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Enhanced detection of *BRAF*-mutants by pre-PCR cleavage of wild-type sequences revealed circulating melanoma cells heterogeneity

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ABSTRACT

Purpose: Characterisation of circulating melanoma cells (CMC) for *BRAF* status can provide a strategy for non-invasive serial genotype monitoring in patients receiving BRAF inhibitors. We aimed to establish a method for *BRAF* mutation analysis at CMC level and we applied it in a cohort of CMC-positive patients.

Methods: We established a sensitive method for detection of *BRAF* mutations at codon 600 in whole blood samples of patients with melanoma, positive for presence of CMC. The method based on selective cleavage of wild-type sequences by taking the advantage of the presence of a TspR1 enzyme restriction site located at the site of mutation.

Results: In a serial dilution experiment spiking *BRAF* mutated cDNA into *BRAF* wild-type cDNA the method allowed to detect mutated cDNA till a dilution of $1:10^4$. Peripheral blood (PB) samples resulting positive for CMC and matched tissue specimens from 21 different AJCC stage IV melanoma patients were analysed. A 91% (19/21) correspondence between *BRAF* status in tissue and PB specimens was observed. In a patient (whose melanoma showed to bear the *BRAF* V600E mutation in blood, but not at tissue level) our analysis showed that blood samples with PCR evidence for CMC were heterogeneous for *BRAF* status under limiting-dilution conditions, suggestive of heterogeneity of CMC.

Conclusion: The method reported here represents a rapid approach for determination of *BRAF* status in the blood of patients with CMC.

1. Introduction

Over the last decade, advances in melanoma translational research have attempted to identify key components in molecular and genetic alterations that affect progression and prognosis of the disease and that can possibly be therapy targets at the same time.

BRAF mutations are well characterised in melanoma. *BRAF* encodes a serine/threonine kinase downstream for RAS that tranduces the regulatory signal from RAS through MAPK.[1,2] *BRAF* mutations occur in around 40% of primary melanomas (range: 11–69%) and in 50% of metastatic lesions (range: 27–68%).[3] The most common *BRAF* alteration is the codon 600 valin to glutamate mutation (c.1799 T > A; p.V600E) resulting in increased kinase activity. It occurs in more than 90% of the cases, whereas the mutations GT > AA (c.1798/99; p.V600K) and GT > AG (c.1798/99; p.V600R) occurring at the same codon represent less then 10% of *BRAF*-mutated melanoma.

Some studies suggested an impact of *BRAF* mutation on prognosis in melanoma; in particular the *BRAF* V600E

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mutation was significantly associated with diminished duration of response to chemotherapy or with shortened survival from diagnosis of stage IV melanoma patients.[4] Lymph node metastases with *BRAF* mutations and/or alterations of multiple tumour suppressor genes were found to be associated with reduced survival.[5] Similarly *BRAF*-mutated form was shown to be associated with significantly worse overall survival in patients receiving biochemotherapy.[6] However, others did not find any effect on overall disease free survival.[7,8]

The multi-kinase inhibitor sorafenib inhibits BRAF activity and it has been therefore investigated in melanoma, but results are controversial.[9,10] PLX4032 (PDB ID: 3C4C) is a *BRAF* kinase inhibitor for the treatment of melanoma harbouring the V600E mutation. In a phase II study 32 patients with metastatic melanoma with the V600E *BRAF* mutation were treated at the recommended dose of 960 mg. A total of 26 of the 32 patients had a response (81%), with a complete response in 2 patients and a partial response in 24 patients. The estimated median progression-free survival among these patients was more than 7 months. In the phase I study no treatment response was observed in patients without the mutation, and the progression-free survival was less than 2 months, consistent with historical data.[11]

The introduction in clinical practice of molecularly targeted agents is a promising strategy, but limited by the fact that expression of targeted proteins is usually defined at cancer diagnosis, while mutations, some of which lead to acquired drug resistance, may emerge during treatment. To address this issue, the characterisation of circulating tumour cells (CTC) would be a useful tool to monitor tumour plasticity over time without needing serially invasive biopsies. Direct RT-PCR analysis of blood would permit detection of transcriptome of CTC, but this approach is limited by the difficulty to obtain a relatively pure population after enrichment for CTC. If detection of mutant forms is of interest, elimination of the wild-type sequences, or at least their substantial reduction in the original cDNA sample, can minimise the mutant/wild-type sequences competition problems and it would be therefore helpful for identification of mutant sequences. A possibility consists of a selective cleavage of the wild-type sequences prior to PCR.

We established a sensitive method for detection of *BRAF* mutations at codon 600 in whole blood samples of patients affected by melanoma who resulted positive for the presence of circulating melanoma cells (CMC). The method based on selective cleavage of wild-type sequences by taking the advantage of the presence of a TspR1 enzyme restriction site located at the site of the mutation.

2. Material and methods

2.1. Sample selection, RNA extraction and reverse transcription

Twenty-one tissue samples of metastatic cutaneous ($n = 16$) or uveal ($n = 5$) melanoma lesions and matched blood samples were collected according to approved guidelines of our Institution. Patients received informed consent. Total cellular RNA was extracted from 10 mL of EDTA-stabilised blood peripheral samples by acid guanidinium thiocyanate/phenol chloroform isolation,[12] including Phase Lock Gel Heavy (Eppendorf, Hamburg, Germany) and further purified using the High Pure Isolation Kit (Roche Diagnostics, Mannheim, Germany) as outlined by the manufacturer. Total RNA of tissues was isolated by RNeasy Mini Kit, including RNase-Free DNase Set (Qiagen, Hilden, Germany). RNA integrity was checked eletrophoretically and RNA concentration and purity was determined by spectrophotometry. Samples were stored at −80 °C until analysis. Two micrograms of RNA were diluted in 15 μL of RNase-free water, incubated for 5 min at 65 °C and placed on ice for further 5 min. A 7.5 μL mixture containing 2 μL of oligo-p$(dT)_{15}$ primer (0.8 μg/μL), 2 μL of deoxynucleoside triphosphate (5 mM), 0.5 μL of RNAsin (40 units/μL), 1 μL of Omniscript Reverse Transcriptase (4.5 units/μL), and 2 μL of reverse transcriptase buffer (×10) was prepared and added to the diluted RNA and incubated for 1 h at 37 °C. Omniscript Reverse Transcriptase was then inactivated for 5 min at 95 °C and cDNA was stored at −20 °C. All reverse transcriptase reagents, except oligo p$(dT)_{15}$ primers and RNAsin (Roche Diagnostics), were purchased from Qiagen.

2.2. Pre-Real Time PCR treatment

cDNA templates were amplified by a short conventional RT-PCR using a gradient Cycler (DNA Engine Thermal Cycler, BIORAD Laboratories, Munich, Germany). Primers sequences for *BRAF* (NM_004333.4) were designed as follows: forward: 5′-TTTCTTCATGAAGACCTCACAGTAA-3′; reverse: 5′-GCATA TACATCTGACTGAAAGC-3′. Primers amplified a fragment of 176 bp which include codon 600. Primers were purchased from Metabion (Martinsried, Germany). Four microlitres of cDNA were diluted to a volume of 50 μL of PCR mix containing each PCR primer (0.1 pmol), 2 mM dNTPs (Roche), 2 mM MgCl2, 1× OptiBuffer, 4 U BIO-X-ACT Short polymerase (Bioline, Luckenwalde, Germany). Samples were amplified with a pre-cycling hold at 95 °C for 5 min, followed by 15 cycles of denaturation at 95 °C, annealing for 30 s at 55 °C, elongation at 72 °C for 30 s and a final extension at 72 °C for 10 min.

Ultra-pure cDNA was recovered by DNA Clean and Concentrator (Zymo Research, Freiburg, Germany) in 10 μL volume of a mix containing 1X NEB Buffer 4 (New England Biolabs, Frankfurt am Main, Germany) supplemented with 100 μL/mL Bovine Serum Albumin (New England Biolabs). Samples were enriched for mutants by digestion with 6 U TspR1 (restriction site = NNCASTGNN; New England Biolabs) at 65 °C for 4 h. After 2 h of incubation, samples were shortly centrifuged in order to settle down the condension water.

2.3. Quantitative real-time PCR

BRAF RT-PCR assays were performed using a LightCycler (Roche Diagnostic). Primers sequences were described above. Probes sequences were designed as follows: Sensor: 5′-LC Red 640-ACCCACTCCATCGAGATTTCACTG-Pho-3′; Anchor: 5′-ACAAAATGGATCCAGACAACTGTTCAAACTGATG-Fluo-3′. The sensor probe was designed to bind the wild-type form (Fig. 1). Fluorophore probes were purchased from TIB Molbiol (Berlin, Germany). Two microlitres of cDNA were diluted to a volume of 20 μL of PCR mix (LightCycler Faststart DNA Master Hybridisation Probes; Roche Diagnostic) containing 0.5 pmol of

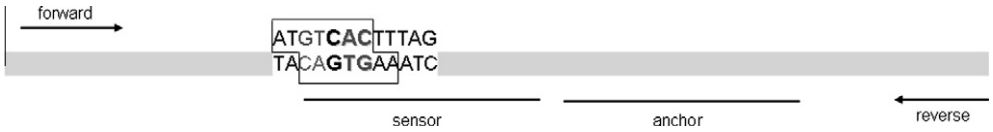

Fig. 1 – Annealing sites of primers and probes on the *BRAF* sequence. (Codon 600 bases are marked in bold; bases in grey are the bases recognised by the enzyme TspR1 and the sequence cut by the enzyme is marked in box).

primer and 0.2 pmol of probe and a final $MgCl_2$ concentration of 5 mM. Samples were amplified with a pre-cycling hold at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C, annealing for 10 s at 64 °C, elongation at 72 °C for 7 s and a final extension at 72 °C for 2 min. The melting curve consisted of 95 °C for 15 s, 45 °C for 1 min with transition rate 20 °C/s and an acquisition step from 45 to 90 °C with transition rate 0.3 °C/s. All the samples were analysed in duplicate and the presence of the mutations was assessed by melting curve analysis (LightCycler software version 3, Roche).

Only blood samples that resulted positive for the presence of CMC have been analysed for *BRAF* status. Presence of CMC has been assessed by Real Time PCRs routinely employed in our laboratory for detection of the two melanocyte transcripts tyrosinase (*TYR*) and Melan-A (*MLANA*) as described elsewhere.[13] The PCR consented detection of a single melanoma cells in 1 mL blood. A sample was considered positive for presence of melanoma cells when it resulted positive for any of the two markers.

3. Results

3.1. *Overview of the protocol*

The final experimental protocol is illustrated in Fig. 2. After a brief conventional PCR for amplification of *BRAF* sequences containing the site of eventual mutations (codons 599 and 600), PCR products were cleaned up and enriched for mutants by cleavage of the wild-type forms. The presence of a TspR1 restriction site (NNCASTGNN) located at codon 600 in *BRAF* wild-type form gene allowed us to efficiently digest selectively the wild-type *BRAF* sequences (TACAGTGAA) as none of the other possible V600 mutations were substrates for TspR1 (Fig. 1). The digested material was then re-amplified in a Real Time RT-PCR using the same primer sequences employed for the conventional PCR and a sensor probe binding to codons 599–607 of the wild-type form in order to practically allow detection within a single PCR of all sort of mutants at codons 599–600. Presence of mutations was subsequently assessed by melting curve analysis: *BRAF* wild-type control cDNA showed a melting peak at 68 °C whereas the *BRAF* V600E mutant control showed a melting peak at 64 °C.

3.2. *Sensitivity of melting curve analysis for BRAF genotyping*

To test the method a conventional PCR has been performed with cDNA from the breast cancer cell line MCF-7 (known to be wild-type for *BRAF* codon 600), with cDNA from the melanoma cell line SK-Mel28 (known to bear the *BRAF* V600E mutation) and with a 50% mix of both. cDNA was previously obtained by transcribing 2 μg of RNA. After digestion by TspR1 the amount of wild-type sequences was significantly reduced as shown in Fig. 3A.

In a serial dilution experiment spiking cDNA of the cell line SK-Mel28 into cDNA of the cell line MCF-7 the method allowed to detect mutated cDNA till a dilution of $1:10^4$ (Fig. 3B).

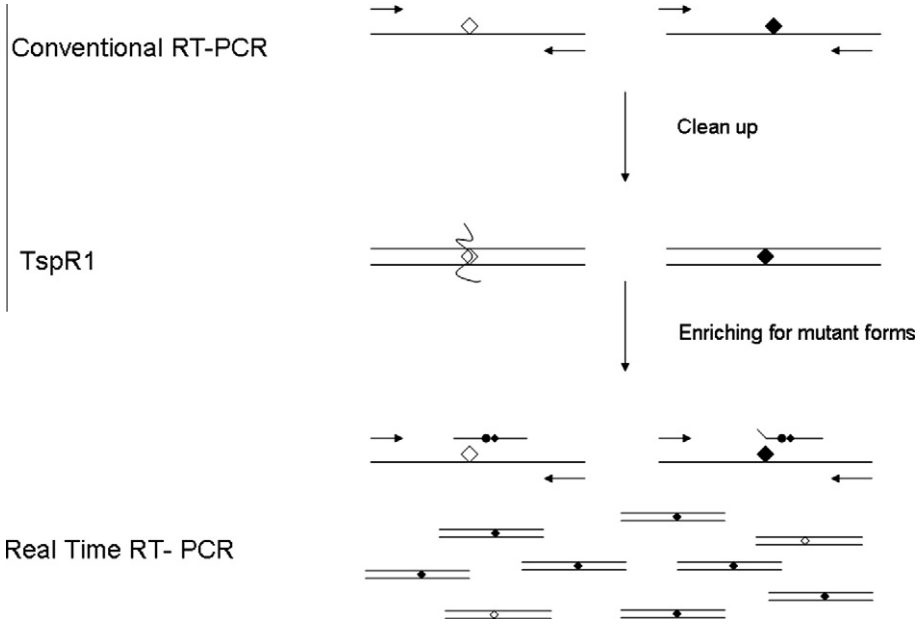

Fig. 2 – Overview of the protocol.

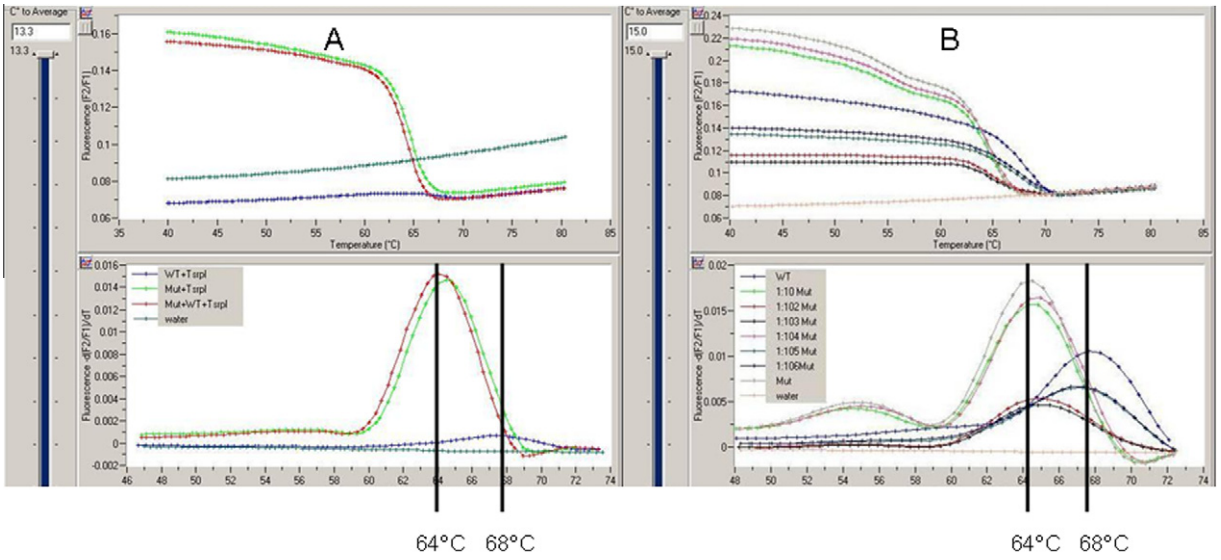

Fig. 3 – (A) Melting curve analysis by employing cDNA from the breast cancer cell line MCF-7 (known to be wild-type for *BRAF* codon 600), with cDNA from the melanoma cell line SK-Mel28 (known to bear the *BRAF* V600E mutation) and with a 50% mix of both. Digestion by TspR1 reduced the amount of wild-type sequences significantly. (B) Serial dilution experiment spiking cDNA of the cell line SK-Mel28 into cDNA of the cell line MCF-7. The method allowed to detect mutated cDNA till a dilution of 1:10^4.

3.3. *BRAF* analysis in peripheral blood (PB) and matched tissue samples

PB samples resulting positive for presence of CMC (*TYR* and/or *MLANA* positive by Real Time PCR [13]) and matched tissue specimens from 21 different AJCC stage IV melanoma patients were analysed. Correct tissue sampling was also verified by evaluating expression of *TYR* and *MLANA* by Real Time PCR. A 91% (19/21) correspondence between *BRAF* status in tissue and PB specimens was observed as presented in Table 1. Seven (33%) tissue and nine (43%) MLANA and/or TYR-positive blood samples showed the *BRAF* point mutation V600E. In two patients *BRAF* resulted to be wild-type in tissue, but mutated in peripheral blood. RNA from tissue specimens of the two discordant cases was isolated two further times confirming the results.

Table 1 – *BRAF* status in 21 tissues and in peripheral-blood matched samples.

Patient	Tissue origin	Mutation Analysis	
		Tissue	Peripheral Blood
#1 M, 37 yrs, UM	Liver	WT	WT
#2 F, 43 yrs, CM	Subcutis	WT	Mut (V600E)
#3 M, 36 yrs, CM	Lymphnode	Mut (V600E)	Mut (V600E)
#4 M, 63 yrs, CM	Lung	WT	WT
#5 F, 41 yrs, UM	Liver	WT	WT
#6 M, 33 yrs, CM	Subcutis	Mut (V600E)	Mut (V600E)
#7 M, 54 yrs, CM	Lymphnode	WT	WT
#8 F, 57 yrs, CM	Subcutis	WT	WT
#9 F, 50 yrs, UM	Liver	Mut (V600E)	Mut (V600E)
#10 F, 43 yrs, CM	Lymphnode	WT	WT
#11 M, 48 yrs, CM	Lung	Mut (V600E)	Mut (V600E)
#12 M, 59 yrs, UM	Liver	Mut (V600E)	Mut (V600E)
#13 M, 73 yrs, CM	Liver	WT	WT
#14 M, 65 yrs, CM	Lymphnode	WT	WT
#15 F, 49 yrs, CM	Subcutis	Mut (V600E)	Mut (V600E)
#16 M, 53 yrs, UM	Liver	WT	WT
#17 M, 89 yrs, CM	Liver	WT	WT
#18 F, 31 yrs, CM	Subcutis	WT	WT
#19 F, 57 yrs, CM	Lymphnode	Mut (V600E)	Mut (V600E)
#20 F, 58 yrs, CM	Subcutis	WT	Mut (V600E)
#21 M, 51 yrs, CM	Lymphnode	WT	WT

Yrs: years; UM: uveal melanoma; CM: cutaneous melanoma; wt: wild-type; Mut: mutated form.

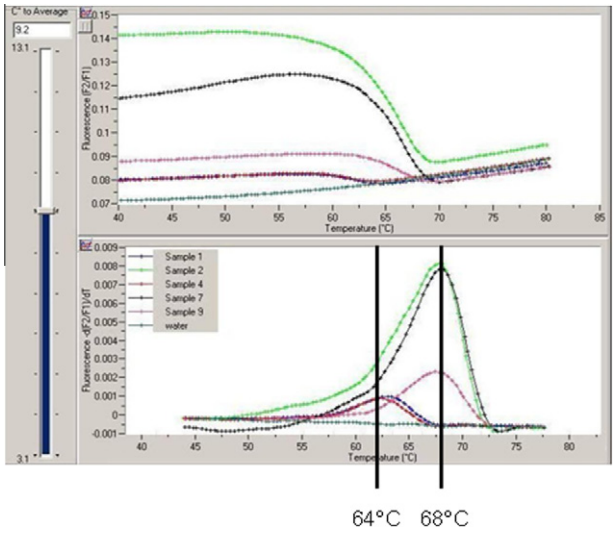

Fig. 4 – Melting curve analysis of five peripheral blood samples (assumed to have a single melanoma cell per sample) of patient #21, who showed *BRAF* wild-type genotype in tissue and *BRAF* V600E mutant in blood.

3.4. *Circulating melanoma cells heterogeneity*

We then asked whether contradictory results between blood and tissue might be due to heterogeneous expression of the mutated form V600E by CMC. From patient #20 (whose melanoma showed to bear the *BRAF* V600E mutation in blood, but not at tissue level) and from patient #21 (whose tumour resulted to be wild-type both at tissue and blood level) 20 mL blood was collected in order to estimate the number of CMC in blood as described elsewhere.[14,15] Further 10 mL blood was drawn and thereafter split in aliquots in order to have single or very few melanoma cells per sample to allow heterogeneity testing. Specimens were analysed for *TYR* and *MLANA* expression by Real Time PCR and samples which resulted positive for the melanoma markers were further analysed for *BRAF* mutation. In patient #20 the results suggested heterogeneity among CMC as in two out of five *TYR* and/or *MLANA* positive samples *BRAF* was mutated, and non-mutated in the other samples (Fig. 4), whereas in patient #21 seven out of seven *TYR* and/or *MLANA* positive samples revealed wild-type *BRAF* (not shown). PCR products were commercially sequenced confirming the findings obtained by melting curve analysis.

4. Discussion

The current study described the development and initial application of a specific and highly sensitive method for detection of *BRAF* V600 mutation in excess of wild-type *BRAF* forms. *BRAF* sequences were amplified in a conventional RT-PCR and digested by the restriction enzyme TspR1 targeting the wild-type form. Digested PCR products were further amplified on a Real Time cycler and evaluated for presence of mutation by Melting Curve Analysis.

The enzyme TspR1 had been previously used to detect *BRAF* mutations at codon 600.[16–18] Panka et al. recently reported on a similar method for detection of *BRAF* mutation in tumour biopsies and blood.[18] Their protocol consisted of a series of PCR amplifications and restriction digestions that take advantage of exclusive characteristics of the wild-type and V600E-mutated form at codon 600 of the gene *BRAF*. Differently from our method they did not use a Real Time PCR for detection of *BRAF* mutants and they needed to include a further amplification step using a unique nested forward oligonucleotide which created a restriction site for the enzyme Xba1 in the amplified product only with the mutant sequence. Mutant products could be detected in the presence of a thousandfold excess of wild-type RNA. We were able to detect the *BRAF* V600E mutant form till a dilution step of 1:10,000 as showed by diluting *BRAF* V600E mutated cDNA (derived obtained transcribing 2 μg RNA) into wild-type cDNA. The higher sensitivity of a fluorescence-based PCR products detection compared to conventional PCR followed by separation on acrylamide gel might explain the difference. The method established by Panka allowed detection of the sole V600E form whereas the method we established could practically detect all sorts of mutants at codons 599–600 since different mutations can be differentiated and identified with melting curve analysis by characteristic melting temperature shifts. Similarly to Panka, we also observed an incomplete digestion by TspR1, but the relatively higher abundance of mutated form after digestion let Real Time PCR detection of the mutated form in the presence of a ten-thousandfold excess of wild-type cDNA in the starting material.

In clinical specimens we observed a *BRAF* status correspondence of 91% comparing tissue and blood matched specimens. Two patients (#2 and #20) showed a discrepancy concerning *BRAF* status between tissue and blood; in particular they both showed a wild-type genotype at tissue level and the mutant V600E in the blood. In both patients a mutated tumour subclone can be postulated, that led to shedding of CTC, although these initial findings cannot provide a formal proof. Similarly discrepancies have been observed by others,[6,19] but *BRAF* mutations were evaluated in circulating DNA in serum and, whether mutated DNA actually derives from melanoma cells remains to be determined. We evaluated *BRAF* at RNA level and even in the case of the presence of mutated DNA in the RNA samples, the sequence would have been not amplified since primers overlap two different exons. A discrepancy in phenotype and genotype between CTC and tissue has been observed in other tumour entities.[20,21] In patients with primary breast cancer CTC showed to express higher level of Her-2 and significantly lower level of ER- and PR-receptor compared to primary tumour suggesting that the biology of the primary tumour directed the spread of CTC.[21] Biological differences between tumour and circulating tumour cell compartment might therefore explain the discrepancy of the findings. In our case, a relatively naturally enriched population of melanoma cells bearing the *BRAF* V600 mutant in blood might have allowed us to detect melanoma cells bearing the mutation. In case of patient #20 our analysis showed that blood samples with PCR evidence for CMC were heterogeneous under limiting-dilution conditions, suggestive of heterogeneity of CTC with wild-type as well as mutated *BRAF* subpopulations, as already reported for other cancer entities.[22,23] In order to exclude any bias in the

procedure, we repeated the same experiment in another patient, who resulted to bear a wild-type *BRAF* genotype both at tissue and at circulating melanoma cell level, validating the robustness of our detection method.

Characterisation of CTC can provide a strategy for non-invasive serial monitoring of tumour genotypes during treatment and may be useful in monitoring treatment response. The use of BRAF inhibitors to target *BRAF* mutated cells in patients with melanoma seemed to be effective, but genotypic changes during time or presence of a heterogeneous population of cells for *BRAF* status may limit the efficacy of the treatment. The method reported here represents a rapid approach for determination of *BRAF* status in blood of patients with CMC. Further studies involving serial analysis of blood and tissue specimens are needed for more detailed estimation of the clinical utility of this assay.

Conflict of interest statement

None declared.

Role of the funding source

None.

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REFERENCES

1. Davies H, Bignelli GR, Cox C, et al. Mutations of the *BRAF* gene in human cancer. *Nature* 2002;**417**:949–54.
2. Kolch W. Meaningful relationship: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. *Biochem J* 2000;**351**(Pt. 2):289–305.
3. Platz A, Egyhazi S, Ringborg U, Hansson J. Human cutaneous melanoma; a review of NRAS and *BRAF* mutation frequencies in relation to histogenic subclass and body site. *Mol Oncology* 2008;**1**:395–405.
4. Houben R, Becker JC, Kappel A, et al. Constitutive activation of the Ras-Raf signaling pathway in metastatic melanoma is associated with poor prognosis. *J Carcinog* 2004;**3**:6.
5. Daniotti M, Oggionni M, Ranzani T, et al. *BRAF* alterations are associated with complex mutational profiles in malignant melanoma. *Oncogene* 2004;**23**:5968–77.
6. Shinozaki M, O'Day SJ, Kitago M, et al. Utility of circulating B-RAF DNA mutation in serum for monitoring melanoma patients receiving biochemotherapy. *Clin Cancer Res* 2007;**13**:2068–74.
7. Board RE, Ellison G, Orr MC, et al. Detection of *BRAF* mutations in the tumour and serum of patients enrolled in the AZD6244 (ARRY-142886) advanced melanoma phase II study. *Br J Cancer* 2009;**101**:1724–30.
8. Akslen LA, Angelini S, Straume O, 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**:312–7.
9. Hauschild A, Agarwala SS, Trefzer U. Results of a phase III, randomized, placebo-controlled study of sorafenib in combination with carboplatin and paclitaxel as second-line treatment in patients with unresectable stage III or stage IV melanoma. *J Clin Oncol* 2009;**27**:2823–30.
10. McDermott DF, Sosman JA. Gonzalez R et al: Double-blind randomized phase II study of the combination of sorafenib and dacarbazine in patients with advanced melanoma: a report from the 11715 Study Group. *J Clin Oncol* 2008;**26**:2178–85.
11. Flaherty KT, Puzanov I, Kim KB, et al. Inhibition of mutated, activated BRAF in metastatic melanoma. *N Engl J Med* 2010;**363**:809–19.
12. Chomczynski P, Sacchi N. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;**162**:156–9.
13. Keilholz U, Goldin-Lang P, Bechrakis NE, et al. Quantitative detection of circulating tumor cells in cutaneous and ocular melanoma and quality assessment by real-time reverse transcriptase-polymerase chain reaction. *Clin Cancer Res* 2004;**10**:1605–12.
14. Fusi A, Liu Z, Schmittel A, Tinhofer I, Trefzer U, Keilholz U. Monitoring of circulating tumor cells in a patient with synchronous metastatic melanoma and colon carcinoma. *Ann Oncol* 2010;**21**:1734–5.
15. Fusi A, Reichelt U, Busse A, et al. Expression of the stem cell markers nestin and CD133 on circulating melanoma cells. *J Invest Dermatol* 2011;**131**:487–94.
16. Domingo E, Laiho P, Ollikainen M, et al. BRAF screening as a low-cost effective strategy for simplifying HNPCC genetic testing. *J Med Genet* 2004;**41**:664–8.
17. Zatelli MC, Trasforini G, Leoni S, et al. BRAFV600E mutation analysis increases diagnostic accuracy for papillary thyroid carcinoma in fine-needle aspiration biopsies. *Eur J Endocrinol* 2009;**161**:467–73.
18. Panka DJ, Sullivan RJ, Mier JW. An inexpensive, specific and highly sensitive protocol to detect the BRAFV600E mutation in melanoma tumor biopsies and blood. *Melanoma Res* 2010;**20**:401–7.
19. Pinzani P, Salvianti F, Cascella R, et al. Allele specific Taqman-based real-time PCR assay to quantify circulating BRAFV600E mutated DNA in plasma of melanoma patients. *Clin Chim Acta* 2010;**411**:1319–24.
20. Maheswaran S, Sequist LV, Nagrath S, et al. Detection of mutations in EGFR in circulating lung-cancer cells. *N Engl J Med* 2008;**359**:366–77.
21. Fehm T, Hoffmann O, Aktas B, et al. Detection, characterization of circulating tumor cells in blood of primary breast cancer patients by RT-PCR, comparison to status of bone marrow disseminated cells. *Breast Cancer Res* 2009;**11**:59.
22. Ignatiadis M, Kallergi G, Ntoulia M, et al. Prognostic value of the molecular detection of circulating tumor cells using a multimarker reverse transcription-PCR assay for cytokeratin 19, mammoglobin A, and HER2 in early breast cancer. *Clin Cancer Res* 2008;**14**:2593–600.
23. Laversha MA, Han J, Asgari Z, et al. Fluorescence in situ hybridization analysis of circulating tumor cells in metastatic prostate cancer. *Clin Cancer Res* 2009;**15**:2091–7.