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BRAF-V600E expression in primary nodular melanoma is associated with aggressive tumour features and reduced survival

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Background: Around 50% of primary melanomas harbour *BRAF* mutations, but their prognostic impact has not been clear. Recently, a *BRAF-V600E* mutation-specific antibody has become available for immunohistochemistry. Here, we investigated for the first time the prognostic impact of *BRAF-V600E* protein expression in primary melanoma.

Methods: In a patient series of 248 nodular melanomas, *BRAF-V600E* and total *BRAF* expression were assessed by immunohistochemistry using tissue microarray sections of paraffin-embedded archival tissue. Mutation status was assessed by real-time PCR in cases with sufficient tumour tissue ($n=191$).

Results: Positive *BRAF-V600E* expression was present in 86 (35%) of the cases, and was significantly associated with increased tumour thickness, presence of tumour ulceration and reduced survival. Further, *BRAF-V600E* expression was an independent prognostic factor by multivariate analysis, whereas *BRAF* mutation status was not significant. There was 88% concordance between *BRAF-V600E* expression and mutation status.

Conclusions: Our findings indicate that *BRAF-V600E* expression is a novel prognostic marker in primary melanoma.

The incidence of cutaneous melanoma has increased in fairly skinned populations worldwide during the last decades (Nikolaou and Stratigos, 2014). The prognosis is poor for patients with metastatic disease, and therapeutic opportunities have been limited. Recently, new targeted and immunotherapeutic agents that prolong progression-free and overall survival for patients with advanced melanoma have become available (Shah and Dronca, 2014). Detection of *BRAF-V600* mutations is now mandatory to select patients with unresectable stage III and stage IV melanoma to targeted treatment with *BRAF* inhibitors. *BRAF* is the most frequently mutated oncogene in cutaneous melanoma, and nearly 50% of primary and metastatic melanomas harbour mutations in *BRAF* (Davies *et al*, 2002; Long *et al*, 2011; Colombino *et al*, 2012).

A mutation-specific antibody has been reported to have high sensitivity and specificity for immunohistochemical detection of

the *BRAF-V600E* mutation in melanoma. Reported sensitivities range from 72% to 100%, and specificities range from 47% to 100% (Long *et al*, 2013; Ehsani *et al*, 2014; Liu *et al*, 2014; Thiel *et al*, 2014; Ritterhouse and Barletta, 2015). In a comparison of Sanger sequencing, pyrosequencing, real-time PCR and immunohistochemistry (IHC), IHC was comparable to pyrosequencing in detection of the mutation with a sensitivity and specificity of 100% (Colomba *et al*, 2013). This study recommended IHC as a first line method for detection of the *BRAF-V600E* mutation in melanoma, and to use DNA-based tests in addition for *BRAF-V600E* negative or uninterpretable cases.

The proto-oncogene, *BRAF*, is part of the mitogen-activated protein kinase (MAPK) pathway. Presence of an activating *BRAF* mutation in melanoma cells is believed to be constitutively stimulate and drive the MAPK-pathway, resulting in increased

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cell proliferation (Smalley, 2003). The *BRAF-V600E* mutation accounts for ~80% of the *BRAF* mutations, and the *BRAF-V600K* mutation accounts for nearly 20%. Other less common mutations are the V600R and V600D mutations (Long *et al*, 2011; Bucheit *et al*, 2013).

Since the discovery of frequent *BRAF* mutations in melanoma by Davies *et al* (2002), multiple studies have investigated their association with distinct melanoma phenotypes and survival. The most common findings in previous investigations have been associations with younger age at diagnosis, melanoma arising on intermittently sun-exposed body-sites, and the superficially spreading melanoma subtype (Liu *et al*, 2007; Devitt *et al*, 2011; Long *et al*, 2011; Meckbach *et al*, 2014a). Regarding tumour features, two studies demonstrated an association with tumour thickness (Ellerhorst *et al*, 2011; Garcia-Casado *et al*, 2014), and some studies indicate a relation between *BRAF* mutation and increased proliferation such as higher mitotic count (Long *et al*, 2011; Meckbach *et al*, 2014a). Also, associations with presence of ulceration have been found in previous studies (Akslen *et al*, 2005; Ellerhorst *et al*, 2011; Si *et al*, 2012; Garcia-Casado *et al*, 2014).

Along with these findings, a prognostic value of *BRAF* mutation in primary melanoma has been discussed. Nagore *et al* (2014) found an independent prognostic impact of *BRAF* mutation on disease-free survival, but not for overall survival. Two other studies found an association with reduced survival, but multivariate analysis was not performed (Si *et al*, 2012; Wu *et al*, 2014). Recently, an independent impact of *BRAF* mutation on melanoma-specific survival has been demonstrated (Mar *et al*, 2015; Thomas *et al*, 2015). Still, there are previous studies reporting no association with survival (Akslen *et al*, 2005; Devitt *et al*, 2011; Ellerhorst *et al*, 2011; Meckbach *et al*, 2014a). For stage III and IV melanoma, the findings have also been diverse. *BRAF* mutation has been associated with reduced survival in some studies (Long *et al*, 2011; Mann *et al*, 2013; Barbour *et al*, 2014), although others found no association (Ellerhorst *et al*, 2011; Carlino *et al*, 2014; Meckbach *et al*, 2014b). One study has examined the predictive value of *BRAF* expression and response to *BRAF* inhibitors, without finding any association (Wilmott *et al*, 2013). In summary, the prognostic role of *BRAF* mutation in melanoma remains undetermined.

All previous studies analysing clinico-pathologic associations and prognostic impact of *BRAF* mutation in primary melanoma have used DNA-based tests for detection of the mutation, whereas the prognostic influence of *BRAF-V600E* protein expression has not yet been analysed. Here, we sought to determine whether *BRAF-V600E* protein expression in cutaneous melanoma is associated with clinico-pathologic features and disease-specific survival.

MATERIALS AND METHODS

Patients. This patient series consists of 255 consecutive cases of nodular cutaneous melanomas diagnosed at the Department of Pathology, Haukeland University Hospital (Bergen, Norway) during 1998–2008. The presence of a vertical growth phase and absence of a radial growth phase, that is adjacent *in situ* or microinvasive components, were used as inclusion criteria. Cases with minor secondary involvement of the adjacent epidermis up to three epidermal ridges were included. There was no known history of familial occurrence. During this time period, the sentinel node procedure was not performed in Norway. This patient series therefore lacks complete staging.

Median age was 70 years, and the median tumour thickness was 3.6 mm (range 0.7–29.0 mm). Complete information on patient survival, time and cause of death was available in all 255 cases. Last date of follow-up was 31 December, 2008, and median follow-up

Table 1. Patient characteristics

Age (years)	
Median (range)	70 (21–98)
Sex	
Men	138
Women	117
Tumour anatomic site	
Trunc	77
Non-trunc	175
Causes of death	
Alive	155
Melanoma-specific death	60
Other death causes	40

time for survivors was 31 months (range 0–131 months). During the follow-up period, 60 patients (23.5%) died of malignant melanoma and 40 (16%) died of other causes. A summary of patient characteristics is given in Table 1.

In the present study, previously reported information on clinico-pathologic characteristics, the mitotic marker PHH3 and survival data was included for comparison (Ladstein *et al*, 2012a,b). The Norwegian Data Inspectorate and the Regional Committee for Ethics in Research (Health Region III; 178.05) have approved this project. The study was performed in accordance with the Declaration of Helsinki Principles.

Clinico-pathologic variables. Previously recorded clinical data and histologic variables included in this study were: date of histologic diagnosis, sex, age at diagnosis, tumour anatomic site, tumour thickness according to Breslow (Breslow, 1970), level of invasion according to Clark *et al* (1969), mitotic count, microscopic tumour ulceration (Ladstein *et al*, 2012a) and tumour necrosis (Ladstein *et al*, 2012b).

Tissue microarray (TMA). The TMA technique has been described and validated in several studies (Kononen *et al*, 1998; Nocito *et al*, 2001; Straume and Akslen, 2002). Three tissue cylinders from representative tumour areas identified on H&E stained slides, generally at the supra-basal area of the melanoma, were punched and mounted into a recipient paraffin block using a custom made precision instrument (Beecher Instruments, Silver Spring, MD, USA). The diameter of the tissue cylinders was 1.0 mm. Sections of the resulting TMA blocks (5 μ m) were made by standard technique.

Immunohistochemistry. The immunohistochemical staining was performed on 5- μ m-thick TMA sections of paraffin-embedded archival tissue. Sufficient tumour tissue for IHC was available in 248 of the 255 cases. For detection of *BRAF-V600E* expression we used the Ultraview Red procedure (catalogue # 760-501) (Ventana Medical Systems, Tucson, AZ, USA) on the Ventana Benchmark Ultra immunostainer (Ventana Medical Systems). The slides were deparaffinized with Ventana Ez-prep (catalogue # 950-102, Ventana Medical Systems) before antigen retrieval with Ventana Ultra Cell Conditioner 1 solution (catalogue # 950-224, Ventana Medical Systems) at pH 8–9 for 64 min at 95 °C. The slides were then incubated with undiluted anti-*BRAF-V600E* clone VE1 mouse monoclonal primary antibody (catalogue # 790-4855, Ventana Medical Systems) for 32 min in room temperature. Detection was done using the Ventana Ultraview Universal Alkaline Phosphatase Red detection kit (catalogue # 760-501, Ventana Medical Systems) with Ventana Amplification kit (catalogue # 760-080, Ventana Medical Systems). The slides were finally counterstained with hematoxylin. Two nodular melanomas with known *BRAF-V600E* negative and positive status confirmed by Therascreen PCR mutation analysis were used as negative and positive controls.

For detection of total BRAF protein expression, the slides were dewaxed with xylene/ethanol before microwave antigen retrieval for 20 min in Target Retrieval Solution (DAKO 1699, Glostrup, Denmark) (pH=6). Endogenous peroxidase activity was prevented by treating the slides with peroxidase block (DAKO S2001) for 8 min. The slides were incubated with a polyclonal anti-BRAF antibody (dilution 1:200) (catalogue # B1687) (Sigma, St Louis, MO, USA) for 1 h at room temperature. The staining procedure was performed using the anti-rabbit EnVision labelled polymer method (DAKO K4011) with 3-amino-9-ethylcarbazole (AEC) (DAKO K3469) as substrate chromogen. Brief counterstaining was performed with hematoxylin (DAKO S2020). Negative controls were obtained by omitting the primary antibody.

Evaluation of staining. Expression of both BRAF-V600E and total BRAF was characterised by cytoplasmic staining of melanoma cells. The staining intensity was recorded as either negative, weak, moderate or strong (0–3). Proportion of tumour cells stained was recorded as either 1 (<10%), 2 (10–50%) or 3 (>50%). A staining index (SI) was calculated as the product of staining intensity and area score (proportion of tumour cells stained) (Straume and Akslen, 1997). For additional evaluation of the staining, a subset of cases ($n=69$) was scored blindly by two observers (EH, RGL) showing very good inter-observer agreement ($\kappa=0.85$, $P<0.01$) for BRAF-V600E staining and good inter-observer agreement ($\kappa=0.68$, $P<0.01$) for total BRAF staining. Evaluation of the cases was done blindly for patient characteristics and outcome.

DNA extraction. The tumour area was identified and marked by the pathologist (LAA) on hematoxylin and eosin stained slides. Marked tumour tissue was manually dissected from five slides (10 μm thick) of formalin-fixed, paraffin-embedded (FFPE) tissue for each case. DNA was extracted using the QIAmp DNA FFPE Tissue Kit (Qiagen 56404, Hilden, Germany) for the first 130 cases, and the E.Z.N.A tissue DNA kit (Omega Biotek D3396, Norcross, GA, USA) for the remaining cases (with no significant difference in mutation frequency). The DNA concentration was determined by the NanoDrop Spectrophotometer.

Real-time PCR. The *Therascreen* BRAF RGQ PCR Kit (Qiagen, Manchester, UK) was used for the *BRAF* mutation analysis. The assay detects five somatic mutations in *BRAF* codon 600: *V600E*, *V600E complex (V600Ec)*, *V600D*, *V600K* and *V600R*, but cannot distinguish between *V600E* and *V600Ec*. *Therascreen* applies Amplification Refractory Mutation System (ARMS) and Scorpion technologies to ensure the specific amplification of mutated DNA. The real-time PCR was performed on the Rotator-Gene Q 5plex HRM instrument (Quiagen, Manchester, UK). All procedures were performed according to the manufacturer's protocol.

Sanger sequencing. PCR amplification was performed using the AmpliTaq Gold PCR Master Mix (Applied Biosystems, Foster City, CA, USA). PCR products were enzymatically treated by IllustraExoProStar 1-Step (GE Healthcare Life Sciences, Little Chalfont, UK) before sequenced in both directions using the BigDye Terminator Cycle Sequencing Kit version 1.1 (Applied Biosystems). The sequence reactions were analysed on the Applied Biosystems 3500xL Genetic Analyzer using Sequencing Analysis software, version 6 (Applied Biosystems), and the electropherograms were examined manually.

Statistics. Statistical analyses were performed using the IBM Statistical package for the Social Sciences version 19 (SPSS Inc., Chicago, IL, USA). Associations between different categorical variables were assessed by Pearson's χ^2 test. Non-parametric correlations were tested by the Spearman's ρ correlation coefficient. Comparison of two or more continuous variables not following the normal distribution was performed using the Mann–

Whitney *U* or Kruskal–Wallis tests. Kappa (κ) statistics was used in analyses of inter-observer agreement of categorical data.

Univariate analyses of time to death due to malignant melanoma were performed using the product-limit procedure (Kaplan–Meier method), and differences between categories were estimated by the log-rank test, with date of histological diagnosis as the starting point. Patients who died of other causes were censored at the date of death. The influence of co-variables on patient survival was analysed by the proportional hazards method, and tested by the likelihood ratio (lratio) test. The variables were tested by log–log plot to determine their ability to be incorporated in multivariate models. All results were considered significant if $P\leq 0.05$. In the statistical analyses, the cut-off points for categorisation were determined after considering the frequency distribution curve, size of subgroups and number of events.

RESULTS

IHC for BRAF-V600E-VE1 and total BRAF protein expression.

Staining obtained with the BRAF-V600E-VE1 antibody was assessed in 248 cases. The staining was cytoplasmic and generally homogenous, and differences in staining intensity between tumour areas were rarely observed. In statistical analyses, BRAF-V600E expression was categorised according to staining index as either negative (SI 0–2) ($n=57$; 23%), borderline/weak (SI 3) ($n=105$; 42%) or strongly positive (SI 4–9) ($n=86$; 35%), with cut-off points based on the lower quartile (SI 0–2) and median values (SI 3) (Figure 1). In the analyses of BRAF-V600E expression categorised in two groups (negative and weak vs positive), the cut-off point was based on the median value (SI 3).

Staining obtained with the antibody for total BRAF protein was assessed in 248 cases. The staining was cytoplasmic and generally

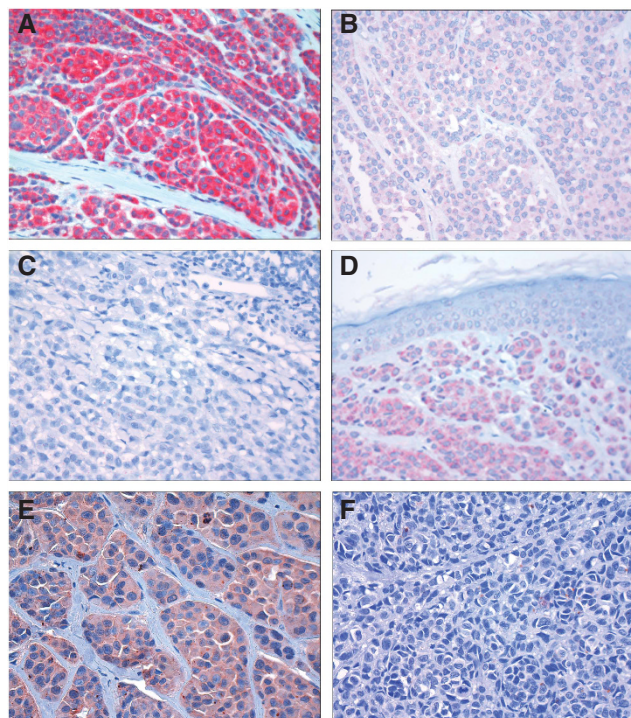


Figure 1. Immunohistochemical staining. Immunohistochemical staining showing nodular melanomas with positive (A), weakly positive (B) and negative (C) BRAF-V600E expression. Positive BRAF-V600E expression is shown in a BRAF-V600E mutation-positive benign naevus (D). Nodular melanoma with positive (E) and negative (F) total BRAF expression.

homogenous, and differences in staining intensity between tumour areas were rarely observed. In the statistical analyses, total BRAF expression was categorised as either negative (SI 0–2) ($n=37$; 15%) or positive (SI 3–9) ($n=211$; 85%) (Figure 1); the cut-off point was based on the distribution of the median values of tumour thickness and mitotic count, which both showed a shift in value between staining index 2 and 3.

Real-time PCR analysis of BRAF mutation status. BRAF mutation status could be determined in 191 of the 248 cases (77%) with sufficient tissue left in the paraffin blocks. Of these, 67 cases (35%) were BRAF-V600E/Ec-mutation positive, 9 cases (5%) were BRAF-V600K-mutation positive, and 2 cases (1%) were BRAF-V600R-mutation positive (Table 2). Cases with sufficient tissue for PCR analysis ($n=191$) were significantly thicker, had higher frequency of ulceration, higher mitotic count and were significantly associated with positive BRAF-V600E expression, when compared with the others (all $P<0.05$, χ^2 test; data not shown).

Table 2. Summary of BRAF mutation status by PCR and BRAF-V600E expression

	BRAF-V600E expression (SI) ^a	
	Negative (0–3)	Positive (4–9)
BRAF mutation status		
Negative	99 (86%)	14 (18%)
BRAF-V600E	8 (7%)	59 (78%)
BRAF-V600K	6 (5%)	3 (4%)
BRAF-V600R	2 (2%)	0 (0%)
Total	115	76

^aCut-off point median.

BRAF protein expression in association with BRAF mutation status and clinico-pathologic characteristics. Positive BRAF-V600E expression was significantly associated with increased tumour thickness, presence of tumour ulceration, truncal anatomic site, and younger age at diagnosis ($P<0.05$, Kruskal–Wallis or χ^2 tests), and a tendency to increased mitotic count ($P=0.062$, Kruskal–Wallis test) (Table 3). In pairwise analyses, the weakly stained (borderline) subgroup had significantly increased tumour thickness, presence of tumour ulceration and higher mitotic count when compared to the completely negative subgroup (all $P<0.05$, Mann–Whitney test and χ^2 test) (data not shown).

Assessing the combination of mutation negative and BRAF-V600E mutated cases ($n=180$), there was 88% concordance between BRAF-V600E protein expression and mutation status; 22 of the 180 cases (12%) were discordant: 8 cases (4%) were BRAF-V600E expression negative and BRAF-V600E-mutation positive, whereas 14 cases (8%) were BRAF-V600E expression positive and BRAF-V600E-mutation negative (Table 2). Positive BRAF-V600E expression was significantly associated with BRAF mutation status (negative vs V600E) ($P<0.01$, χ^2 test) (not shown). Of the 9 BRAF-V600K mutated cases, 1 case was BRAF-V600E expression negative, 5 cases were BRAF-V600E weakly positive, and 3 cases were BRAF-V600E positive. Of BRAF-V600R mutated cases, 2 of 2 were BRAF-V600E expression weakly positive (Supplementary Table S1). There were significantly more V600K mutations in the BRAF-V600E weakly stained subgroup compared to BRAF-V600E positive cases ($P<0.01$, χ^2 test) (data not shown). There were no associations between BRAF-V600E mutation status (negative vs V600E vs non-V600E mutations) and clinico-pathologic variables, except for significantly younger patient age in the V600E mutant cases ($P<0.01$, Kruskal–Wallis test) (Table 3).

Sanger sequencing was performed for the 14 discordant cases with positive BRAF-V600E expression and negative mutation status by real-time PCR analysis. One of these 14 cases showed a

Table 3. BRAF-V600E and total BRAF protein expression ($n=248$) and BRAF mutation status ($n=191$) in association with histopathologic variables

Variables	BRAF-V600E expression (SI) ^a				Total BRAF expression (SI) ^b			Mutation status by PCR			
	0–2	3	4–9	P	0–2	3–9	P	Negative	V600E	Non-V600E	P
Tumour thickness (mm)				0.01 ^c			<0.01 ^d				ns ^e
Median	2.2	3.9	3.8		2.0	3.9		4.3	4.0	4.0	
Ulceration^g					0.02 ^f			ns ^f			ns ^f
Absent (n)	35	48	33		20	96		51	27	3	
Present (n)	21	56	52		16	113		62	39	7	
Mitotic count (no./mm²)				0.06 ^c			<0.01 ^d				ns
Median	3.8	4.7	4.7		1.9	4.7		5.7	4.7	4.7	
Tumour necrosis				ns ^f			ns ^f				ns ^f
Absent (n)	44	77	65		32	154		79	50	8	
Present (n)	13	28	21		5	57		34	17	3	
Age (years)				<0.01 ^c			ns ^d				<0.01 ^c
Median	70	75	63		70	71		73	64	78	
Anatomic site^h				0.04 ^g			ns ^f				ns ^f
Truncus (n)	14	30	33		14	63		29	25	3	
Other (n)	43	75	50		23	145		84	39	8	

Abbreviation: SI = staining index.
^aCut-off point lower quartile and median.
^bCut-off point see text.
^cKruskal–Wallis test.
^dMann–Whitney U test.
^e3/248 and 2/191 missing cases; information not available.
^f χ^2 test.
^g χ^2 test SI 0–3 vs 4–9.
^h3/248 and 3/191 missing cases; information not available.

Table 4. Total BRAF expression in correlation with BRAF-V600E expression

	BRAF-V600E expression (SI) ^a			P
	Negative (0–2)	Weakly positive (3)	Positive (4–9)	
Total BRAF expression (SI)^b				<0.01 ^c
Negative (0–2)	21 (37%)	7 (7%)	9 (10%)	
Positive (3–9)	36 (63%)	98 (93%)	77 (90%)	

^aCut-off point lower quartile and median.
^bCut-off point see text.
^c χ^2 test.

BRAF-V600E mutation by sequencing (GTG/GAG), the others were mutation negative by this method.

Total BRAF protein expression was significantly associated with increased tumour thickness and mitotic count when looking at all cases ($P < 0.01$, Mann–Whitney U test) (Table 3), as well as when restricting the analysis to BRAF-V600E negative or weakly stained cases ($P < 0.01$) (not shown). There was a weak but significant correlation between BRAF-V600E-VE1 and total BRAF expression (Spearman’s ρ correlation coefficient 0.17, $P < 0.01$), also when analysed as categorical variables ($P < 0.01$, χ^2 test) (Table 4). Including the BRAF-V600E expression negative and weakly stained cases, total BRAF protein expression was increased in the subgroup with weak BRAF-V600E expression ($P < 0.01$, χ^2 test) (not shown). When looking at the 14 discordant cases with positive BRAF-V600E expression and negative mutation status, all of these except one had positive total BRAF expression (not shown).

Survival analyses. Univariate analysis showed significantly reduced survival for cases with strongly positive BRAF-V600E expression, both when categorised as two groups (negative and weak vs positive) ($P = 0.001$) (log-rank test) (Figure 2), and three groups (negative vs weak vs positive) ($P = 0.001$) (Supplementary Figure S1). Within the 191 cases in which mutation status could be assessed, BRAF-V600E protein expression was still associated with reduced survival ($P = 0.01$) (not shown). Univariate analysis of total BRAF protein expression showed a tendency to reduced survival for cases with positive total BRAF expression ($P = 0.082$) (data not shown). Univariate analysis of BRAF mutation status (negative vs V600E vs non-V600E) was not significantly associated with patient survival ($P = 0.35$) (Figure 2).

In multivariate analysis (proportional hazards method), we first examined the variables included in the pT classification from 2010 (Balch, 2009): tumour thickness, ulceration and mitotic count. In this basic model, tumour thickness > 4.0 mm compared to tumours ≤ 2.0 mm (HR 5.7, CI 1.7–19.3, $P = 0.003$) and presence of ulceration (HR 2.3, CI 1.2–4.3, $P = 0.005$) were independent predictors of reduced survival, whereas mitotic count did not obtain statistical significance ($P = 0.10$). Next, the same variables were included together with BRAF-V600E protein expression. Tumour thickness > 4.0 mm (HR 6.6, CI 1.5–29.0, $P = 0.007$), presence of ulceration (HR 2.0, CI 1.1–3.8, $P = 0.026$), mitotic count (HR 2.7, CI 0.9–7.7, $P = 0.036$) and positive BRAF-V600E expression HR 2.3, CI 1.4–4.0, $P = 0.001$) were all significant and independent predictors of prognosis in the final model (Table 5).

DISCUSSION

The detailed role of BRAF alterations in the progress and prognosis of cutaneous melanoma is still being discussed. To our knowledge, BRAF-V600E protein expression in association with patient survival has not been previously reported for these tumours. Here, we found that strong BRAF-V600E protein expression, present

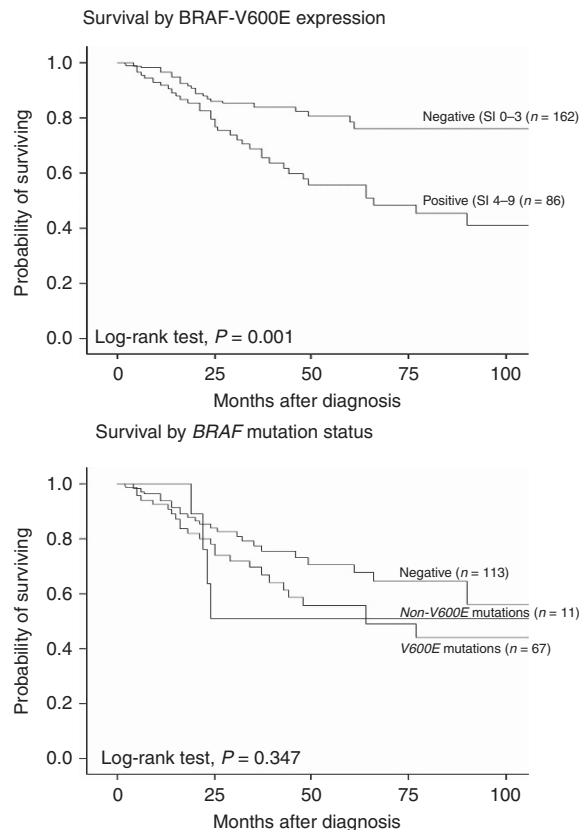


Figure 2. Survival curves. Survival curves by BRAF-V600E expression ($n = 248$) and by BRAF mutation status ($n = 191$) (Kaplan–Meier method, log-rank significance test). SI in parenthesis.

Table 5. Multivariate survival analysis (Cox’ proportional hazards method), with the final model after inclusion of tumour thickness, ulceration, mitotic count and BRAF-V600E protein expression ($n = 245$)

Variable	N	HR	95% CI	P-value ^a
Tumour thickness (mm)				0.007
≤ 2.0	58	1		
2.1–4.0	86	6.1	1.4–27.0	
> 4.0	101	6.5	1.5–29.0	
Ulceration				0.026
Absent	116	1		
Present	129	2.0	1.1–3.8	
Mitotic count (no./mm²)^b				0.036
≤ 1.9	56	1		
> 1.9	189	2.7	0.9–7.7	
BRAF-V600E expression (SI)^c				0.001
Negative (0–3)	162	1		
Positive (4–9)	85	2.3	1.4–4.0	

Abbreviations: HR = hazard ratio; CI = confidence interval.
^aLikelihood ratio.
^bCut-off point lower quartile.
^cCut-off point median.

in 35% of the cases, was associated with aggressive tumour features like increased thickness and presence of ulceration, and represented an independent prognostic factor in a large cohort of nodular cutaneous melanoma. This indicates a strong relationship between BRAF-V600E protein in the primary tumour and long-term outcome in terms of melanoma-related deaths, and

expression status should be determined on the primary tumours at the time of diagnosis, at least for this aggressive melanoma subtype.

The frequency of positive BRAF-V600E expression and the BRAF-V600E mutation rate were both slightly lower than expected from most previous reports on BRAF mutation rate in primary melanoma (Colombino *et al*, 2012), although the mutation rate was almost the same as the one we found in our previous study using Sanger sequencing on a different but similar cohort (Akslen *et al*, 2005). This might be related to the relatively high median age of our cases, which is likely to be a reflection of the nodular subtype (Moreno-Ramirez *et al*, 2015).

Previous studies have been diverse regarding the impact of BRAF mutation on patient survival in primary melanoma. However, population differences, patient selection and melanoma subtypes, methods of BRAF mutation detection, and subtypes of BRAF mutations varied in these studies making direct comparisons difficult. Here, we found no prognostic value of BRAF mutation status by PCR, similarly to our previous study using Sanger sequencing on a different but similar cohort (Akslen *et al*, 2005). Regarding the prognostic value, it is not clear why BRAF-V600E protein expression, but not mutation status, is associated with reduced survival, and this finding needs to be studied in more detail.

Methodological issues have to be considered. Mutation status was analysed by real-time PCR on all cases with sufficient DNA. Assessing the combination of BRAF mutation negative and BRAF-V600E positive cases ($n = 180$), 22 cases showed discordancy compared to BRAF-V600E protein expression; 14 cases were BRAF-V600E-expression positive but mutation negative, and 8 cases were BRAF-V600E expression negative but V600E-mutation positive. This discordance rate is slightly higher than in previous studies. One possible explanation for the discordant cases might be intra-tumoral heterogeneity. Due to limited amount of tissue for many cases in our series, sections used for IHC and mutation analysis could be derived from different paraffin blocks in some cases. In support of this are reports on heterogeneity of the BRAF-V600E mutation within individual melanoma specimens (Miller *et al*, 2004; Yancovitz *et al*, 2012). Further, false-negative interpretation of BRAF-V600E protein expression might be due to impaired antigenicity of the epitope because of tissue coagulation or early necrosis (Capper *et al*, 2011), although this was not a frequent finding in our melanoma series. In a study by Ehsani *et al* (2014), 32% of melanoma tumours were BRAF-V600E-expression positive by IHC and mutation negative by PCR. Lade-Keller *et al* (2013) reported 4 of 13 melanoma cases as IHC positive and PCR negative. In an analysis of circulating melanoma cells, Hofman *et al* (2013) reported 15% of cases to be IHC positive and pyrosequencing negative. The mechanism behind these false-positive cases is not clear. False-positive interpretation might be due to antibody cross-reactivity to an unknown epitope. Another explanation for the discordancy in our study could be that the PCR method is not as sensitive as IHC, although it is proved to be more sensitive than Sanger sequencing (Colomba *et al*, 2013). Still, we found that one of the 14 discordant cases (7%) with negative mutation status by PCR was V600E-mutation positive by Sanger sequencing. Thus, a positive IHC result could represent the presence of a mutated BRAF-V600E protein even in the absence of a positive molecular test.

Importantly, our data do not allow for a conclusion as for which method is the best for detection of the BRAF-V600E mutation. It is likely, based on our findings, that both IHC and PCR have missed a few BRAF-V600E mutated cases, and the two methods might therefore be combined in the work-up of these tumours. This is also in line with the conclusion of previous studies assessing BRAF mutation status by IHC and molecular methods (Ihle *et al*, 2014; Uguen *et al*, 2015).

In a subgroup of the cases, weak BRAF-V600E staining was observed. Interestingly, these cases had similar thickness and mitotic rate as the BRAF-V600E-positive cases, and the survival was intermediary between positive and completely negative cases. In total, 67 of 79 BRAF-V600E expression weakly positive cases were mutation negative (85%), 5 had a V600E mutation (6%) and 7 (9%) had a non-V600E mutation. We hypothesised that the characteristics of the BRAF-V600E weakly positive subgroup could be caused by an upregulation of wild-type BRAF, and this was supported by our findings that weak BRAF-V600E expression was significantly associated with increased total BRAF. Further, total BRAF expression was associated with increased tumour thickness and mitotic count. Previously, an association of increased total BRAF protein expression and reduced survival has been reported (Safae Ardekani *et al*, 2013). Our findings do suggest that cases with upregulation of wild-type BRAF might demonstrate a weakly positive staining also for BRAF-V600E. Another possible explanation for the findings could be the presence of non-V600E mutations in the group with weak or borderline BRAF-V600E expression, and this was found. In comparison with BRAF-V600E mutations, BRAF-V600K mutations have previously been found to be associated with shorter time from diagnosis of primary melanoma to first distant metastasis, but with no difference in survival thereafter (Menzies *et al*, 2012). We found significantly more non-V600E mutations in the BRAF-V600E expression weakly positive subgroup compared to the BRAF-V600E-expression positive subgroup. Further, the survival plot for the non-V600E mutations shows an early and steep fall which could represent early metastasis in these cases.

In summary, we find that BRAF-V600E protein expression detected by IHC is independently associated with reduced survival, whereas this was not the case for BRAF mutation status. Our data do not allow for a conclusion as for which method is currently the best for detection of BRAF-V600E mutation in melanomas, but indicate that IHC should be used complementary to molecular detection methods. Our findings suggest that BRAF-V600E expression is a novel prognostic marker in primary melanoma, and additional studies should be performed to confirm this finding.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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