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Rare BRAF mutations in melanoma patients: implications for molecular testing in clinical practice

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Background: The detection of V600E BRAF mutation in melanoma is fundamental since here BRAF inhibitors represent an effective treatment. Non-V600E BRAF mutations that may also respond are not detected by certain screening methods. Thus, knowledge about detection of these mutations is needed.

Methods: A total of 276 tumour samples from 174 melanoma patients were investigated for BRAF mutations by pyrosequencing. Rare mutations were confirmed by capillary sequencing and compared with findings from COBAS test and immunohistochemistry using a novel BRAF antibody. Melanoma type, localisation, and survival were summarised.

Results: BRAF mutations were found in 43% of patients (124 tumours in 75 patients). Among those, 14 patients (18.7%) exhibited rare mutations. The V600EK601del and V600DK601del mutations have never been described before in melanoma. Furthermore, V600K, V600E2, and V600D, V600G, V600R, and L597S mutations were detected. Mutations were not detected by COBAS test in 7 out of these 14 patients and immunohistochemistry only reliably detected patients with the V600E2 and V600EK601del mutation.

Conclusion: Accurate diagnosis of rare BRAF mutations is crucial. We show that pyrosequencing is accurate, highly sensitive, reliable, and time saving to detect rare BRAF mutations. Missing these rare variant mutations would exclude a subset of patients from available effective BRAF-targeting therapy.

Discovering the activating V600E BRAF mutation that is present in ~41–50% of melanomas (Houben *et al*, 2004; Curtin *et al*, 2005) has paved the way to targeted therapy with BRAF inhibitors. The first BRAF inhibitor to gain approval, vemurafenib (Zelboraf, Roche, Grenzach-Wyhlen, Germany), has demonstrated improvement of survival in patients with metastatic melanoma who have the V600E mutation (Chapman *et al*, 2011). Another BRAF inhibitor, dabrafenib, has also shown promising results (Hauschild *et al*, 2012). Other rare variant BRAF V600 mutations, for example V600K, have been described and were shown to be associated with distinct clinicopathological features including differences in age distribution (higher rates in older patients), localisation (higher rates of presentation on head and neck) and a worse distant metastasis-free survival (Menzies *et al*, 2012). However, these mutations might not be detected with certain mutation-specific detection methods (Anderson *et al*, 2012) and consequently these patients might be excluded from clinical trials with BRAF inhibitors or regular treatment with vemurafenib (Flaherty *et al*, 2010). Depending on the study, ~6–30% of all BRAF mutations were described to be distinct from the V600E genotype (Rubinstein *et al*, 2010; Beadling *et al*, 2011; Long *et al*, 2011; Lovly *et al*, 2012). In fact, among BRAF V600 mutations, 79%, 12%, 5%, and 4% were

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V600E, V600K, V600R, and V600M, respectively (Lovly *et al*, 2012). Interestingly, *in vitro* and *in vivo* data indicate that BRAF inhibitors could be effective in these patients (Rubinstein *et al*, 2010; Chapman *et al*, 2011).

Since treatment of BRAF-mutated patients has a profound impact on disease and overall survival (Long *et al*, 2011) the correct identification of the mutation status is crucial. A variety of technologies is currently available to detect the V600E mutant, which describes the most common sequence variant p.Val600Glu from formalin-fixed paraffin-embedded (FFPE) tumour samples including PCR with and without fluorescence monitoring, dideoxysequencing, direct capillary sequencing, and pyrosequencing (Grossmann *et al*, 2012). Although all platforms start with a careful selection of a tumour specimen, they differ remarkably with respect to sensitivity and specificity for the V600E and especially for the rare (actionable) mutations V600K, V600D, V600R, and others. It has recently been reported that the FDA-approved COBAS test does not reliably detect rare variant mutations nor can distinguish variant mutations, that is, V600K from V600E (Anderson *et al*, 2012; Halait *et al*, 2012). Sanger sequencing, mass spectrometry, and next-generation sequencing can detect all possible mutations but they are rather labour intensive and expensive (Beadling *et al*, 2011; Dutton-Regester *et al*, 2012). Furthermore, Sanger sequencing has a lower analytical sensitivity compared with pyrosequencing (Colomba *et al*, 2013). Especially in small biopsies from metastasis with few tumour cells in the background of normal fibroblasts and inflammatory cells this could present a serious problem. The minimal tumour percentage that can be detected is 15–20% by pyrosequencing and 40% by Sanger sequencing, respectively (Spittle *et al*, 2007). COLD-PCR (co-amplification at lower denaturation temperature) has been described to enrich mutant alleles before the application of these methods (Pinzani *et al*, 2011). High Resolution Melting Analysis (HRMA) detects unequivocal abnormal melting curves in mutant amplification products but it cannot identify the exact type of mutation (Willmore-Payne *et al*, 2005). Thus, HRMA can be used only as a pre-screening technique to identify the presence or absence of a mutation. Allele-specific/mutation-specific PCR has a very high sensitivity because the used primers contain the specific mutation of interest (Yancovitz *et al*, 2012). This has some advantages in specimen with a high content of non-tumorous tissue but non-specific priming and amplification might also generate false positive results. Mutation-specific probes recognise only the mutant sequence in a pool of amplified products and their sensitivity is limited due to the different amounts of non-tumour cells in the samples. Although there is a recent report about the successful use of a new antibody to detect the V600E mutation its potential for detecting rare BRAF variants is not yet clear (Feller *et al*, 2012; Skorokhod *et al*, 2012).

Another interesting question that is not fully understood to date is the consistency or heterogeneity among BRAF genotypes in different melanoma metastases of an individual patient. Although there seems to exist a certain consistency in BRAF mutation status of multiple metastases within one patient variation of mutations between distant metastases, lymph-node metastases, or the primary tumour has been observed with, for example, higher mutation rates in metastases (41–55%) compared with primary tumours (33–47%; Long *et al*, 2011). Interestingly, variation is also dependent on the site of the tumour with mutations being more frequent in skin metastases compared with visceral lesions (Colombino *et al*, 2012). Branched evolution in metastatic disease has been shown to create a remarkable genetic heterogeneity among different metastases of one patient (Gerlinger *et al*, 2012; Yancovitz *et al*, 2012) and within single metastases (Lin *et al*, 2011).

This study investigates the frequency, type, and intraindividual concordance of rare V600 BRAF mutations in primary tumours and different metastases of melanoma patients, compares different detection methods, and correlates the BRAF genotype with clinical characteristics.

MATERIALS AND METHODS

Patients. A total of 276 tumour samples from 174 consecutive patients with metastatic stage IV melanoma consulting the University Hospital Erlangen were analysed within this study excluding patients with uveal melanoma. Data on tumour type, treatment, and course of disease were gathered from patient files. Survival data were obtained from the Clinical Tumor Registry Nürnberg-Erlangen if not accessible from the clinical files. For patients with rare mutations, all tumour tissue samples available were obtained for mutation testing. The investigations were approved by the local ethics committee of the University of Erlangen-Nürnberg.

DNA extraction. Genomic DNA was extracted from 2 to 3 5-μm sections of FFPE tissue blocks. The relevant tumour area was marked by a pathologist (AH) and in some cases by a dermatopathologist (JB). Tumour areas were microdissected manually and yielded a tumour content of $>75\%$. No tumours had to be excluded from analysis due to a too low tumour content. After deparaffinisation, DNA was prepared as described recently using the NucleoSpinTissue kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions (Daniels *et al*, 2011). For COBAS testing, the DNA was extracted by the matched kit.

Mutation analyses

Pyrosequencing. DNA was amplified using the multiplex PCR-kit according to the instructions of the manufacturer (Qiagen, Hilden, Germany) and the following primers: forward: 5′TGAAGA CCTCACAGTAAAAATAGG-3′, and reverse: 5′Biotin AAAA TGGATCCAGACAACTGTTC-3′. The cycling was performed as follows: a single cycle of denaturation at 95°C for 15 min, 42 cycles at 95°C for 20 s, 61°C for 30 s, and 72°C for 30 s, and a final 5 min extension at 72°C. For pyrosequencing (PyroMark Q24; Qiagen) single-stranded DNA was prepared from 40 μl biotinylated PCR product with streptavidin-coated sepharose and 0.5 μM of the sequencing primer: 5′-GGTGATTTTGGTCTAGC-3′ using the PSQ Vacuum Prep Tool (Qiagen). The set-up for the pyrosequencing assay was selected with the following sequence in 'Sequence to Analyze': TACAG<u>A/T</u>GAAA. The underlined A/T describes the hot spot mutation site at codon 600 and primarily describes the V600E with a substitution of GTG (valine) by GAG (glutamic acid). The following dispensation order was used: GTACA<u>C</u>GATG. The underlined 'C' was included as an internal control. The colorectal cancer cell lines HCT116 and HT29 were used as negative and positive BRAF V600E controls, respectively.

Sanger sequencing. For Sanger sequencing, PCR was performed with multiplex PCR-kit according to manufacturer's instruction using the following primers: forward: 5′-TCTTCATGAAGACCT-CACAGT-3′, and reverse: 5′-CCAGACAACTGTTCAAACTGA-3′. The thermal conditions were as follows: initial heating period for 15 min at 95°C, 36 cycles at 95°C for 1 min, 55°C for 1 min and 72°C for 1 min, and finally 10 min at 72°C. To purify PCR products MinElute PCR Purification Kit (Qiagen) was used. Sequencing PCR was performed with the forward primer using the BigDye Terminator v1.1 Cycle Sequencing Kit according to manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Products were purified with Centri-Sep Spin Columns (Princeton Separations, Freehold, NJ, USA) and subsequently analysed on an automatic sequencer (ABI 3500 Dx Genetic Analyzer; Applied Biosystems).

COBAS. All reagents, calibrators, and controls were from the FDA-approved Roche kit (cobas 4800 BRAF V600 Mutation Test; Roche Diagnostics, West Sussex, UK) and used according to manufacturers' recommendations on the appropriate system (cobas z 480 Analyzer; Roche, Basel, Switzerland).

Immunohistochemistry. Sections were stained after deparaffinisation with the BRAF V600E mutation-specific antibody (Spring-bio, Pleasanton, CA, USA), which was applied according to manufacturer's instructions. Enhancing kits were Optiview DAB IHC Detection Kit (Roche). Staining was performed on a Ventana BenchMark XT immuno stainer (Ventana Medical Systems, Tucson, AZ, USA). Three observers evaluated the immunostained slides simultaneously on a multiheaded microscope. All three observers were unaware of the mutational status of the stained tumour specimens. Immunoreactivity was scored positive when moderate to strong cytoplasmatic staining was observed in a substantial fraction of viable tumour cells ($\geqslant$30%). Faint cytoplasmic staining, nuclear staining or weak staining of interspersed single cells was scored negative. Heavily pigmented areas were avoided and melanin granula cross-checked with HE staining.

Statistical analyses. For analysing differences of survival times in the different groups, the Mann–Whitney *U*-test was applied.

RESULTS

A total of 276 melanoma tissue samples from 174 patients were analysed. Tissues included primary tumours, skin metastases, lymph node and distant metastases (lung, liver, gastric, pancreas, brain, intestinal, and soft tissue). Wildtype BRAF was present in 55.1% of the evaluated tumour samples and 64% of patients (152 tumour samples in 112 patients), and mutant BRAF in 44.9% of the evaluated tumour samples and 43% of the patients (124 tumour samples in 75 patients). Some patients showed both metastases with and without the BRAF mutation. Twelve samples yielded too little DNA to be analysed. Therefore, the success rate of DNA preparation for mutation analyses was >95%. Out of the BRAF-mutated patients, 61 were V600E (in a total of 78 tumour probes) whereas rare BRAF mutations were found in 14 patients (Table 1). These rare mutations were V600K (six cases), V600E2 (GAA; two cases), and V600D, V600G, V600DK601 del, V600EK601del, V600R, and L597S one case each, respectively (Figures 1 and 2). These patients comprise 8.0% of all patients analysed at our centre and 18.7% of patients (14 out of 75) with a BRAF mutation.

All rare mutations were cross-checked and confirmed by Sanger sequencing. To facilitate interpretation of pyrosequencing results, an atlas of pyrograms for BRAF mutations was assembled (Figures 1 and 2). Considering the work flow of both techniques starting with the prepared DNA the pyrosequencing reduces the

Table 1. Clinical characteristics of patients with rare Braf mutations

Patient No.	Primary melanoma (SSM/NM)	Gender	Age	Braf mutation	According to HGVS-approved nomenclature[a]	Survival in months from stage IV disease (months)	Treatment with kinase inhibitor (vem = vemurafenib/ sor = sorafenib/none; response)
1	uk	M	70	V600K	c.1798_1799GT>AA	uk	uk
2	NM; 4 mm	M	47	V600EK601del	c.[1799_1800TG>AA(;)1801_1803delAAA]	18	No vem[b]; sor: PD
3	Unknown primary	M	17	V600D	c.1799_1800TG>AT	17	None
4	uk	M	75	V600G	c.1799T>G	5	None
5	SSM; 1.2 mm	F	42	V600K	c.1798_1799GT>AA	1+	None
6	NM	F	81	V600K	c.1798_1799GT>AA	uk	uk
7	SSM; 0.85 mm	M	57	L597S	c.1789_1790CT>TC	9	No vem[b]; no sor
8	NM; 1.6 mm	M	68	V600E2 (GAA)	c.1799_1800TG>AA	23	No vem; sor + temozolomide: SD – PD
9	Unknown primary	F	37	V600DK601del	c.[1799_1800TG>AT(;)1801_1803delAAA]	17	No vem; sor: PD
10	NM; ulcerated; 8 mm	M	65	V600R	c.1798_1799GT>AG	28	No vem; sor + temozolomide: PR
11	Secondary NM; ulcerated; 5.5 mm	M	56	V600K	c.1798_1799GT>AA	23	No vem; sor: PD
12	NM; 3.5 mm	F	68	V600K	c.1798_1799GT>AA	3	No vem; sor: PD
13	NM; 10 mm	M	37	V600K	c.1798_1799GT>AA	22	No vem; sor: PD
14	Unknown primary	F	43	V600E2 (GAA)	c.1799_1800TG>AA	16	No vem; sor: PD

Abbreviations: SSM = superficial spreading melanoma; NM = nodular melanoma; NA = not applicable; uk = unknown; PD = progressive disease; SD = stable disease.
[a]HGVS: Human Genome Variation Society (http://www.hgvs.org/mutnomen).
[b]Due to detection of wildtype at study facility.

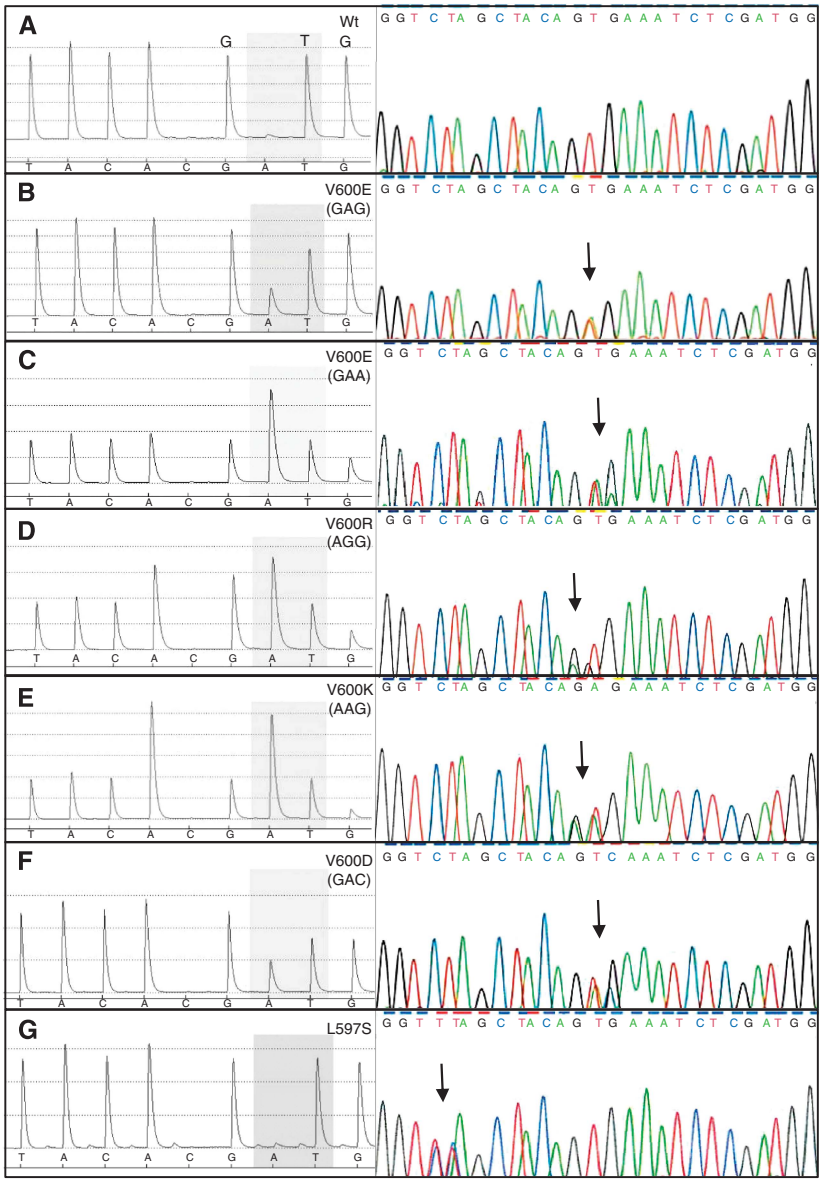

Figure 1. Pyrograms and Sanger sequencing of *rare* BRAF V600 mutations. (**A**) Wildtype, (**B**) V600E, (**C**) V600E2 (GAA variant), (**D**) V600R, (**E**) V600K), (**F**) V600D, and (**G**) L597S. The height of the signal peaks at the **A** position before codon 600 and the **G** signal peak after the codon 600, respectively, as well as the **G** at the third position of codon 600 discriminate between the six mutant variants. The L597S mutation cannot be detected by pyrosequencing. The arrows indicate the mutated codon in the Sanger sequence. The deceptive letter codes in (**B**, **C**, **E**, **G**) above the Sanger sequencing panels indicate the need for careful cross-check to define the final mutation.

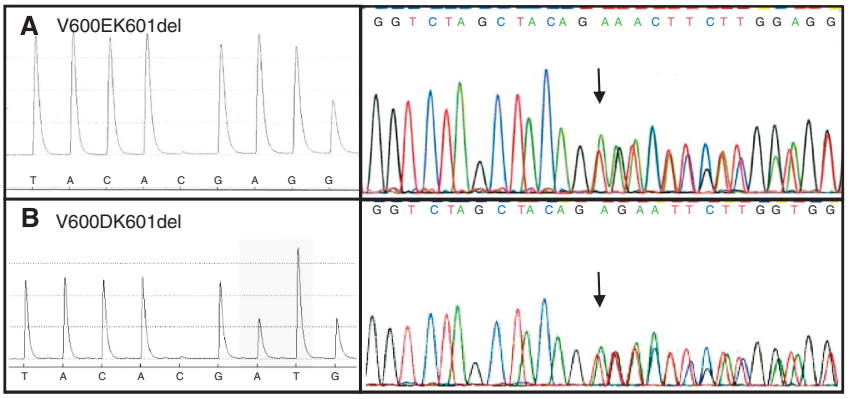

Figure 2. Pyrograms and Sanger sequencing of *novel* BRAF mutations. (**A**) V600EK601del and (**B**) V600DK601del. The V600EK601del pyrogram shows a remarkably aberrant pattern indicating the necessity of Sanger sequencing, the pyrogram of V600DK601del cannot be discriminated from the V600D mutation. The arrows indicate the mutated codon in the Sanger sequence.

Table 2. Distribution of mutation status (mutation landscape)

Patient No.	Mutation status	Organ metastases	Date
1	V600K	Brain	uk
	V600K	uk	uk
2	V600EK601del	Primary tumour	07/2009
	V600EK601del	uk	06/2011
	V600EK601del	Pancreas	05/2011
	V600EK601del	Stomach	05/2011
3	V600D	Lymph node	10/2009
	V600D	Brain	01/2011
5	V600K	uk	09/2011
	V600K	Lymph node	09/2011
7	L597S	uk	02/2011
	L597S	Lymph node	uk
8	V600E2 (GAA)	Primary tumour	01/2005
	V600E2 (GAA)	Soft tissue	04/2005
9	V600DK601del	Skin	06/2006
	V600DK601del	Skin	07/2006
10	V600R	Skin	01/2006
	V600R	Skin	01/2006
	V600R	Skin	05/2006
	V600R	Skin	05/2006
	V600R	Skin	11/2006
	V600R	Skin	11/2006
	V600R	Skin	11/2006
	V600R	Skin	11/2006
	V600R	Skin	01/2007
	V600R	Skin	02/2007
	V600R	Skin	06/2007
	V600R	Skin	10/2007
	V600R	Skin	01/2008
11	V600K	Skin	06/2008
	V600K	Skin	06/2008
	V600K	Skin	12/2009
	V600K	Skin	12/2009
	V600K	Lymph node	04/2009
12	V600K	Skin	08/2007
	V600K	Lung	09/2009
	V600K	Lung	08/2009
	V600K	Skin	08/2008
13	V600K	Skin	03/2009
	V600K	Skin	03/2009
14	V600E (GAA)	Skin	02/2007
	V600E (GAA)	Skin	06/2007
	V600E (GAA)	Skin	10/2007
	V600E (GAA)	Skin	11/2007

Abbreviation: uk = unknown.

turn-around time to ~4 h in comparison with Sanger sequencing that needs a minimum of one full working day to obtain final results. Moreover, Sanger sequencing needs an additional PCR step and two purification steps, which increases the pipetting time needed, mistakes by the experimenter and the risk of contamination.

Patients with rare mutations showed the same survival as other BRAF-mutated patients. Patients with rare mutations showed a median survival of a little >17 months (Table 1; one patient is still alive) as compared with 15 months in patients with V600E mutation. This difference was not statistically significant. The only remarkable feature was that 3 out of 14 patients (21.4%) exhibited a metastatic melanoma with unknown primary tumour (MUP), which is high when compared with previously published data.

No variation in the mutation status within individual patients with rare BRAF mutations. In 12 out of 14 patients with rare BRAF mutations, multiple tumour probes (2–13 biopsies) were available for analysis. Metastatic tissue was from skin, lymph nodes, soft tissue, lung, visceral organs, and brain. All patients with a rare mutation were concordant with respect to mutation status (Table 2) opposed to patients with V600E mutation, which previously showed some discordant results (Houben *et al*, 2004).

Rare mutations are detected incompletely by COBAS test and immunohistochemistry. In all, 7 out of 14 patients with rare mutations detected by pyrosequencing were also characterised as mutated by the COBAS test (with 8 samples out of 18 classified as mutated) whereas in 7 patients mutations were not detected (Table 3). These were V600EK601del, V600E2 (GAA), V600D, V600K, L597S, and V600R. The detected samples were those with the V600K mutation (7 out of 7 samples in 6 patients) and the case with the V600DK601del mutation. Overall detection rates are provided in Table 4.

Immunohistochemistry with the V600E-specific antibody was positive for the tumours with the V600E2 and the V600EK601del mutations and negative for tumours with V600D, L597S, V600R, and V600DK601del mutations (Table 3; Figure 3). Interestingly, one of the V600K patients showed a positive staining whereas all others were negative. Furthermore, interspersed positively stained cells were seen in some of the cases (<10% of tumour cells).

DISCUSSION

This study summarises data on 14 patients with rare BRAF mutations detected in an analysis of 276 tumour samples in a study population of 174 patients with metastatic melanoma. Thus, 18.7% of BRAF-mutated patients showed a rare mutation. While V600K is more frequently found in Australia with up to 20% of BRAF-mutated cases (Long *et al*, 2011) our population showed this mutation in only 8% and Schoenewolf *et al* (2012) did not find any case in a study population of 52 cases. Another 10.7% of our BRAF-mutated patients showed other rare mutations. While in the literature V600E2, V600D, V600G, V600R, and L597S (Beadling *et al*, 2011; Dahlman *et al*, 2012) have been described this is the first study to report more complex mutations such as V600EK601del and V600DK601del in melanoma. Remarkably, 21% of patients with rare mutations analysed in our study presented with melanoma of unknown primary (MUP), which is much higher than the 1–8% in previously documented cases (Katz *et al*, 2005; Cormier *et al*, 2006).

In clinical practice, mutation analyses are performed from a tissue sample available, preferably from a recently detected and resected metastasis. However, heterogeneity in BRAF mutation status has been documented between primary tumour and metastases (Houben *et al*, 2004) and between different metastases (Lin *et al*, 2011; Yancovitz *et al*, 2012). For example, in patients with multiple metastatic specimens, discordant BRAF status among metastases was detected in 26–33% of patients depending

Table 3. Detection of rare BRAF mutations with different methods

Patient ID	Pyrosequencing/Sanger sequencing	COBAS	Immunohistochemistry BRAF V600E	Localisation/organ
1	V600K	√	×	Brain
	V600K	ND	ND	uk
2	V600EK601del	×	ND	Primary tumour
	V600EK601del	ND	√	Stomach
	V600EK601del	ND	√	Pancreas
3	V600D	×	ND	Lymph node
	V600D	ND	×	Lung
	V600D	×	ND	uk
4	V600G	×	ND	Liver
5	V600K	√	ND	uk
	V600K	√	×	Lymph node
6	V600K	√	ND	uk
7	L597S	×	ND	uk
	L597S	ND	×	
	L597S	ND	ND	Lymph node
8	V600E2 (GAA)	Invalid	√	Primary tumour
	V600E2 (GAA)	×	√	Lymph node
9	V600DK601del	√	×	Skin
	V600DK601del	ND	×	Skin
10	V600R	ND	×	Skin
	V600R	ND	×	Skin
	V600R	ND	×	Skin
	V600R	×	×	Skin
	V600R	×	×	Skin
11	V600K	√	ND	Skin
12	V600K	Invalid	ND	Skin
	V600K	ND	×	Skin
	V600K	√	ND	Lung
	V600K	ND	×	Skin
	V600K	Invalid	ND	Skin
13	V600K	√	ND	Skin
	V600K	ND	√	Skin
14	V600E2 (GAA)	×	ND	Skin
	V600E2 (GAA)	×	ND	Skin

Abbreviations: ND = not done; √ = mutation detected; × = mutation not detected; Invalid = no result; uk = unknown.

Table 4. Overall detection rates of rare BRAF mutations with different methods

Reference	Pyrosequencing	Pyrosequencing/Sanger sequencing	COBAS	Immunohistochemistry BRAF V600E
Patients	92.9% (13/14[a])	100% (14/14)	50.0% (7/14)	21.4% (3/14)
Samples	95.5% (42/44[b])	100% (44/44)	44% (8/18)	27.8% (5/18)

[a]Including two patients where mutations could not be fully classified.
[b]Including six samples where mutations could not be fully classified.

on the method being used (Yancovitz *et al*, 2012). Our analysis of the intraindividual mutation spectrum in patients with rare mutations, however, shows no discordance with analysis of 2–13 different metastases in 14 patients. However, a recent report describes a patient with discordant mutation status with a wildtype satellite metastasis and a V600K skin metastasis (Richtig *et al*, 2012). A further problem that has been addressed in a landmark publication on renal cell carcinoma is intratumoral heterogeneity (Gerlinger *et al*, 2012). This would mean that depending on where the section for the DNA extraction is taken results may differ

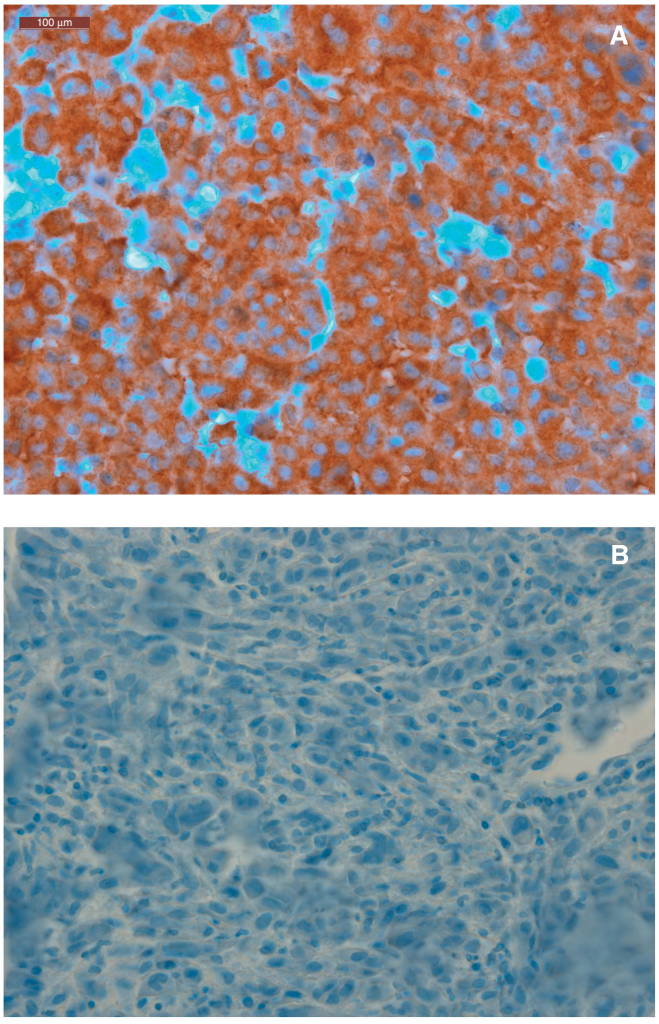

Figure 3. Detection of BRAF mutation by immunohistochemistry. (**A**) Positive staining in a V600E2-mutated melanoma sample of patient 8 (homogenous brown colour indicating mutated cells) and (**B**) negative control.

within one tumour nodule. By now, this has also been demonstrated in melanoma with intratumour heterogeneity in six out of nine cases (Yancovitz *et al*, 2012).

Since BRAF mutations can lead to a constitutive activation of the protein kinase activity and thus downstream activation of MEK and the ERK1/2 kinases with induction of proliferation, BRAF inhibitors have been shown to be effective with tumour responses in about 48% of V600E-mutated melanoma patients (Chapman *et al*, 2011). Whether BRAF inhibitors have the same effectiveness in patients with rare BRAF mutations has to be evaluated. Since the companion assay (COBAS) used in the phase 3 vemurafenib study has a high sensitivity for V600E with partial cross-reactivity to V600K (http://www.accessdata.fda.gov/cdrh_docs/pdf11/P110020b.pdf), 10 patients who were later found to have the BRAF V600K mutation were included in the vemurafenib group. Four out of these 10 patients (40%) showed a partial response (Chapman *et al*, 2011). Since these data indicate that vemurafenib is active in melanoma with the V600K mutation, the assays used in association with the dabrafenib and trametinib trials are now allele specific and thus distinguish V600E from V600K. Interestingly, the MEK inhibitor trametinib appeared to be less active in patients whose tumours had MAP kinase activation by BRAF V600K (Margolin, 2012).

Comparison of results from COBAS and immunohistochemistry proved that rare mutations cannot be reliably detected neither by COBAS nor by immunohistochemistry with the currently available antibody. This is in accordance with the publication of Skorokhod *et al* (2012), which also showed that V600K mutations did not show positive staining with the V600E antibody. Here, we demonstrate that pyrosequencing is an accurate, reliable, and time-saving method to detect rare BRAF mutations, which is decisive for treatment with BRAF inhibitors and thus possibly prognosis. It can be synergistically combined with Sanger sequencing to optimise detection of rare mutations. Further studies are needed to specify response rates in these populations since so far little data exist.

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REFERENCES

Anderson S, Bloom KJ, Vallera DU, Rueschoff J, Meldrum C, Schilling R, Kovach B, Lee JR, Ochoa P, Langland R, Halait H, Lawrence HJ, Dugan MC (2012) Multisite analytic performance studies of a real-time polymerase chain reaction assay for the detection of BRAF V600E mutations in formalin-fixed paraffin-embedded tissue specimens of malignant melanoma. *Arch Pathol Lab Med* **136**(11): 1385–1391.

Beadling C, Heinrich MC, Warrick A, Forbes EM, Nelson D, Justusson E, Levine J, Neff TL, Patterson J, Presnell A, McKinley A, Winter LJ, Dewey C, Harlow A, Barney O, Druker BJ, Schuff KG, Corless CL (2011) Multiplex mutation screening by mass spectrometry evaluation of 820 cases from a personalized cancer medicine registry. *J Mol Diagn* **13**(5): 504–513.

Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, Dummer R, Garbe C, Testori A, Maio M, Hogg D, Lorigan P, Lebbe C, Jouary T, Schadendorf D, Ribas A, O'Day SJ, Sosman JA, Kirkwood JM, Eggermont AM, Dreno B, Nolop K, Li J, Nelson B, Hou J, Lee RJ, Flaherty KT, McArthur GA (2011) Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med* **364**(26): 2507–2516.

Colomba E, Helias-Rodzewicz Z, von DA, Marin C, Terrones N, Pechaud D, Surel S, Cote JF, Peschaud F, Capper D, Blons H, Zimmermann U, Clerici T, Saiag P, Emile JF (2013) Detection of BRAF p.V600E mutations in melanomas: comparison of four methods argues for sequential use of immunohistochemistry and pyrosequencing. *J Mol Diagn* **15**(1): 94–100.

Colombino M, Capone M, Lissia A, Cossu A, Rubino C, De Giorgi V, Massi D, Fonsatti E, Staibano S, Nappi O, Pagani E, Casula M, Manca A, Sini M, Franco R, Botti G, Caraco C, Mozzillo N, Ascierto PA, Palmieri G (2012) BRAF/NRAS mutation frequencies among primary tumors and metastases in patients with melanoma. *J Clin Oncol* **30**(20): 2522–2529.

Cormier JN, Xing Y, Feng L, Huang X, Davidson L, Gershenwald JE, Lee JE, Mansfield PF, Ross MI (2006) Metastatic melanoma to lymph nodes in patients with unknown primary sites. *Cancer* **106**(9): 2012–2020.

Curtin JA, Fridlyand J, Kageshita T, Patel HN, Busam KJ, Kutzner H, Cho KH, Aiba S, Brocker EB, LeBoit PE, Pinkel D, Bastian BC (2005) Distinct sets of genetic alterations in melanoma. *N Engl J Med* **353**(20): 2135–2147.

Dahlman KB, Xia J, Hutchinson K, Ng C, Hucks D, Jia P, Atefi M, Su Z, Branch S, Lyle PL, Hicks DJ, Bozon V, Glaspy JA, Rosen N, Solit DB, Netterville JL, Vnencak-Jones CL, Sosman JA, Ribas A, Zhao Z, Pao W (2012) BRAF(L597) mutations in melanoma are associated with sensitivity to MEK inhibitors. *Cancer Discov* **2**(9): 791–797.

Daniels M, Lurkin I, Pauli R, Erbstosser E, Hildebrandt U, Hellwig K, Zschille U, Luders P, Kruger G, Knolle J, Stengel B, Prall F, Hertel K, Lobeck H, Popp B, Theissig F, Wunsch P, Zwarthoff E, Agaimy A, Schneider-Stock R (2011) Spectrum of KIT/PDGFRA/BRAF mutations and Phosphatidylinositol-3-Kinase pathway gene alterations in gastrointestinal stromal tumors (GIST). *Cancer Lett* **312**(1): 43–54.

Dutton-Regester K, Irwin D, Hunt P, Aoude LG, Tembe V, Pupo GM, Lanagan C, Carter CD, O'Connor L, O'Rourke M, Scolyer RA, Mann GJ, Schmidt CW, Herington A, Hayward NK (2012) A high-throughput panel for identifying clinically relevant mutation profiles in melanoma. *Mol Cancer Ther* **11**(4): 888–897.

Feller JK, Yang S, Mahalingam M (2012) Immunohistochemistry with a mutation-specific monoclonal antibody as a screening tool for the BRAFV600E mutational status in primary cutaneous malignant melanoma. *Mod Pathol* **26**(3): 414–420.

Flaherty KT, Puzanov I, Kim KB, Ribas A, McArthur GA, Sosman JA, O'Dwyer PJ, Lee RJ, Grippo JF, Nolop K, Chapman PB (2010) Inhibition of mutated, activated BRAF in metastatic melanoma. *N Engl J Med* **363**(9): 809–819.

Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, Martinez P, Matthews N, Stewart A, Tarpey P, Varela I, Phillimore B, Begum S, McDonald NQ, Butler A, Jones D, Raine K, Latimer C, Santos CR, Nohadani M, Eklund AC, Spencer-Dene B, Clark G, Pickering L, Stamp G, Gore M, Szallasi Z, Downward J, Futreal PA, Swanton C (2012) Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N Engl J Med* **366**(10): 883–892.

Grossmann AH, Grossmann KF, Wallander ML (2012) Molecular testing in malignant melanoma. *Diagn Cytopathol* **40**(6): 503–510.

Halait H, Demartin K, Shah S, Soviero S, Langland R, Cheng S, Hillman G, Wu L, Lawrence HJ (2012) Analytical performance of a real-time PCR-based assay for V600 mutations in the BRAF gene, used as the companion diagnostic test for the novel BRAF inhibitor vemurafenib in metastatic melanoma. *Diagn Mol Pathol* **21**(1): 1–8.

Hauschild A, Grob JJ, Demidov LV, Jouary T, Gutzmer R, Millward M, Rutkowski P, Blank CU, Miller Jr WH, Kaempgen E, Martin-Algarra S, Karaszewska B, Mauch C, Chiarion-Sileni V, Martin AM, Swann S, Haney P, Mirakhur B, Guckert ME, Goodman V, Chapman PB (2012) Dabrafenib in BRAF-mutated metastatic melanoma: a multicentre, open-label, phase 3 randomised controlled trial. *Lancet* **380**(9839): 358–365.

Houben R, Becker JC, Kappel A, Terheyden P, Brocker EB, Goetz R, Rapp UR (2004) Constitutive activation of the Ras-Raf signaling pathway in metastatic melanoma is associated with poor prognosis. *J Carcinog* **3**(1): 6.

Katz KA, Jonasch E, Hodi FS, Soiffer R, Kwitkiwski K, Sober AJ, Haluska FG (2005) Melanoma of unknown primary: experience at Massachusetts General Hospital and Dana-Farber Cancer Institute. *Melanoma Res* **15**(1): 77–82.

Lin J, Goto Y, Murata H, Sakaizawa K, Uchiyama A, Saida T, Takata M (2011) Polyclonality of BRAF mutations in primary melanoma and the selection of mutant alleles during progression. *Br J Cancer* **104**(3): 464–468.

Long GV, Menzies AM, Nagrial AM, Haydu LE, Hamilton AL, Mann GJ, Hughes TM, Thompson JF, Scolyer RA, Kefford RF (2011) Prognostic and clinicopathologic associations of oncogenic BRAF in metastatic melanoma. *J Clin Oncol* **29**(10): 1239–1246.

Lovly CM, Dahlman KB, Fohn LE, Su Z, Dias-Santagata D, Hicks DJ, Hucks D, Berry E, Terry C, Duke M, Su Y, Sobolik-Delmaire T, Richmond A, Kelley MC, Vnencak-Jones CL, Iafrate AJ, Sosman J, Pao W (2012) Routine multiplex mutational profiling of melanomas enables enrollment in genotype-driven therapeutic trials. *PLoS ONE* **7**(4): e35309.

Margolin K (2012) BRAF inhibition and beyond in advanced melanoma. *Lancet* **380**(9839): 320–322.

Menzies AM, Haydu LE, Visintin L, Carlino MS, Howle JR, Thompson JF, Kefford RF, Scolyer RA, Long GV (2012) Distinguishing clinicopathologic features of patients with V600E and V600K BRAF-mutant metastatic melanoma. *Clin Cancer Res* **18**(12): 3242–3249.

Pinzani P, Santucci C, Mancini I, Simi L, Salvianti F, Pratesi N, Massi D, De Giorgi V, Pazzagli M, Orlando C (2011) BRAFV600E detection in melanoma is highly improved by COLD-PCR. *Clin Chim Acta* **412**(11-12): 901–905.

Richtig E, Schrama D, Ugurel S, Fried I, Niederkorn A, Massone C, Becker JC (2012) BRAF mutation analysis of only one metastatic lesion can restrict the treatment of melanoma: a case report. *Br J Dermatol* **168**(2): 428–430.

Rubinstein JC, Sznol M, Pavlick AC, Ariyan S, Cheng E, Bacchiocchi A, Kluger HM, Narayan D, Halaban R (2010) Incidence of the V600K mutation among melanoma patients with BRAF mutations, and potential therapeutic response to the specific BRAF inhibitor PLX4032. *J Transl Med* **8**: 67.

Schoenewolf NL, Dummer R, Mihic-Probst D, Moch H, Simcock M, Ochsenbein A, Gillessen S, Schraml P, von MR (2012) Detecting BRAF mutations in formalin-fixed melanoma: experiences with two state-of-the-art techniques. *Case Rep Oncol* **5**(2): 280–289.

Skorokhod A, Capper D, von DA, Enk A, Helmbold P (2012) Detection of BRAF V600E mutations in skin metastases of malignant melanoma by monoclonal antibody VE1. *J Am Acad Dermatol* **67**(3): 488–491.

Spittle C, Ward MR, Nathanson KL, Gimotty PA, Rappaport E, Brose MS, Medina A, Letrero R, Herlyn M, Edwards RH (2007) Application of a BRAF pyrosequencing assay for mutation detection and copy number analysis in malignant melanoma. *J Mol Diagn* **9**(4): 464–471.

Willmore-Payne C, Holden JA, Tripp S, Layfield LJ (2005) Human malignant melanoma: detection of. *Hum Pathol* **36**(5): 486–493.

Yancovitz M, Litterman A, Yoon J, Ng E, Shapiro RL, Berman RS, Pavlick AC, Darvishian F, Christos P, Mazumdar M, Osman I, Polsky D (2012) Intra- and inter-tumor heterogeneity of BRAF(V600E))mutations in primary and metastatic melanoma. *PLoS ONE* **7**(1): e29336.