesenfeld are employed by Roche. C Garbe has received research funding from BMS, Novartis, and Roche, honoraria from Amgen, BMS, MSD, Novartis, and Roche, travel, accommodation, and other expenses from Amgen, BMS, MSD, Novartis, and Roche, and payment for consultancy or advisory roles from Amgen, BMS, MSD, Novartis, and Roche.

Ethical approval Ethics approval was obtained at the University of Erlangen-Nürnberg (approval no. 14_13 B dated Feb 19th 2013).

Informed consent Informed consent was not required as all data were collected as part of routine testing and were fully anonymized prior to analysis.

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