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BRAF^{V600E} Expression Is Homogenous and Associated with Nonrecurrent Disease and Better Survival in Primary Melanoma

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Keywords
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Abstract

Background: Superficial spreading melanomas (SSMs) are the most common type of melanoma and cause the majority of skin cancer deaths. More than 50% of cases harbor a mutation in the BRAF gene that activates the mitogen-activated protein kinase (MAPK) cancer signaling pathway. BRAF^{V600E} is the most common BRAF mutation, and it represents an important biomarker that guides treatment selection. However, the relationship between the *BRAF^{V600E}* gene expression and intratumoral protein distribution, on one side, and clinicopathological factors and patient outcomes, on the other, is not fully described. Additionally, whether MAPK cancer signaling activation in melanoma is due to increased biochemical activity of BRAF^{V600E}, increased mRNA levels, or both requires further investigation. Here, we addressed these questions by examining expression patterns of BRAF^{V600E} in primary treatment-naive melanomas and correlating them to clinicopathological factors and patient outcomes. ***Methods:*** In 166 SSM cases, we performed immunohistochemical staining to investigate the protein expression of BRAF^{V600E}, and we measured BRAF mRNA levels using NanoString nCounter system. ***Results:*** Ninety-seven (49%) melanomas stained positive for BRAF^{V600E}, with nearly 100% intratumoral homogeneity observed. Positive BRAF^{V600E} expression was significantly associated with nonrecurrent disease and was found to be an independent predictor of better prognosis in univariate and multivariable analyses. Furthermore, presence of tumor-infiltrating lymphocytes, sentinel lymph node biopsy negativity, and low Breslow thickness were all independent predictors of better prognosis. We observed no difference in the BRAF mRNA levels in BRAF^{V600E}-negative and BRAF^{V600E}-positive melanomas, respectively. Validation in a larger publicly available cohort confirmed that there is only a weak correlation (Spearman 0.4) between BRAF^{V600E} mRNA and protein levels and no differences in mRNA between BRAF^{V600E} mutated and non-mutated patients. ***Conclusion:*** Our findings indicated that BRAF^{V600E} is homogeneously present throughout the whole tumor and is associated with nonrecurrent disease and better survival in primary melanoma. We also showed that BRAF^{V600E} mutation does not result in higher transcriptional levels, suggesting that activation of the MAPK signaling pathway in BRAF^{V600E} mutated patients can be attributed to the increased biochemical activity caused by the mutation.

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Introduction

Cutaneous melanoma arises from the malignantly transformed melanocytes in the skin, and it is the leading cause of skin cancer-related mortality worldwide [1]. Genome-wide sequencing has identified a remarkable genetic complexity in melanoma, and based on the pattern of the most prevalent oncogenes, melanoma can be classified into four genomic subtypes: mutant BRAF, mutant RAS, and mutant NF1, which feature activated mitogen-activated protein kinase (MAPK) cancer signaling pathway, and triple wild-type (WT), which has no mutations in any of the three genes [2]. The presence of mutated BRAF has been documented in more than 50% of melanomas, although it is generally seen at the earlier stages of tumor evolution and considered insufficient to cause the invasive phenotype [3, 4]. Several studies have found an association between BRAF mutation and reduced survival, while other studies report no differences [5–11]. Oncogenic BRAF mutations are more frequently found in younger patients, in anatomical regions of the skin that are not subjected to chronic sun exposure, a risk factor for melanoma [12]. Furthermore, BRAF mutation-positive melanomas very frequently display a superficial spreading or nodular morphology [13]. BRAF inhibitors have emerged as an important class of targeted therapies for malignant melanomas. Unfortunately, patients undergoing treatment with BRAF inhibitors often develop resistance, especially when BRAF inhibitors are used as a monotherapy. Previously, intertumor and intratumor heterogeneity of BRAF mutations have been shown to give rise to different cellular proliferation rates and, more importantly, responses to treatment and resistance [14, 15]. This suggests that BRAF tumor heterogeneity may give rise to rapid emergence of drug resistance. However, a recent meta-analysis suggests that BRAF mutated tumors may display relatively low overall rates of heterogeneity [16].

Furthermore, BRAF mutations in melanoma and other cancers are usually detected on DNA level, but in one study, mRNA was used to synthesize cDNA that was sequenced to detect BRAF mutation [17, 18]. Thus, it is clear that the BRAF mutation is transcribed, but there is limited information about how the mutation affects the BRAF mRNA levels. In a small thyroid cancer study, the mRNA levels of $BRAF^{V600E}$ exhibited surprisingly large variability [19]. In addition to the mRNA level variability, there are reports of typically poor correlation between mRNA and protein levels, leading to the question of the biological translation of these findings in melanoma [20]. Although it is well established that BRAF mutation results in higher kinase activity, it is to our knowledge yet to be reported if BRAF mRNA level is affected in any way by the mutation.

Here, we provide insights into intratumor heterogeneity in primary treatment-naive melanomas by combining immunohistochemistry (IHC), which assesses $BRAF^{V600E}$ mutant protein levels, with mRNA analysis. Our results show that $BRAF^{V600E}$ is expressed homogenously throughout the individual tumors, and the mutation does not result in an increased transcription, measured by BRAF mRNA levels. Additionally, we observed that positive $BRAF^{V600E}$ expression was significantly associated with nonrecurrent disease and better prognosis.

Materials and Methods

Patient Samples and Criteria for Inclusion

A search in the Danish Pathology Register in Region Zealand in the period 1995 to 2015 using search SNOMED Clinical Terms for superficial spreading melanoma identified 1709 melanomas. After applying excluding criteria (Breslow thickness <1.0 mm or >4.0 mm, the presence of regression or ulceration), 166 cases were included in the final cohort (online suppl. Fig. S1; for all online suppl. material, see www.karger.com/doi/10.1159/000528159). We did not include thinner melanomas due to missing sentinel lymph node biopsy (SLNB) status in these patients. Melanomas thicker than 4.0 mm or with ulceration were excluded due to their known association with markedly poorer prognosis. As there is still some uncertainty regarding prognostic importance of histological regression, we excluded all cases with this phenomenon [21].

Clinical and histopathological data were retrieved and included (Table 1). From 1998, all patients in Denmark diagnosed with melanoma over 1 mm thickness had SLNB performed. All patients were staged at the time of diagnosis according to the latest edition of the American Joint Committee on Cancer (AJCC8) melanoma staging manual [22]. Treatment with BRAF/MEK inhibitors and immune checkpoint inhibitors (ICIs) was registered. In Denmark, treatment with CTLA4 inhibitors and PD1 inhibitors was available for advanced disease from 2011 to 2015, respectively. Treatment with BRAF and MEK inhibitors was approved for advanced disease in 2012 and 2013, respectively.

Electronic and paper medical charts were searched until death or until October 01, 2020. Melanoma-specific survival (MSS) and disease-free survival (DFS) were calculated from the date of receiving the specimen to the date of death or recurrence. Patients who did not die nor had recurrence were censored on the date of the last follow-up, and patients who died of other causes were censored at the time of death. The study was approved by the Regional Ethics Committee (SJ-742) and the Danish Data Protection Agency (REG-066-2019).

IHC Analyses of $BRAF^{V600E}$

Formalin-fixed paraffin-embedded (FFPE) sections (3 μm) were stained on the fully automated instrument Omnis (Dako, Carpinteria, CA, USA). Sections were deparaffinized, exposed to

Table 1. Clinicopathological data of 166 melanoma patients

Parameter	Number (%)	Parameter	Number (%)
Patient age at diagnosis, range (mean±SD)	21–84 (57.7±12.5)	BRAF VE1 stain	
		Positive	85 (51.2)
		Negative	81 (48.8)
Sex		BRAF VE1 intensity	
Male	86 (51.8)	Negative	81 (48.8)
		Weak	2 (1.2)
Female	80 (48.2)	Moderate	35 (21.2)
		Strong	48 (28.9)
Tumor site		Stage at diagnosis including SLNB	
Head	7 (4.2)	IA	1 (0.6)
Trunk	83 (50.0)	IB	103 (62.0)
Arm	35 (21.1)	IIA	24 (14.5)
Leg	41 (24.7)	III	38 (22.9)
Age FFPE blocks, range (mean±SD)	8–21 (13.3±3.45)	SLNB status	
		Positive	39 (23.5)
		Negative	127 (76.5)
Breslow thickness, range (mean±SD)	1–4 (1.70±0.65)	Recurrent disease	
		Recurrence	40 (24.1)
		Non-recurrence	126 (75.9)
Clark level			
2	5 (3.0)	Recurrence within 2 years	17 (10)
3	89 (53.6)	Recurrence between 2 and 4 years	10 (6)
4	70 (42.2)	Recurrence after 4 years	13 (7.8)
5	2 (1.2)		
TILs		Cause of death	
Brisk	47 (28.3)	Alive	114 (68.7)
Non-brisk	73 (44.0)	Melanoma-specific death	30 (18.1)
Absent	46 (27.7)	Death of other causes	22 (13.3)
Concurrent cancers (after MM diagnosis)	55 (33.1)	Treatment at recurrence	40 (24.1)
Non-melanoma skin cancer		BRAF/MEK inhib	2 (5.0)
Squamous cell carcinoma	2 (3.6)	BRAF/MEK inhib + ICIs	1 (2.5)
Basal cell carcinoma	14 (25.5)	ICIs	3 (7.5)
Melanocyte derived dysplasia/cancer		Interleukin/interferon	7 (17.5)
Lentigo/dysplastic nevus/MM insitu	4 (7.3)	ICIs + interleukin/interferon	1(2.5)
Later detected primary melanoma<1 mm and negative SLNB	12 (21.8)	Only surgical treatment	3 (7.5)
Other cancers		Other (regional chemo)	4 (10.0)
Carcinoma	19 (34.5)	None b/c of contraindications	3 (7.5)
Hematological cancer	3 (5.5)	None b/c of rapid death	4 (10.0)
Other	1 (1.8)	Pt. refused treatment	2 (5.0)
		Unknown	10 (25.0)
BRAF mutational test at recurrence		12	
PCR BRAF positive		6 (50)	
PCR BRAF positive		6 (50)	
Concordance with VE1		100%	

SLNB, sentinel lymph node biopsy; inhib, inhibitor; ICI, immune checkpoint inhibitor. [a] All patients that received ICIs had negative $BRAF^{V600E}$ status.

antigen retrieval using EnVision FLEX Target Retrieval Solution (3-in-1) pH 9 (cat # GV834; Dako), and heated for 48 minutes at 97°C. Slides were incubated with anti-$BRAF^{V600E}$ clone VE1 (cat # ab228461; Abcam plc, Cambridge, UK) for 30 min at 32°C and diluted at 1:1500 for brown staining or 1:3000 for red staining (2 heavily pigmented cases). Antibody was diluted in Renoir Red (cat # PD904L; Biocare Medical, Pacheco, CA, USA). After washing and blocking of endogenous peroxidase activity, the reactions were visualized using the Envision FLEX + High pH kit (cat # GV800 + GV809/GV821; Dako) with 3,3′-diaminobenzidine as a chromogen for brown color and the Envision Magenta Substrate Chromogen System (cat # GV900; Dako) for red color, according to the manufacturer's instructions. Finally, slides were rinsed in water and counterstained with Mayer's hematoxylin.

The presence of mutated $BRAF^{V600E}$ protein was characterized by cytoplasmic staining of the melanoma cells. General faint diffuse staining, weak staining of single interspersed cells, and only nuclear dot staining were scored as negative. The staining intensity was recorded on a semi-quantitative scale, with 0 = negative, 1 = weakly positive, 2 = moderately positive, and 3 = strongly positive. The proportion of stained tumor cells was scored as 0 (0%), 1 (1–25%), 2 (26–50%), 3 (51–75%), or 4 (76–100%). To assess interobserver variability, all cases were assessed by two pathologists (LMGR and SONA), who were blinded to the patients' clinicopathological and mutation data (BRAF PCR test done at recurrence). In cases with disagreeing scores, the slides were viewed by both observers together, and any discrepancies were resolved by consensus.

Gene Expression Studies Using NanoString nCounter Platform

Total RNA was isolated from five 10-µm sections of FFPE tissue using Roche Hi-Pure FFPET Kit (Roche Life Science, Mannheim, Germany) according to manufacturer's protocol. NanoDrop™ Lite spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA) was used to measure total RNA quality and quantity. From each sample, 100–200 ng of RNA was used for hybridization using the nCounter PanCancer IO 360 Gene Expression Panel, according to the manufacturer's nCounter XT protocol (NanoString Technologies, Seattle, WA, USA). The panel includes 770 genes, providing a 360-degree view of gene expression of tumor, microenvironment, and immune response. In addition, 20 genes of interest, including BRAF, were added to the panel (online suppl. Table S1). Samples were processed on nCounter sample preparation station and nCounter Digital Analyzer as previously described [23].

Statistical Analyses

Statistical analysis was performed using SPSS version 24 software (IBM Corporation, Armonk, NY, USA) and GraphPad Prism (version 8.1.1, GraphPad Software, San Diego, CA, USA). Baseline characteristics were described using means, standard deviation (SD) with ranges for the continuous variables, and numbers with percentage frequencies for the categorical variables. To evaluate the association between two variables, Pearson's χ^2 test (χ^2), Fisher's exact test, or Mann-Whitney U test were used as appropriate. MFS and DFS curves were calculated according to the Kaplan-Meier method and compared using the log-rank test. For univariate and multivariate recurrence and survival analyses, univariate and multivariate Cox proportional hazards regression models were used. A significance level of 5% ($\alpha = 0.05$) was considered statistically significant.

TCGA Dataset

Skin Cutaneous Melanoma (TCGA Firehose Legacy) dataset at the cBioPortal site was used for validation [24, 25]. This cohort is based upon data generated by the TCGA Research Network: https://www.cancer.gov/tcga. The dataset consisted of 471 patients with several available molecular profiles: methylation (HM450), mRNA expression (RNA Seq V2 RSEM), mRNA expression z-scores relative to all samples (log RNA Seq V2 RSEM), mutations, protein expression z-scores (reverse-phase protein array [RPPA]), and protein expression (RPPA). Metadata included ethnicity, sex, Breslow thickness, tumor site, Clark level, adjuvant postoperative pharmaceutical therapy administered, and staging according to the American Joint Committee on Cancer (AJCC). The Onco Query Language tool was used to query for BRAF mutation and subsequently comparison/survival and plot features were used to investigate DFS and overall survival (OS) and to correlate mRNA and protein data. Group comparison feature was used to create custom groups and compare their clinical and genomic features.

Results

Patient Cohort Characteristics

Samples from 166 patients diagnosed with melanoma in Region Zealand between 1995 and 2015 were included in the study. Clinicopathological data of all patients are shown in Table 1. Distribution of male and female patients was almost equal, with 80 (48.2%) women and 86 (51.8%) men. The mean age was 57.7 years (range 21–84 years). The primary tumor site was predominantly on the trunk ($n = 83$, 50.0%) or leg ($n = 41$, 24.7%). The majority of patients had localized disease (N0 and M0), but 22.9% had at least one lymph node metastasis (N1-N3), and none had distance metastasis at the time of diagnosis. Information on patient survival, time, and cause of death was available in all cases. The last date of follow-up was December 1, 2020, and median follow-up time for survivors was 121 months (range 0–255 months). During the follow-up time, 40 (24.1%) patients experienced recurrence and 30 (18.1%) patients died of melanoma. Of the 40 patients with recurrence, 22.2% experienced recurrence within 1 year and 42.5 % within 2 years of diagnosis, and only 17.5 % experienced recurrent disease after 5 years (online suppl. Fig. S2). All treatment modalities were recorded for patients with recurrence, except in 10 patients (25%), due to destroyed medical charts. At recurrence, only 12 out of 40 patients had performed BRAF mutational status by PCR test, of which 6 was positive. Only 3 of these 6 patients received treatment with BRAF/MEK inhibitors alone or combined with ICIs. Of the 3 other patients, one received treatment with temozolomide (alkylating agent), one received treatment with interleukin, and one did not receive treatment because of poor physical health. In total, 3 patients received ICI monotherapy, and all of them had negative $BRAF^{V600E}$ expression.

Protein Expression of $BRAF^{V600E}$ in Melanoma Patient Samples

Staining with $BRAF^{V600E}$ antibody was assessed in all 166 cases, with 85 (51.2%) being positive and 81 (48.8%) being negative. The staining was cytoplasmic, and the staining intensity was assessed in all positive cases, with

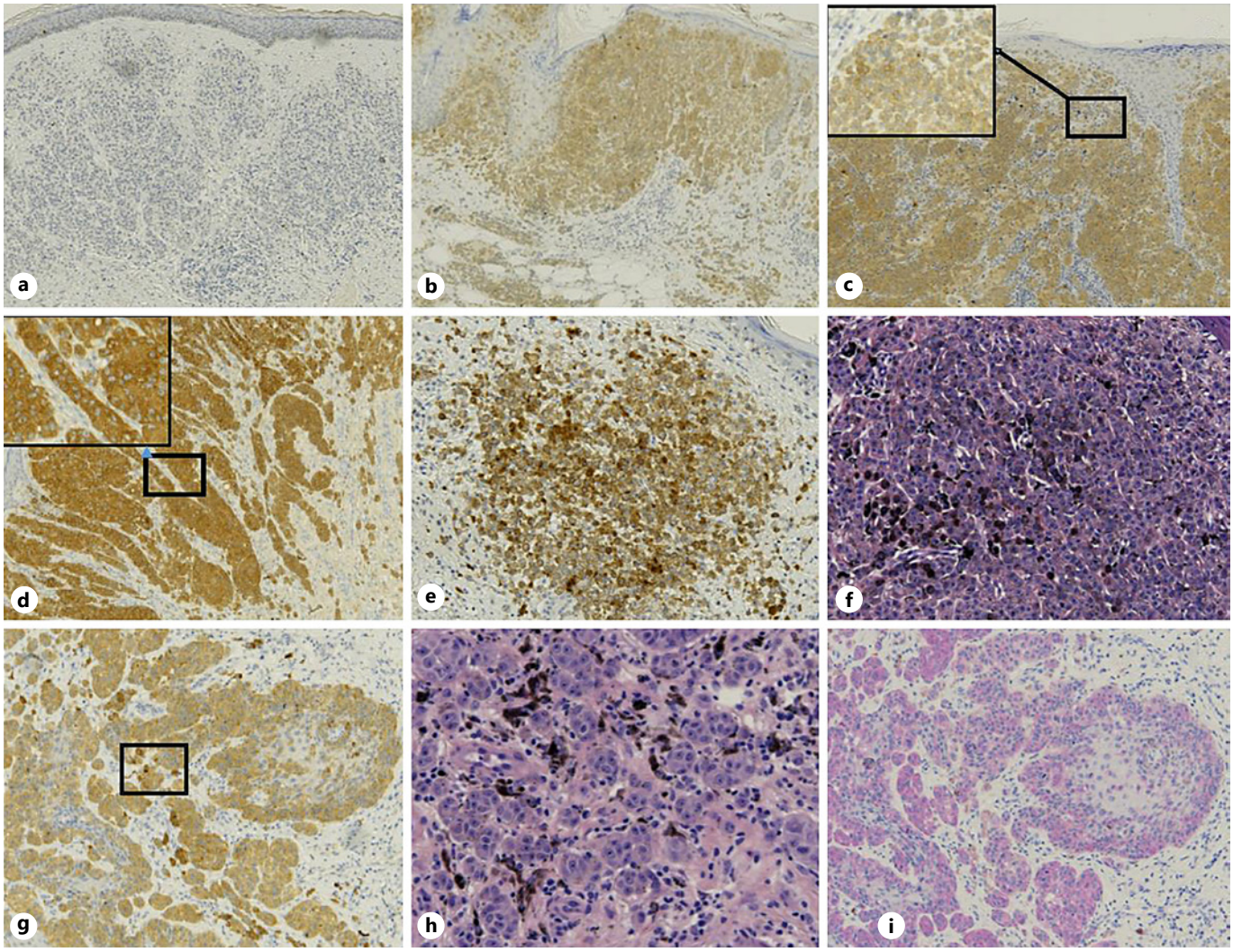


Fig. 1. IHC staining for $BRAF^{V600E}$. Skin melanoma with negative (**a**), weak (**b**), moderate (**c**), and strong (**d**) $BRAF^{V600E}$ expression with magnification for all ×10. High-powered view (×25) of the rectangular area in (**c**) and (**d**) shows approximately 100% homogeneous $BRAF^{V600E}$ expression across tumor cells. **e** Melanoma with abundant melanin deposition complicating evaluation of $BRAF^{V600E}$ expression. **f** HE stain of the same area as in **e** confirms that the brown color observed in VE1 stain was solely representing melanin and the melanoma was considered "negative "for $BRAF^{V600E}$. **g** Melanoma with weak VE1 staining, melanin deposition in macrophages, and some background staining. **h** HE stain with high-powered view (×25) of the rectangular area in **g** showing abundant pigment-laden macrophages, still representing uncertainty. **i** VE1 stain with magenta of the same area as in **g** reveals a convincing $BRAF^{V600E}$ staining of the tumor cells, thus melanoma is regarded as "moderate positive."

only 2 (2.4% of positives) cases staining weakly, 35 (41.1%) cases staining moderately, and 48 (56.5%) cases staining strongly (Fig. 1b–d). In pigmented cases, it was difficult to differentiate the positive signals of IHC from melanin deposition since the 3,3′-diaminobenzidine and melanin have a similar brown color, causing possible misjudgment of IHC results. In these cases, we compared the VE1 staining with already available hematoxylin and eosin stain (HE) which in most cases enabled us to clearly distinguish VE1 staining from melanin (Fig. 1g, h). In three challenging cases, we used a red chromogen (magenta) that easily discriminated VE1 stain from the brown pigment (Fig. 1i). The VE1 staining was clearly present in tumor cells from both the radial growth phase and vertical growth phase (Fig. 1g). Difference in staining intensity between tumor areas was rarely observed and linked to the presence of necrosis, fixation artifacts, or difference in morphology.

Table 2. Clinicopathological factors associated with $BRAF^{V600E}$ protein positivity

Parameters	$BRAF^{V600E}$		*p* value
	positive	negative	
Sex			
Male	51	35	$p = 0.005^{a,*}$
Female	30	50	
TILS			
Non-brisk	37	36	$p = 0.799^{a}$
Brisk	21	26	
Absent	23	23	
Recurrent disease	28	12	$p = 0.002^{a*}$
Nonrecurrent disease	53	73	
SLNB positive	17	22	$p = 0.457^{a}$
SLNB negative	64	63	
Stage at diagnosis incl. SLNB			
IA	1	0	$p = 0.551^{a}$
IB	53	50	
IIA	11	13	
III	16	22	
Tumor site			
Head	5	2	$p = 0.124^{bd}$
Arm	22	13	
Trunk	35	48	
Leg	19	22	
Age at diagnosis, years (mean ± SD)	60.73±11.38	54.74±12.96	$p = 0.003^{c,*}$
Breslow thickness, mm (mean ± SD)	1.68±0.64	1.69±0.66	$p = 0.822^{c}$
Medical treatment at recurrence			
Received	13	5	$p = 0.781^{a}$
Not received	15	7	

TILs, tumor infiltrating lymphocytes; SLNB, sentinel lymph node biopsy. [a] Pearson's χ^2 test. [b] Fisher's exact test. [c] Mann-Whitney U test. [d] Greater than 20% of the cells have expected count less than 5. * Significant values.

Next, we analyzed the proportion of stained tumor cells present in each VE1 positive case to examine intertumoral heterogeneity of $BRAF^{V600E}$ distribution. All samples were scored as 4 (76–100% of cells positive), and no notable intratumoral heterogeneity was observed. At recurrence, 12 patients had known BRAF mutational status, and correlating these findings to our VE1 staining results, we found 100% concordance (Table 1). Taken together, these results indicate that $BRAF^{V600E}$ molecules are homogeneously distributed within $BRAF^{V600E}$-positive melanomas.

Association between $BRAF^{V600E}$ Expression and Clinicopathological Factors

To examine potential association between $BRAF^{V600E}$ expression and routine clinicopathological factors, we combined results of our protein expression analysis with the patient-cohort data (results of the analysis shown in Table 2). Among the factors we examined, including the presence of tumor-infiltrating lymphocytes (TILs), SLNB status, stage at diagnosis, primary tumor site, and Breslow thickness, only female sex ($p = 0.0059$), younger age at diagnosis ($p = 0.003$), and nonrecurrent disease ($p = 0.002$) were significantly associated with $BRAF^{V600E}$ expression. Surprisingly, we observed lower risk of recurrence in $BRAF^{V600E}$-positive cases, and patients with $BRAF^{V600E}$-positive melanoma had significantly longer MSS than those with $BRAF^{V600E}$-negative melanoma ($p = 0.0041$, 5-year MSS 92 % and 82 %, respectively) (Fig. 2a). In addition, patients with $BRAF^{V600E}$-positive melanoma had longer DFS ($p = 0.0021$, 5-year DFS 89 % and 69%, respectively) (Fig. 2b). Subgroup analysis comparing patients with moderate staining intensity with strong intensity showed no significant difference in MSS or DFS (online suppl. Fig. S3A, B). In addition, we investigated if patients with $BRAF^{V600E}$ expression had received medical treatment at higher frequency and if this could account for their better prognosis compared to

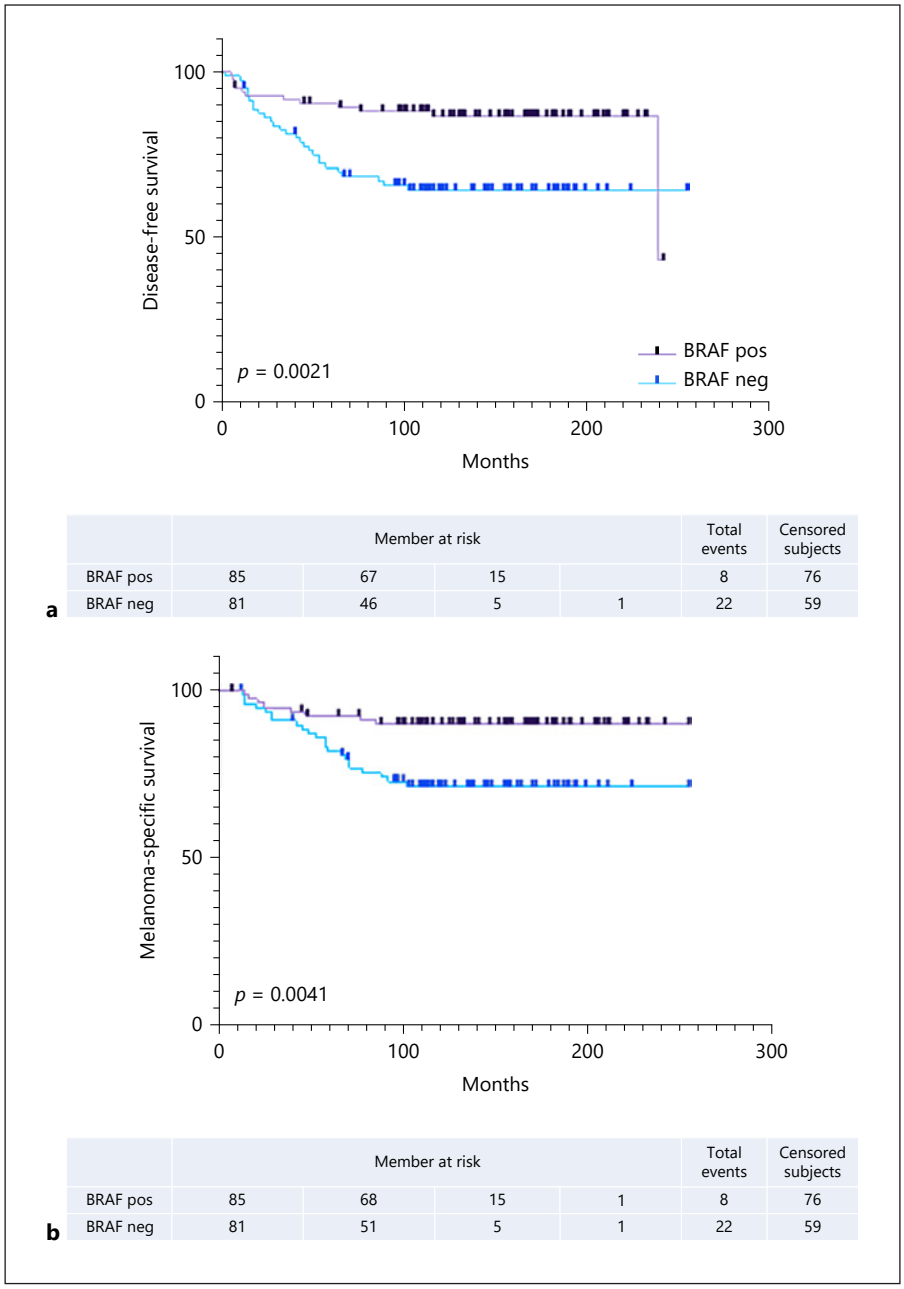


Fig. 2. a, **b** Kaplan-Meier curves for MSS and DFS according to $BRAF^{V600E}$ expression (log-rank test).

$BRAF^{V600E}$-negative patients. No difference was found, with 5 out of 12 patients (41.6%) with positive VE1 stain and 13 out of 28 patients (46.4%) with negative VE1 stain receiving medical treatment ($p = 0.781$) (Table 2). Only 3 patients received ICI monotherapy, and all of them had negative $BRAF^{V600E}$ staining (Table 1). As ICIs have promising clinical benefit, the advantage of the targeted therapy that was given to the 3 patients with BRAF mutation was reduced. To validate our results, we investigated the association between $BRAF^{V600E}$ mutational status at DNA level and prognosis in a larger cohort consisting of 471 cutaneous melanoma samples (TCGA Firehose Legacy) at cBioPortal. In line with our results, patients with a $BRAF^{V600E}$ mutation had a significantly better OS ($p = 0.00334$) and DFS ($p = 0.0232$) when compared to patients with no $BRAF^{V600E}$ mutation (online suppl. Fig. S4A, B). In the TCGA dataset, 18.3% (67/366) of the total patients with available mutation data received non-specified adjuvant postoperative pharmaceutical therapy. In comparison, 10.8% (18/166) of patients in the cur-

Table 3. Cox proportional hazards regression models of variables associated with MSS

Variable	HR	Univariate 95% CI	*p* value	HR	Multivariate 95% CI	*p* value
Patient age	1.022	0.991–1.053	0.160			
Female sex	0.473	0.221–1.011	0.053			
TILs present	0.439	0.213–0.903	0.025*	0.360	0.146–0.887	0.026*
TILs non-brisk[a]	0.402	0.172–0.940	0.035*			
TILs brisk[a]	0.587	0.243–1.416	0.236			
BRAF VE1 positive	0.325	0.145–0.730	0.006*	0.280	0.123–0.638	
BRAF VE1 intensity						
Weak[b] (2 patients)	0.000094	0000–0000	0.979			
Moderate[b]	0.390	0.134–1.131	0.083			
Strong[b]	0.290	0.1–0.841	0.023*			
SLNB positive	3.238	1.579–6.637	0.001*	3.681	1.769–7.659	<0.001*
Breslow thickness	1.780	1.112–2.849	0.016 *	1.588	0.968–2.604	

CI, confidence interval; HR, hazards ratio; TILs, tumor infiltrating lymphocytes; SLNB, sentinel node lymph node biopsy. [a] TILs absent as reference category [b] BRAFVE1 negative as reference category * Significant values

Table 4. Cox proportional hazards regression models of variables associated with disease free survival

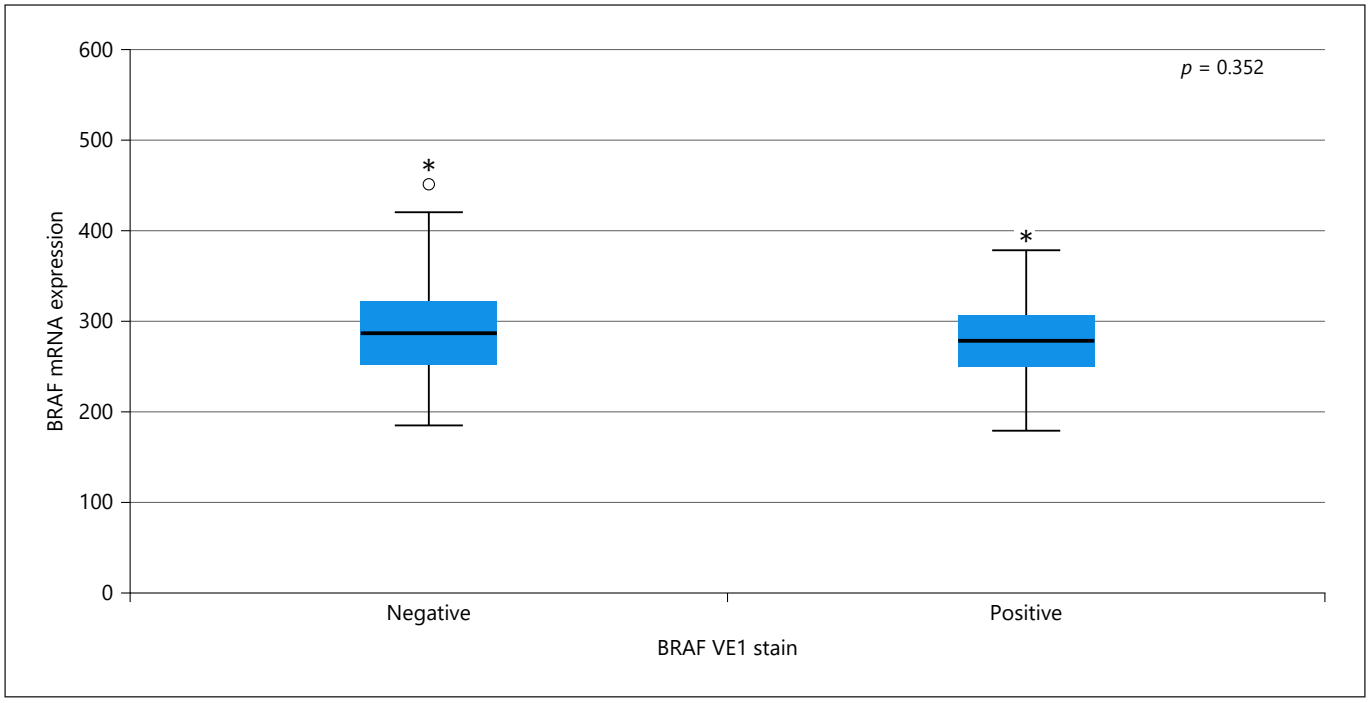
Variable	HR	Univariate 95% CI	*p* value	HR	Multivariate 95% CI	*p* value
Patient age	1.026	0.999–1.054	0.057			
Female sex	0.466	0.239–0.908	0.025*	0.577	0.293–1.134	0.110
TILs present	0.644	0.366–1.233	0.184			
TILs non-brisk[a]	0.595	0.297–1.190	0.142			
TILs brisk[a]	0.460	0.197–1.076	0.073			
BRAFVE1	0.361	0.183–0.712	0.003*	0.375	0.189–0.747	0.005*
SLNB positive	2.844	1.517–5.332	0.001*	2,875	1,521–5.435	0.001*
Breslow thickness	1.846	1.846–2.744	0.002*	1.845	1,219–2.794	0.004*

CI, confidence interval; HR, hazards ratio; SLNB, sentinel lymph node biopsy. [a] TILs absent as reference category * Significant values

rent cohort received adjuvant pharmaceutical therapy. Furthermore, in the TCGA dataset, we observed that a higher percentage of patients with a BRAF mutation received adjuvant postoperative pharmaceutical therapy compared to the patients with no BRAF mutation. Difference in treatment frequency could possibly explain the better OS in the dataset. There was, however, no difference in OS and DFS between the patients that received adjuvant therapy compared to those that did not (online suppl. Fig. S5A, B). Taken together, we observed no significant association between $BRAF^{V600E}$ and the presence of TILs, SLNB status, stage at diagnosis, primary tumor site, and Breslow thickness. However, significant association was observed between the presence of $BRAF^{V600E}$ and female sex, younger age at diagnosis, and nonrecurrent disease. Furthermore, $BRAF^{V600E}$-positive patients exhibited better OS.

Cox Univariate and Multivariate Analysis for Patient Survival

Having noted that mutant $BRAF^{V600E}$ protein expression is associated with MSS and DFS, we wanted to examine if it was an independent prognostic factor for MSS and DFS (Tables 3, 4). The presence of TILs, $BRAF^{V600E}$ positivity, SLNB negativity, and low Breslow thickness were significantly associated with better survival in univariate analysis of MSS, and all variables except Breslow thickness remained significant in final multivariable analysis. In the univariate analysis, we also included TILs and VE1 staining intensity as categories and observed significant asso-



Color version available online

Fig. 3. Box plot of BRAF mRNA levels across samples with positive and negative expression of $BRAF^{V600E}$, Mann-Whitney U test, $p = 0.352$.

ciation only when comparing non-brisk TILs with total absence of TILs and when comparing strong VE1 staining intensity with no staining. $BRAF^{V600E}$ positivity, SLNB negativity, and low Breslow thickness were significantly associated with improved DSF in univariate analysis and remained significant and independent predictors of better prognosis in the multivariable analysis. Therefore, $BRAF^{V600E}$ status represents an independent predictor of patient's survival.

$BRAF^{V600E}$ Protein Expression and Correlation to mRNA Levels

We next examined whether $BRAF^{V600E}$-positive and -negative tumors exhibit significantly different BRAF mRNA levels. As shown in Figure 3, no difference between mRNA levels of the two groups was observed ($p = 0.352$). We next correlated the mRNA levels to $BRAF^{V600E}$ protein expression intensity, finding no significant changes (online suppl. Table S2). We compared our results to those of a larger melanoma cohort (TCGA Firehose Legacy), comparing the mRNA levels across samples with and without $BRAF^{V600E}$ mutation. We could not identify substantial changes between these two groups, $p = 0.052$ (online suppl. Fig. S6A). A weak correlation was found comparing the protein levels of BRAF measured by RPPA to mRNA levels in the same cohort (Spearman correlation 0.38, $p = 9.83e{-}14$) (online suppl. Fig. S6B).

Taken together, these analyses suggest that melanoma tumors express similar levels of BRAF mRNA, regardless of the mutational status. Therefore, any changes in MAPK signaling pathway activity are likely due to well-documented increased enzymatic activity of $BRAF^{V600E}$ protein and not increased mRNA levels.

Discussion

Here we describe results of a regional population-based study aimed at characterizing the interpatient intratumor heterogeneity with respect to the most frequent BRAF mutation (V600E) in melanoma. We employed IHC staining and correlated the results to clinicopathological factors, prognosis, and BRAF mRNA levels. We observed that $BRAF^{V600E}$ displayed a 100% homogenous staining pattern throughout the tumor when present and that these patients did not have higher corresponding mRNA levels when compared to those with no $BRAF^{V600E}$ expression. Furthermore, the presence of $BRAF^{V600E}$ predicted an improved MSS and DFS after adjustments for other prognostic fac-

tors. Our results contribute new knowledge to melanoma biology and may have important implications for melanoma treatment and management, as we discuss below.

It is well known that melanomas harbor heterogeneous molecular profiles, and BRAF intratumor heterogeneity has been suggested as one of the several causes of drug resistance [26]. However, numerous BRAF testing methods, including PCR DNA based tests, next-generation sequencing, and qPCR, do not create clear in situ visualization of tumor heterogeneity. Therefore, we chose to employ IHC as it provides a very fast indication of mutational status, is less expensive, and is readily available in most pathology departments. Furthermore, we chose to focus on visualizing $BRAF^{V600E}$ because (1) this mutation is the most frequently reported mutation in melanomas and (2) a mutation-specific, highly specific (1.00) and highly sensitive (0.96) antibody (VE1) is readily available [27, 28], thus ensuring highly reliable IHC analysis. In this study, we used VE1 antibody to characterize the $BRAF^{V600E}$ expression in 166 patients with primary superficial spreading melanomas. The staining was positive in just over half (51.2%) of the cases, in accordance with other reports [2]. We found a homogenous $BRAF^{V600E}$ expression in all cases, similar to some previous studies [29, 30] and contrary to other studies [31, 32]. It is known that $BRAF^{V600E}$ arises early during melanoma development, and this could help to explain the homogenous immunolabelling of the majority of tumor cells.

In addition to the high level of quality control that went into this work, the strength of the current study is a large and well-characterized patient cohort. This allowed us to perform in-depth analysis of association between $BRAF^{V600E}$ positivity and clinicopathological factors. We observed no association between $BRAF^{V600E}$ status and important clinicopathological parameters that could potentially effect survival, such as presence of TILs, SLNB status, stage at diagnosis, primary tumor site, and Breslow thickness. We did observe a higher frequency of positive $BRAF^{V600E}$ staining in women, although previous reports have shown no gender differences [9, 33]. Biological differences (e.g., hormonal) or different behaviors between men and women (e.g., level of sun exposure) could explain our results. Additionally, we also noted higher frequency in younger patients in line with other studies [12, 13].

Generally, patients with BRAF mutated tumors have been reported to have a higher risk of recurrence [34, 35], but surprisingly, we report a lower risk of recurrence in patients with $BRAF^{V600E}$ expression. Our survival analysis also supported the positive relationship between $BRAF^{V600E}$ positivity and MSS and DFS. Overall, our study indicates that $BRAF^{V600E}$ positivity, SLNB negativity, and low Breslow thickness represent independent predictors for better survival considering both MSS and DFS. Furthermore, we report that the presence of TILs is an independent predictor for longer MSS. Our results are consistent with a large body of research concluding that TILs can predict melanoma survival and identify patients at risk for metastatic recurrence using both IHC and molecular analyses [36, 37]. However, in our cohort, TILs were not associated with DFS, consistent with the results from a larger meta-analysis that failed to show a prognostic effect of TILs of any grade on DFS [38].

Nevertheless, given that the current literature report contradictory results on this point, including a meta-analysis assessing 52 studies (7,519 patients) revealing poorer prognosis and OS for patients with mutant BRAF versus WT patients and several studies that reported no difference in survival between the different mutation profiles [39–43], at this point, we argue that the question regarding correlation of BRAF mutational status and clinical behavior remains open.

Of note, in the meta-analysis by Ny et al. [39], studies with lower stage melanomas either reported no difference in OS or better but not statistically significant OS in BRAF mutated melanomas. The majority of included studies, where BRAF mutation was associated with poor survival, involved patient samples from stage III and IV melanomas. However, the meta-analysis did not adjust for Breslow thickness, the presence of TILs, or ulceration status – factors that are known to affect prognosis in early-stage melanoma [44]. For advanced melanoma, on the other hand, the survival is associated with factors such as the number of positive regional lymph nodes and site of metastases [22]. In our cohort, 77.1% (128/166) of the patients had stage I-II disease at time of diagnosis. Our prediction of the clinical impact of BRAF mutation is based on a multivariate survival analyses, thereby adjusting for Breslow thickness and presence of TILs.

Another challenge in interpreting the previous results from Ny et al. [39] is that there is no consensus in regards to WT melanomas. In the aforementioned meta-analysis, WT was defined as dual BRAF/NRAS WT in 19/52 studies and, in the remaining 33 studies, WT included solely BRAF WT tumors. According to the TCGA research network, WT is defined as triple BRAF/NRAS/

NF1 WT, as all of them exert their effect in the MAPK pathway [2]. Furthermore, differences in the level of BRAF subtyping also represent limitations, as some of the studies measure all the known BRAF mutations and while others map the most prevalent, complicating direct comparisons between studies.

A potential limitation to the current study could also have been different treatment regimens depending on mutational status. We therefore investigated if patients with positive $BRAF^{V600E}$ had the advantage of better treatment at recurrence and found no differences compared to patients with negative $BRAF^{V600E}$. We also validated our results in online melanoma cohort with known $BRAF^{V600E}$ DNA mutational status, showing similar results. The validation cohort is larger than our cohort, broadly inclusive and comprises extensive clinical and molecular data. In the TCGA dataset, patients with a BRAF mutation received adjuvant therapy at a higher frequency, but the adjuvant therapy did not seem to affect OS and DFS. Another issue with interpreting prognosis from various studies is the different populations, patient selection, and percentage of histologic subtypes, genetic variation, level of UV exposure, skin pigmentation, and other unknown factors effecting the progression of melanoma [45]. Therefore, going forward, studies correlating BRAF mutational status to survival in large cohorts including different regions in Denmark or countries in Europe should be conducted to clarify whether correlations identified by us and others are influenced by the variance of different populations.

High sensitivity and specificity of VE1 allowed us to bypass DNA testing and to, for the first time, correlate $BRAF^{V600E}$ results to mRNA levels. Positive VE1 staining in our cohort, which is representative of $BRAF^{V600E}$ mutation, was not associated with higher mRNA levels. Moreover, subsequent analysis in a larger online melanoma cohort also showed lack of association between mRNA levels and $BRAF^{V600E}$ mutation. Many factors are involved in regulation of mRNA levels and protein levels, such as translation and stability of mRNA and protein half-life. In addition, the majority of BRAF mutations constitute missense mutations that change the amino acid sequences of the protein and not necessarily result in a higher mRNA transcription and protein levels. Although BRAF mRNA may have no current clinical application value, it gives important knowledge about biology and may be useful for researchers studying gene expression in melanoma by targeted gene expression profiling, RNA seq., etc. Our results indicate that upregulation of MAPK kinase signaling pathway in $BRAF^{V600E}$ melanoma is not due to increased levels of $BRAF^{V600E}$ mRNA but to increased enzymatic activity of the mutant BRAF, which is known to be 500-fold more active than the WT in vitro [46]. This theory is also supported by the fact that no association was found when correlating mRNA to $BRAF^{V600E}$ protein expression intensity in our cohort.

In conclusion, we have shown that $BRAF^{V600E}$ is expressed homogenously throughout the individual SMM tumors and the mutation does not result in an increased transcription, as measured by mRNA levels. We also identified associations between several important clinicopathological factors and $BRAF^{V600E}$ positivity, including a surprising positive association between $BRAF^{V600E}$ levels and MSS and DFS. The common view that BRAF mutation promotes poor prognosis is based on studies of advanced stage melanomas. Surprisingly, in our cohort of primarily low stage melanomas, we found a better prognosis in patients with $BRAF^{V600E}$ mutation. By controlling for other factors known to affect survival, including Breslow thickness and presence of TILs, we strengthen the power of $BRAF^{V600E}$ as an independent predictor for better survival. However, gene expression studies in melanoma have also indicated that additional factors act in concert with the BRAF mutation to produce a prognostic phenotype [47, 48]. These observations are in accordance with the fact that the progression of melanoma is a complex process, involving several interconnected pathways and multiple oncogenes [3]. Although BRAF is arguably an important driver of tumor growth, underlined by the efficacy of targeted treatments, there is still a need for a better understanding of the biological translation of these mutations in melanoma and clinical biomarkers to predict and monitor disease progression and treatment efficacy.

Key Message

$BRAF^{V600E}$ expression in melanoma predicts better prognosis.

Statement of Ethics

The study protocol was reviewed and approved by the data protection agency in Region Zealand, Denmark, approval number (REG-066-2019) and by the Ethics Committee in Region Zealand, Denmark, approval number (SJ-742). The approval from the Ethics Committee in Region Zealand, also included exemption from informed consent.

Conflict of Interest Statement

Lise Mette Rahbek Gjerdrum has received funding from NanoString Technologies. The remaining authors state no conflict of interest.

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Author Contributions

Lise Mette Rahbek Gjerdrum and Beatrice Dyring-Andersen designed the research; Soraya Naimy, Michael Bzorek, and Jens Ole Eriksen performed experiments; Soraya Naimy, Lise Mette Rahbek Gjerdrum, Beatrice Dyring-Andersen, and Jens Ole Eriksen analyzed the data; Soraya Naimy, Lise Mette Rahbek Gjerdrum, Beatrice Dyring-Andersen, and Michael Bzorek wrote the paper. All authors read and approved the manuscript.

Data Availability Statement

Dataset related to this article have been deposited in the Gene Expression Omnibus (GEO) with accession number GSE193802 and were made publicly available on August 31, 2022. Further inquiries can be directed to the corresponding author.

1 Shain AH, Joseph NM, Yu R, Benhamida J, Liu S, Prow T, et al. Genomic and transcriptomic analysis reveals incremental disruption of key signaling pathways during melanoma evolution. Cancer Cell. 2018;34(1):45–55.e4.
2 The cancer Genome Atlas Network, Akbani R, Akdemir KC, Aksoy BA, Albert M, Ally A, et al. Genomic classification of cutaneous melanoma. Cell. 2015 Jun;161(7):1681–96. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0092867415006340.
3 Paluncic J, Kovacevic Z, Jansson PJ, Kalinowski D, Merlot AM, Huang MLH, et al. Roads to melanoma: key pathways and emerging players in melanoma progression and oncogenic signaling. Biochim Biophys Acta. 2016;1863(4):770–84.
4 Shain AH, Yeh I, Kovalyshyn I, Sriharan A, Talevich E, Gagnon A, et al. The genetic evolution of melanoma from precursor lesions. N Engl J Med. 2015;373(20):1926–36. Available from: http://www.nejm.org/doi/10.1056/NEJMoa1502583.
5 Nagore E, Requena C, Traves V, Guillen C, Hayward NK, Whiteman DC, et al. Prognostic value of BRAF mutations in localized cutaneous melanoma. J Am Acad Dermatol. 2014;70(5):858–62.e1–2.
6 Si L, Kong Y, Xu X, Flaherty KT, Sheng X, Cui C, et al. Prevalence of BRAF V600E mutation in Chinese melanoma patients: large scale analysis of BRAF and NRAS mutations in a 432-case cohort. Eur J Cancer. 2012;48(1):94–100.
7 Wu S, Kuo H, Li WQ, Canales AL, Han J, Qureshi AA. Association between BRAFV600E and NRASQ61 Rmutations and clinicopathologic characteristics, risk factors and clinical outcome of primary invasive cutaneous melanoma. Cancer Causes Control. 2014;25(10):1379–86.
8 Mar VJ, Liu W, Devitt B, Wong SQ, Dobrovic A, McArthur GA, et al. The role of BRAF mutations in primary melanoma growth rate and survival. Br J Dermatol. 2015;173(1):76–82.
9 Thomas NE, Edmiston SN, Alexander A, Groben PA, Parrish E, Kricker A, et al. Association between NRAS and BRAF mutational status and melanoma-specific survival among patients with higher-risk primary melanoma. JAMA Oncol. 2015 Jun;1(3):359–68.
10 Maldonado JL, Fridlyand J, Patel H, Jain AN, Busam K, Kageshita T, et al. Determinants of BRAF mutations in primary melanomas. JNCI J Natl Cancer Inst. 2003 Dec 17;95(24):1878–90.
11 Houben R, Becker JC, Kappel A, Terheyden P, Bröcker EB, Goetz R, et al. Constitutive activation of the Ras-Raf signaling pathway in metastatic melanoma is associated with poor prognosis. J Carcinog. 2004;3:6.
12 Long GV, Menzies AM, Nagrial AM, Haydu LE, Hamilton AL, Mann GJ, et al. Prognostic and clinicopathologic associations of oncogenic BRAF in metastatic melanoma. J Clin Oncol. 2011 Apr 1;29(10):1239–46.
13 Spathis A, Katoulis AC, Damaskou V, Liakou AI, Kottaridi C, Leventakou D, et al. BRAF mutation status in primary, recurrent, and metastatic malignant melanoma and its relation to histopathological parameters. Dermatol Pract Concept. 2019 Jan;9(1):54–62.
14 Lebbe C, How-kit A, Battistella M, Sadoux A, Podgorniak MP, Sidina I, et al. BRAF(V600) mutation levels predict response to vemurafenib in metastatic melanoma. Melanoma Res. 2014;24(4):415–8.
15 Bradish JR, Richey JD, Post KM, Meehan K, Sen JD, Malek AJ, et al. Discordancy in BRAF mutations among primary and metastatic melanoma lesions: clinical implications for targeted therapy. Mod Pathol. 2015 Apr;28(4):480–6.
16 Grzywa TM, Paskal W, Włodarski PK. Intratumor and intertumor heterogeneity in melanoma. Transl Oncol. 2017;10(6):956–75.
17 Betancourt LH, Szasz AM, Kuras M, Rodriguez Murillo J, Sugihara Y, Pla I, et al. The hidden story of heterogeneous B-raf V600E mutation quantitative protein expression in metastatic melanoma-association with clinical outcome and tumor phenotypes. Cancers. 2019 Dec 9;11(12):1981.
18 Cheng L, Lopez-Beltran A, Massari F, MacLennan GT, Montironi R. Molecular testing for BRAF mutations to inform melanoma treatment decisions: a move toward precision medicine. Mod Pathol. 2018;31(1):24–38.
19 Tran TV, Dang KX, Pham QH, Nguyen UD, Trinh NTT, Van Hoang L, et al. Evaluation of the expression levels of BRAFV600E mRNA in primary tumors of thyroid cancer using an ultrasensitive mutation assay. BMC Cancer. 2020 May 1;20(1):368.
20 Wegler C, Ölander M, Wiśniewski JR, Lundquist P, Zettl K, Åsberg A, et al. Global variability analysis of mRNA and protein concentrations across and within human tissues. NAR Genom Bioinform. 2020 Mar;2(1):lqz010.
21 Aung PP, Nagarajan P, Prieto VG. Regression in primary cutaneous melanoma: etiopathogenesis and clinical significance. Lab Invest. 2017 Feb 27.

22 Gershenwald JE, Scolyer RA, Hess KR, Sondak VK, Long GV, Ross MI, et al. Melanoma staging: evidence-based changes in the American Joint Committee on Cancer eighth edition cancer staging manual. CA Cancer J Clin. 2017 Nov;67(6):472–92.
23 Nielsen PR, Eriksen JO, Lindahl LM, Wehkamp U, Bzorek M, Andersen G, et al. Diagnostic two-gene classifier in early-stage mycosis fungoides: a retrospective multicenter study. J Invest Dermatol. 2021;141(1):213–7.e5.
24 Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov. 2012 May;2(5):401–4.
25 Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal. 2013;6(269):1–34.
26 Kakadia S, Yarlagadda N, Awad R, Kundranda M, Niu J, Naraev B, et al. Mechanisms of resistance to BRAF and MEK inhibitors and clinical update of US Food and Drug Administration-approved targeted therapy in advanced melanoma. Onco Targets Ther. 2018;11:7095–107. Available from: https://www.dovepress.com/mechanisms-of-resistance-to-braf-and-mek-inhibitors-and-clinical-updat-peer-reviewed-article-OTT.
27 Anwar MAF, Murad F, Dawson E, Abd Elmageed ZY, Tsumagari K, Kandil E. Immunohistochemistry as a reliable method for detection of BRAF-V600E mutation in melanoma: a systematic review and meta-analysis of current published literature. J Surg Res. 2016;203(2):407–15.
28 Capper D, Preusser M, Habel A, Sahm F, Ackermann U, Schindler G, et al. Assessment of BRAF V600E mutation status by immunohistochemistry with a mutation-specific monoclonal antibody. Acta Neuropathol. 2011 Jul 3;122(1):11–9.
29 Riveiro-Falkenbach E, Villanueva CA, Garrido MC, Ruano Y, García-Martín RM, Godoy E, et al. Intra- and inter-tumoral homogeneity of BRAF(V600E) mutations in melanoma tumors. J Invest Dermatol. 2015 Dec;135(12):3078–85.
30 Verlinden I, van den Hurk K, Clarijs R, Willig AP, Stallinga CMHA, Roemen GMJM, et al. BRAFV600E immunopositive melanomas show low frequency of heterogeneity and association with epithelioid tumor cells: a STROBE-compliant article. Medicine; 2014 Dec;93(28):e285.
31 Valachis A, Ullenhag GJ. Discrepancy in BRAF status among patients with metastatic malignant melanoma: a meta-analysis. Eur J Cancer; 2017 Aug;81:106–15.
32 Ito T, Tanaka Y, Murata M, Kaku-Ito Y, Furue K, Furue M. BRAF heterogeneity in melanoma. Curr Treat Options Oncol. 2021;22(3):20.
33 Ito T, Kaku-Ito Y, Murata M, Furue K, Shen CH, Oda Y, et al. Immunohistochemical BRAF V600E expression and intratumor BRAF V600E heterogeneity in acral melanoma: implication in melanoma-specific survival. J Clin Med. 2020 Mar 4;9(3):690.
34 Adler NR, Wolfe R, McArthur GA, Kelly JW, Haydon A, McLean CA, et al. Tumour mutation status and melanoma recurrence following a negative sentinel lymph node biopsy. Br J Cancer. 2018;118(10):1289–95.
35 Adler NR, Wolfe R, Kelly JW, Haydon A, McArthur GA, McLean CA, et al. Tumour mutation status and sites of metastasis in patients with cutaneous melanoma. Br J Cancer. 2017 Sep 26;117(7):1026–35.
36 Fortes C, Mastroeni S, Mannooranparampil TJ, Passarelli F, Zappalà A, Annessi G, et al. Tumor-infiltrating lymphocytes predict cutaneous melanoma survival. Melanoma Res. 2015;25(4):306–11.
37 Pruessmann W, Rytlewski J, Wilmott J, Mihm MC, Attrill GH, Dyring-Andersen B, et al. Molecular analysis of primary melanoma T cells identifies patients at risk for metastatic recurrence. Nat Cancer. 2020 Feb 20;1(2):197–209.
38 Fu Q, Chen N, Ge C, Li R, Li Z, Zeng B, et al. Prognostic value of tumor-infiltrating lymphocytes in melanoma: a systematic review and meta-analysis. OncoImmunology. 2019 Jul 3;8(7):e1593806. Available from: https://www.tandfonline.com/doi/full/10.1080/2162402X.2019.1593806.
39 Ny L, Hernberg M, Nyakas M, Koivunen J, Oddershede L, Yoon M, et al. BRAF mutational status as a prognostic marker for survival in malignant melanoma: a systematic review and meta-analysis. Acta Oncol. 2020 Jul;59(7):833–44.
40 Ellerhorst JA, Greene VR, Ekmekcioglu S, Warneke CL, Johnson MM, Cooke CP, et al. Clinical correlates of NRAS and BRAF mutations in primary human melanoma. Clin Cancer Res. 2011 Jan 15;17(2):229–35.
41 Akslen LA, Angelini S, Straume O, Bachmann IM, Molven A, Hemminki K, et al. BRAF and NRAS mutations are frequent in nodular melanoma but are not associated with tumor cell proliferation or patient survival. J Invest Dermatol. 2005;125(2):312–7.
42 Devitt B, Liu W, Salemi R, Wolfe R, Kelly J, Tzen CY, et al. Clinical outcome and pathological features associated with NRAS mutation in cutaneous melanoma. Pigment Cell Melanoma Res. 2011;24(4):666–72.
43 Meckbach D, Bauer J, Pflugfelder A, Meier F, Busch C, Eigentler TK, et al. Survival according to BRAF-V600 tumor mutations: an analysis of 437 patients with primary melanoma. PLoS One. 2014;9(1):e86194.
44 Kim SY, Kim SN, Hahn HJ, Lee YW, Choe YB, Ahn KJ. Metaanalysis of BRAF mutations and clinicopathologic characteristics in primary melanoma. J Am Acad Dermatol. 2015 Jun;72(6):1036–46.e2.
45 Morita SY, Markovic SN. Molecular targets in melanoma: time for "ethnic personalization". Expert Rev Anticancer Ther. 2012 May;12(5):601–8.
46 Wan PTC, Garnett MJ, Roe SM, Lee S, Niculescu-Duvaz D, Good VM, et al. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. Cell. 2004;116(6):855–67.
47 Kannengiesser C, Spatz A, Michiels S, Eychène A, Dessen P, Lazar V, et al. Gene expression signature associated with BRAF mutations in human primary cutaneous melanomas. Mol Oncol. 2008 Apr;1(4):425–30.
48 Jönsson G, Busch C, Knappskog S, Geisler J, Miletic H, Ringnér M, et al. Gene expression profiling-based identification of molecular subtypes in stage IV melanomas with different clinical outcome. Clin Cancer Res. 2010 Jul 1;16(13):3356–67.