

Mutation analysis of *BRAF* and *KIT* in circulating melanoma cells at the single cell level

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BACKGROUND: The availability of molecular-targeted therapies for the treatment of melanoma has emphasised the need to identify mutations in target genes such as *BRAF* and *KIT*. Circulating tumour cells (CTC) are present in the peripheral blood of a significant proportion of cancer patients.

METHODS: High molecular weight melanoma-associated antigen (HMW-MAA) was used to isolate melanoma cells from peripheral blood as it is selectively expressed at high levels on melanomas. The HMW-MAA-positive cells were isolated using immunomagnetic beads. After removing CD45⁺ cells, CTC were identified by staining with MART-1- and gp100-specific antibodies (HMW-MAA⁺, CD45⁻, MART-1/gp100⁺). Single, isolated CTC were then subjected to *BRAF* and *KIT* mutational analysis.

RESULTS: CTC (HMW-MAA⁺, CD45⁻, MART-1/gp100⁺) were isolated from the blood of 11 patients and *BRAF* and *KIT* were sequenced in nine and four patients, respectively. The *BRAF* sequences identified in the CTC were inconsistent with those identified in autologous melanoma tumours in three patients and the *KIT* sequences were inconsistent in three patients. In addition, polyclonal *BRAF* mutations were identified in one patient and concomitant mutations in *BRAF* and *KIT* were identified in another patient.

CONCLUSION: Melanoma cells show clonal heterogeneity. Therefore, CTC genotyping may be crucial for successful molecular-targeted therapy.

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Therapies that target specific molecules markedly inhibit cancer growth in several malignancies, and provide valuable strategies for the treatment of advanced melanoma (Romano *et al*, 2011). In recent years, *BRAF* and *KIT* have become established therapeutic targets in melanoma patients showing activating mutations in these oncogenes. Significant clinical benefits have been achieved using the RAF kinase inhibitor, vemurafenib, to treat melanomas showing *BRAF* mutations and the receptor protein kinase inhibitor, imatinib, to treat melanomas showing *KIT* mutations (Flaherty *et al*, 2010; Carvajal *et al*, 2011; Chapman *et al*, 2011; Guo *et al*, 2011). Somatic missense activating mutations in *BRAF* have been identified in about 60% of primary melanoma lesions (Davies *et al*, 2002), and *KIT* mutations in 14% and 18% of acral lentiginous and mucosal melanomas, respectively (Woodman and Davies, 2010). However, it is crucial that genetic mutations present in the melanoma lesions are identified if we are to design tailor-made therapies for individual patients. The tumour genotypes that determine the selection of molecular-targeted therapies are usually identified in primary tumours; however, primary tumours are not always representative of metastases. For example, Terheyden *et al* (2010) reported the case of a melanoma patient

with a primary lesion and lymph node metastases that showed a wild-type *KIT* genotype, but with lung metastasis harbouring a *KIT*^{V559A} mutation.

Circulating tumour cells (CTC) have been identified in peripheral blood from a significant proportion of patients with recurrent disease, and may still be detectable following removal of the primary tumour (Allard *et al*, 2004; Mocellin *et al*, 2006). It may be a source of valuable information because they can be obtained via routine blood sampling, they provide real-time information about a patient's current disease state, and their features may mirror those of recurrent tumours. However, CTC from melanoma patients have only been characterised to a limited extent. This limitation reflects, at least in part, difficulties in isolating these rare cells from the many circulating blood cells. Recently, melanoma cells were isolated from melanoma patients using immunomagnetic beads coated with antibodies specific for chondroitin sulphate proteoglycan (CSPG) 4 (Ulmer *et al*, 2004; Kitago *et al*, 2009; Suesskind *et al*, 2011). This tumour antigen, also known as high molecular weight melanoma-associated antigen (HMW-MAA), can be used to identify and isolate CTC from peripheral blood because it is expressed on the melanoma cell membrane in >85% of primary and metastatic melanoma lesions (Campoli *et al*, 2004). High affinity CSPG 4-specific antibodies are available, and the corresponding epitopes are not detectable on normal blood cells.

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Therefore, the aim of the present study was to isolate intact CTC from peripheral blood using a three-step purification procedure incorporating positive selection with a pool of three HMW-MAA-specific monoclonal antibodies (mAbs) followed by negative selection with a CD45-specific mAb. The purity of the isolated CTC was monitored according to the expression of HMW-MAA and MART-1/gp100 (melanoma markers), and lack of CD45 expression (a haematopoietic cell marker). *BRAF* and *KIT* mutations in CTC were then examined at the single cell level and the genotypes compared with those of the primary and metastatic lesions.

MATERIALS AND METHODS

Melanoma cell lines, reagents, mAbs, and clinical specimens

Detailed procedures and patient information are described in Supplementary Information.

Isolation of melanoma cells from peripheral blood

Heparinised blood samples (5 ml) were treated with RBC lysis solution (Qiagen, Germantown, MD, USA), after which $>4 \times 10^6$ mononuclear cells were obtained. Circulating tumour cells were isolated from the mononucleated cells using HMW-MAA-specific mAb-coated immunomagnetic beads (Dynabeads CELlection Pan Mouse IgG Kit; Invitrogen, Oslo, Norway) according to manufacturer's instructions with minor modifications. Melanoma cells were labelled with mAb before capture using immunomagnetic beads because previous work showed that this indirect technique is better than direct techniques for separating melanoma cells from the blood (Kitago *et al*, 2009). In brief, 6 μ l of a cocktail containing HMW-MAA-specific mAbs 763.74, VF1-TP41.2, and VT80.12 (final concentration of each mAb: 33 μ g ml⁻¹) was added to 4×10^6 cells suspended in 294 μ l phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). Samples were incubated with rotation at 22 r.p.m. at room temperature (Invitrogen) for 20 min. After washing with PBS/0.1% BSA, the samples were mixed with 25 μ l of immunomagnetic beads (anti-mouse IgG-coated), which included 1×10^7 beads. The mixtures were incubated with rotation at 22 r.p.m. for an additional 30 min. Samples were then placed in a DYNAL MPC-S magnetic device (Invitrogen) for 2 min. Following removal of the fluid by careful pipetting and washing twice with PBS/0.1% BSA, cells were released from the beads by incubation with releasing buffer (Invitrogen) for 20 min on a mixing device. The mixture was then vigorously pipetted and replaced in the DYNAL MPC-S magnetic device for 2 min to isolate HMW-MAA⁺ cells. Human CD45-specific mAb-coated immunomagnetic beads (Invitrogen) were then used to remove contaminating white blood cells. After incubation and washing, the HMW-MAA⁺, CD45⁻ cell population was collected. The HMW-MAA⁺, CD45⁻ cells were then smeared onto a silane-coated glass slide (Dako, Carpinteria, CA, USA) and stained (EnVision System; Dako) with a cocktail of MART-1 and gp100 antibodies. The stained cells were then subjected to laser-capture microdissection using a PALM-MB microdissection system (PALM Microlaser Technologies, Bernried, Germany) and scored as positive when a clear signal was observed over at least 80% of the cell surface. MART-1/gp100⁺ (HMW-MAA⁺, CD45⁻, MART-1/gp100⁺) cells were then isolated individually and placed separately in adhesive tubes (Meiwafosis, Tokyo, Japan). These cells were referred to as CTC.

DNA extraction

For extraction of genomic DNA from single CTC, 3 μ l of lysis buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mg ml⁻¹ proteinase K (Roche Diagnostics, Basel, Switzerland), and 3% Tween-20) was

added to each tube. The tubes were subsequently vortexed. After centrifugation, each CTC was incubated for 16 h at 50°C, after which proteinase K was heat inactivated at 95°C for 10 min. As a negative control, empty tubes were incubated with lysis buffer. In addition, DNA was extracted from formalin-fixed, paraffin-embedded surgically resected tumour tissues. Three 6- μ m sections were cut from paraffin-embedded tissue blocks with a sterile microtome blade and mounted on glass slides. After deparaffinisation, tissues stained with methylene blue were dissected manually on an inverted microscope to select melanoma lesions. The dissected tissues were placed in sterile tubes and digested with a mixture containing 20 μ l of proteinase K and 180 μ l of 25 mM Tris-HCl at 37°C for 12 h. After completion of the incubation, proteinase K was heat inactivated at 80°C for 20 min.

Analysis of *BRAF* and *KIT* mutations

Primers were designed to amplify exon 15 of *BRAF* and exons 11, 13, and 17 of *KIT*, all of which include mutational hot spots (Davies *et al*, 2002; Woodman and Davies, 2010). Exon 15 of *BRAF* was amplified using a hemi-nested PCR, and exons 11, 13, and 17 of *KIT* were amplified using nested PCR (Buttner *et al*, 1998; Hofmann *et al*, 2009; Lin *et al*, 2009). The primer sequences and PCR cycling conditions are shown in Supplementary Table S1. The first PCR amplification for exon 15 of *BRAF* was conducted in a 20- μ l reaction volume containing Ex Taq buffer with 2.0 mM MgCl₂, 0.2 mM dNTP mixture, 0.3 μ M primers, 0.5 U of Ex Taq (Takara, Shiga, Japan), and 2 μ l of DNA template. Then, 0.2 μ l of the first PCR amplification product was used as template for the second PCR amplification, which was conducted in a 20- μ l reaction volume. *KIT* exons were amplified in singleplex reactions. Nested PCR for *KIT* exons, both the first and second PCR amplifications, was conducted in 20 μ l reaction volumes including 2 μ l of DNA template. The PCR amplification was performed in iCycler (Bio-Rad Laboratories, Hercules, CA, USA). After confirming the size of the PCR products on agarose gels, the products were purified using the QIAquick PCR Purification Kit (Qiagen). Direct sequencing was performed to identify mutations within exon 15 of *BRAF* and exons 11, 13, and 17 of *KIT*. The primer used for the forward reading reaction of *BRAF* exon 15 was 5'-TCATAATGCTTGCTCTGATAGGA-3'. The primers used for the forward reading reaction of *KIT* exons 11, 13, and 17 were the same as the forward inner primers used for nested PCR. Each sequencing reaction was carried out using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) in a 20- μ l volume containing purified PCR product and sequence primer. The temperature for the sequencing reaction was 96°C for 1 min, which was followed by 25 cycles at 96°C for 10 s, 54°C for 5 s, and 60°C for 4 min. The reaction products were precipitated with 95% ethanol and 3 mM sodium acetate, washed with 70% ethanol, resuspended in 20 μ l Hi-Di formamide and loaded onto an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The analysis was conducted following the accepted PCR guidelines (Bustin *et al*, 2009).

RESULTS

Melanoma cell separation in an *in-vitro* model experiment

To establish the optimal conditions for isolating CTC from peripheral blood, cultured melanoma cells were mixed with peripheral blood and isolated using immunomagnetic beads. Briefly, the melanoma cell lines 888mel, 928mel, or MMG1, which express HMW-MAA and MART-1/gp100, were spiked into healthy human peripheral blood samples at concentrations ranging from 1×10^2 to 1×10^5 cells per 5 ml blood. The mixtures were sequentially stained with mAbs specific for HMW-MAA- and

CD45 and fractionated using immunomagnetic beads. A mixture of mAbs that recognise distinct and spatially distant epitopes on HMW-MAA was used to minimise false-negative results caused by differential expression of HMW-MAA epitopes, and to increase the detection level of the system (Kitago *et al*, 2009). To confirm that the isolated HMW-MAA⁺, CD45⁻ cells were melanoma cells, the cells were stained with MART-1- and gp100-specific mAbs and observed under a microscope (Figure 1A). Isolated HMW-MAA⁺, CD45⁻, MART-1/gp100⁺ cells comprised 1–24% of the melanoma cells initially mixed with the peripheral blood (Table 1). The three melanoma cell lines showed different recovery rates. The expression level of HMW-MAA was measured by reverse transcription PCR (RT-PCR) and flow cytometry, and that of MART-1/gp100 by immunostaining. Results showed no correlation between the recovery rates and the level of HMW-MAA or MART-1/gp100 (data not shown).

The possibility that mutant alleles may ‘drop-out’ during PCR, a known problem when using single cells, was assessed (Piyamongkol *et al*, 2003). The *BRAF* mutation was analysed in single HMW-MAA⁺, CD45⁻, MART-1/gp100⁺ cells isolated from blood mixed with 928mel cells, which harbour the heterozygous *BRAF*^{V600E} mutation (Lin *et al*, 2009). DNA was extracted from each cell and *BRAF* exon 15, which is the most common mutation site (Platz *et al*, 2008), was sequenced in each cell individually. The *BRAF*^{V600E} mutation was detected in 88.9% of the isolated 928mel cells (48 of 54 cells sequenced). The drop-out of mutant alleles during single-cell PCR is likely to be a relatively rare event; a previous study using a melanoma cell line showed that the phenomenon occurs at a frequency of only 2–14% (1–7 out of 50 single melanoma cells) (Lin *et al*, 2009). Thus, in this *in-vitro* model experiment, the *BRAF* mutation was successfully detected in individual melanoma cells isolated from peripheral blood.

CTC separation from the peripheral blood of melanoma patients

Next, CTC were isolated from the peripheral blood of 11 patients with stage IIIC/IV melanoma (Table 2), all of which expressed HMW-MAA and MART-1/gp100, as detected by immunohistochemical

analysis. Peripheral blood was incubated with the mAb cocktail and magnetic beads as described in the previous section. The HMW-MAA⁺, CD45⁻ cells were isolated and the cellular phenotype was confirmed by MART-1/gp100 expression. The HMW-MAA⁺, CD45⁻, MART-1/gp100⁺ cells were detected in all the patients. The isolated cells were larger and showed an abnormal morphology (Figure 1B). The yield of CTC from 5 ml of blood ranged from 1 to 20 (Table 2). Because MART-1/gp100⁺ cells could be identified microscopically, contamination by non-melanoma cells non-specifically bound to the immunomagnetic beads was unlikely. As controls, peripheral blood samples obtained from healthy individuals were included in every experiment. No HMW-MAA⁺, CD45⁻, MART-1/gp100⁺ CTC were isolated from the peripheral blood of >50 healthy donors.

BRAF mutations in CTC from the peripheral blood of melanoma patients

CTC were captured individually. *BRAF* exon 15 was amplified from single CTC from 9 out of 11 melanoma patients and *BRAF* exon 15 sequences were obtained from 14 individual CTC (Table 3). Single CTC PCR occasionally failed; the success rate of PCR amplification from single CTC ranged from 20% to 100%. In three cases in which primary melanomas showed *BRAF*^{V600E} mutation, CTC shared a similar *BRAF* genotype with the primary tumours (Nos. 4, 5, and 6; Table 3). By contrast, lymph node metastases from patient No. 9 showed a *BRAF*^{V600E} mutation while two CTC showed wild-type *BRAF*. This result should be interpreted with caution because of the possibility of mutant allele drop-out in CTC; however, that phenomenon is likely to be relatively rare, as discussed earlier. Interestingly, CTC from patient No. 5 showed a *BRAF*^{V600K} mutation and a wild-type sequence in addition to the *BRAF*^{V600E} mutation (which was also identified in the primary tumour and lymph node metastasis; Figure 2A). This suggests clonal heterogeneity in terms of *BRAF* mutations during melanoma progression.

The *BRAF* genotype in metastases of patient Nos. 5, 6, and 9 was assessed further because these three cases showed a mismatched *BRAF* genotype between metastasis and CTC. Clusters of 30–50

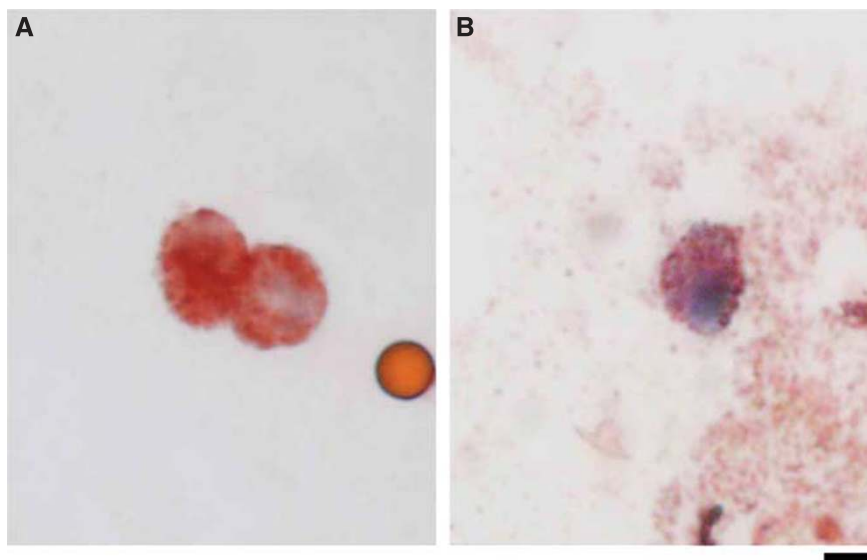


Figure 1 Melanoma cells isolated from peripheral blood. HMW-MAA⁺, CD45⁻ cells were isolated on immunomagnetic beads and stained with a mixture of MART-1 and gp100 antibodies. MART-1/gp100-positive cells stained brownish-red with aminoethylcarbazole. (A) Spiked MMG-1 cells isolated from the peripheral blood of a healthy individual. The small light-brown spheroid is an immunomagnetic bead. (B) CTC detected in a patient with melanoma. Scar bar: 5 μ m.

melanoma cells were microdissected from different areas within metastatic lesions to detect minor populations of tumour cells, since conventional sequencing usually detects genetic mutations at a rate of >20% (Ellison *et al*, 2010). The microdissected lesions showed both *BRAF*^{V600E} and wild-type *BRAF* in Nos. 5 and 6, but not in No. 9 (Supplementary Table S2). Although the macrodissected lesions showed *BRAF*^{V600E} in No. 5 and wild type in No. 6, some microdissected samples showed different genotypes (wild type in No. 5 and *BRAF*^{V600E} in No. 6), suggesting that a substantial number of cells harboured different *BRAF* genotypes. Metastatic lesions may occasionally include heterogeneous tumour cells. The *BRAF*^{V600K} mutation was not detected in the metastasis of No. 5 nor was wild-type *BRAF* in that of No. 9. These data

suggest that CTC with mismatched genotypes may be derived from minor populations within metastatic lesions.

KIT mutations in CTC isolated from the peripheral blood of melanoma patients

The sequences within three exons (exons 11, 13, and 17) of *KIT*, which are reported to harbour mutations (Woodman and Davies, 2010), were also examined. The PCR amplification success rate ranged from 0% to 50% (Table 3). To optimise PCR conditions from single cells, several conditions were tested, including primers, enzymes, and buffers; however, the success rate did not improve sufficiently. *KIT* genotypes were analysed in only four patients (Table 3). Unlike *BRAF* genotypes, *KIT* genotypes were poorly matched between primary tumours, metastases, and CTC (Table 3). The primary tumours from two patients (Nos. 1 and 10) showed *KIT* mutations, but no *KIT* mutations were detectable in lymph node metastases and/or CTC. However, *KIT* mutations were detected in either metastases or CTC in patient Nos. 4 and 11, whereas the primary lesions showed wild-type *KIT*. It is interesting to note that patient No. 4 showed not only a *KIT*^{V560G} mutation in CTC, but also a *BRAF*^{V600E} mutation in the primary tumour, as well as in another CTC (Figure 2B); this is unusual because mutations in *BRAF* and *KIT* are thought to be mutually exclusive (Curtin *et al*, 2006; Carvajal *et al*, 2011). In addition, *KIT*^{V560G} has not been reported in melanomas, but is prevalent in gastrointestinal stromal tumours and mastocytosis (Tarn *et al*, 2005; Lanternier *et al*, 2008).

Table 1 Recovery of melanoma cells mixed with peripheral blood

No. of melanoma cells mixed in 5 ml of peripheral blood	Observed cell count		
	888mel	928mel	MMGI
10 ⁵	7600 (7.6%)	2420 (2.4%)	1920 (1.9%)
10 ⁴	1910 (19.1%)	260 (2.6%)	162 (1.6%)
10 ³	140 (14%)	20 (2%)	19 (1.9%)
10 ²	24 (24%)	1 (1%)	3 (3%)
0	0 (0%)	0 (0%)	0 (0%)

Results represent the average of two independent experiments.

Table 2 Clinical characteristics of the 11 melanoma patients

No.	Sex	Age (years)	Histopathological type	AJCC stage	Metastatic sites	No. of CTC in 5 ml of PB
1	M	54	Mucosal	IV	Lymph nodes, lung, brain, spinal cord	2–5
2	F	78	Mucosal	IV	Lymph nodes, lung, liver	2
3	M	67	Mucosal	IIIC	Lymph nodes	5
4	F	68	Mucosal	IV	Lymph nodes, lung	1–5
5	F	64	Non-CSD	IV	Lymph nodes, lung, liver, skin	1–20
6	M	61	Non-CSD	IV	Lymph nodes, lung, brain, skin	2–7
7	F	62	Non-CSD	IV	Lymph nodes, lung, peritoneum	1–3
8	M	73	Non-CSD	IV	Lymph nodes, digestive tract, pleura, abdominal cavity, skin	1
9	M	69	Unknown	IV	Lymph nodes, liver, digestive tract, bone, adrenal, skin	8
10	F	62	Acral	IIIC	Lymph nodes	6
11	M	84	Acral	IV	Lymph nodes, lung, brain	7

Abbreviations: CTC = circulating tumour cells; non-CSD = melanoma on non-chronic sun-damaged skin; PB = peripheral blood.

Table 3 Genotypes of *BRAF* and *KIT* in melanoma patients

No.	<i>BRAF</i> (exon 15)				<i>KIT</i> (exons 11, 13, and 17)			
	Tissue		Single CTC		Tissue		Single CTC	
	Primary	Metastasis	No. of cells sequenced	Success rate of PCR	Primary	Metastasis	No. of cells sequenced	Success rate of PCR
1	Wild type	Wild type	Wild type (1)	1/1	D820Y	Wild type	UR	0/3
2	Wild type	NA	Wild type (1)	1/1	Wild type	NA	UR	0/1
3	Wild type	NA	Wild type (1)	1/5	Wild type	NA	NE	NE
4	V600E	UR	V600E (1)	1/1	Wild type	UR	V560G (1)	1/2
5	V600E	V600E	V600E (1), V600K (1), wild type (1)	3/3	Wild type	Wild type	Wild type (1)	1/3
6	V600E	Wild type	V600E (3)	3/3	Wild type	Wild type	NE	NE
7	Wild type	Wild type	Wild type (1)	1/2	UR	Wild type	NE	NE
8	Wild type	Wild type	Wild type (1)	1/1	Wild type	Wild type	NE	NE
9	NA	V600E	Wild type (2)	2/4	NA	Wild type	NE	NE
10	Wild type	Wild type	NE	NE	N822Y	Wild type	Wild type (1)	1/6
11	Wild type	Wild type	NE	NE	Wild type	N822Y	Wild type (1)	1/2

Abbreviations: CTC = circulating tumour cells; NA = tissues not available; NE = not examined; UR = sequence unreadable due to PCR failure.

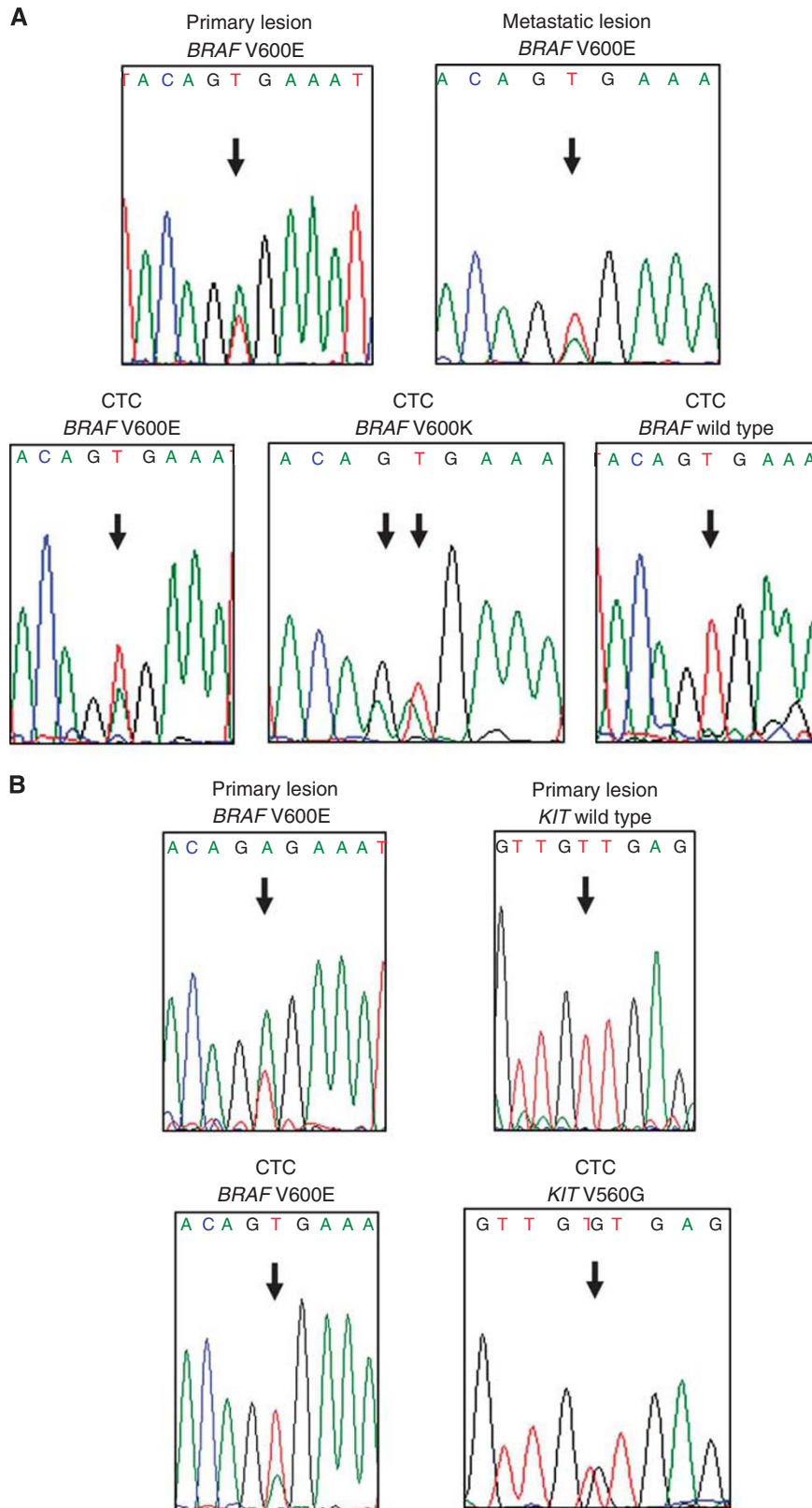


Figure 2 *BRAF* and *KIT* mutations detected in CTC isolated from melanoma patients. **(A)** Polyclonal *BRAF* mutations detected in CTC from patient No. 5. Primary and metastatic lesions showed *BRAF*^{V600E} (upper). Two CTC showed *BRAF*^{V600E} and *BRAF*^{V600K} mutations, respectively (lower left and middle), and another CTC possessed wild-type *BRAF* (lower right). **(B)** Concomitant detection of *BRAF*^{V600E} (lower left) and *KIT*^{V560G} (lower right) mutations in CTC obtained from patient No. 4. The primary lesion showed *BRAF*^{V600E} and wild-type *KIT* (upper).

DISCUSSION

This study describes the purification of CTC from the peripheral blood of melanoma patients using immunomagnetic beads coated with HMW-MAA-specific antibodies followed by immunohistochemical laser dissection techniques. The *BRAF* and *KIT* genotypes of the isolated CTC were then analysed; the results showed that the genotypes of the CTC differed from those of the primary tumours and metastatic lesions. The method outlined in this study presents a new opportunity to characterise the genetic mutations expressed by spreading melanoma cells.

CTC represent a promising prognostic biomarker for melanoma recurrence or progression. Since subclinical, distant metastases occur during the early stages of melanoma development (Ossowski and Aguirre-Ghiso, 2010), detecting melanoma cells released from metastatic tumours may help to identify those melanoma patients at high risk of developing systemic metastatic disease. However, clinical detection of these cells is often extensively delayed after removal of the primary melanoma lesions.

CTC in the epithelial cancers are frequently isolated using immunomagnetic separation techniques incorporating antibodies against epithelial-specific antigens, such as EpCAM, BerEP4, and cytokeratins (Paterlini-Brechot and Benali, 2007; Krebs *et al*, 2010; Negin and Cohen, 2010; Riethdorf and Pantel, 2010). However, somewhat conflicting results have been published regarding the prognostic value of CTC, although it appears that increased CTC numbers are associated with more diffuse cancers, a higher risk of relapse, and a poor prognosis in breast, colon, and other epithelial cancers (Paterlini-Brechot and Benali, 2007; Krebs *et al*, 2010; Negin and Cohen, 2010; Riethdorf and Pantel, 2010). As epithelial-specific antibodies are of no use when attempting to isolate CTC from melanoma patients, CTC have been examined at the mRNA level using PCR-based methods. The presence of melanoma cell-specific mRNA seems to be associated with a poor outcome (Mellado *et al*, 1999; Palmieri *et al*, 1999; Pantel *et al*, 1999; Koyanagi *et al*, 2005); however, such approaches do not permit the genotypic or phenotypic characterisation of melanoma CTC. Therefore, cellular approaches to CTC detection were developed using immunomagnetic cell sorting or other methods (Ulmer *et al*, 2004; Galanzha *et al*, 2009; Kitago *et al*, 2009; De Giorgi *et al*, 2010; Suesskind *et al*, 2011). The HMW-MAA-specific mAbs have been used for the immunomagnetic separation of melanoma CTC (Ulmer *et al*, 2004; Kitago *et al*, 2009; Suesskind *et al*, 2011), but single step selection methods using HMW-MAA still result in blood cell contamination. The HMW-MAA⁺, CD45⁻, MART-1/gp100⁺ cells are morphologically different from blood cells and show atypical features, which can be used to confirm that isolated HMW-MAA⁺, CD45⁻, MART-1/gp100⁺ cells are, in fact, CTC. A potential drawback of this method is the low yield, which may be attributable to the immunomagnetic-negative selection of CD45⁺ cells, since a partial loss of CTC was observed during that process in patients with colon cancer (Ausch *et al*, 2007). Further technical improvements regarding the isolation procedure should aim at improving this aspect.

There are several powerful examples of the application of CTC genotyping to targeted cancer therapy. In lung cancer, molecular analysis of the epidermal growth factor receptor gene in CTC provides important information regarding therapeutic response and prognosis (Maheswaran *et al*, 2008). The human epidermal growth factor receptor-2 (*HER2*) gene is examined in CTC from breast cancer patients because *HER2* antagonists are available for therapy. However, the *HER2* status of the CTC is sometimes different from that of the primary tumours and metastatic lesions (Wulfing *et al*, 2006; Pestrin *et al*, 2009; Flores *et al*, 2010). Furthermore, a subset of patients with *HER2*-negative primary tumours developed *HER2*-positive CTC as the tumour progressed (Meng *et al*, 2004; Pestrin *et al*, 2009). Similarly, the results of the present study show that, although the *BRAF* genotype of the CTC was similar to that of the resected primary tumours, there were still some important

mismatches. The *KIT* genotype showed the greatest variability between primary melanomas, lymph node metastases, and CTC. Since the features of CTC are not always concordant with those of other lesions, it is necessary to pay attention to the genotypes of both the primary/metastatic lesions and the CTC.

CTC from one patient (No. 5) showed three *BRAF* genotypes (*BRAF*^{V600E}, *BRAF*^{V600K}, and wild type). Analysis at the single cell level allowed further genetic dissection, as conventional sequencing techniques fail to detect minor mutations (Ellison *et al*, 2010). The polyclonal genotype implies that melanoma cells have heterogeneous features. It is noteworthy that oesophageal cancer cells disseminate to the bone marrow and lymph nodes at an early stage. Whole genome analysis at the single cell level showed that these disseminated tumour cells are genomically heterogeneous (Stoecklein *et al*, 2008). It is conceivable that, as in oesophageal cancer, heterogeneous melanoma cells may disseminate to various tissues at an early stage. *BRAF* genotyping from single melanoma cells showed heterogeneity within metastatic lesions and primary tumours (Lin *et al*, 2011). In the present study, *BRAF* heterogeneity was also detected within metastatic melanomas through the analysis of DNA isolated from dozens of tumour cells. Intralesional heterogeneity within metastatic lesions is likely to cause mismatched genotypes between CTC and macrodissected metastatic lesions. The CTC features may reflect the cellular state of active melanoma cells migrating from metastatic lesions.

BRAF heterogeneity has significant implications when using *RAF* kinase inhibitors because, in contrast to *BRAF*^{V600E} melanoma cells, *BRAF* wild-type cells are resistant to (and may even be stimulated by) *RAF* kinase inhibitors (Hatzivassiliou *et al*, 2010; Heidorn *et al*, 2010). Furthermore, most patients with *BRAF*^{V600E} mutations develop secondary resistance and show subsequent disease progression. Based on the heterogeneity of melanoma cells, secondary resistance may be attributable to *BRAF* heterogeneity. In this case, the number of wild-type *BRAF* CTC may alter after treatment with kinase inhibitors, and CTC analysis may help to identify the point at which cells 'escape', leading to resistance and relapse.

The CTC analysis also has potential benefits in terms of treating *KIT* mutant melanomas. Recent phase II clinical trials involving patients with metastatic melanoma harbouring *KIT* mutations reported significant clinical responses to imatinib in a subset of patients (Carvajal *et al*, 2011; Guo *et al*, 2011). However, tyrosine kinase inhibitors show little or no activity in melanoma cells harbouring wild-type *KIT* (Becker *et al*, 2007). In a particular case of melanoma with metastases in the lung and lymph nodes, imatinib treatment led to a rapid response in lung metastases harbouring the *KIT*^{V559A} mutation, but no response in lymph node metastases without the mutation (Terheyden *et al*, 2010). Thus, mutation screening is critical if we are to identify those patients that may benefit from the kinase-targeted therapy.

The present study has several limitations. First, the number of melanomas examined was small. Second, PCR amplification from single CTC occasionally failed; melanoma cells may include substances that inhibit PCR. Further improvement of single cell PCR methods may eliminate this limitation. Third, the metastatic potential of CTC is not clear. Furthermore, we do not have data to allow us to examine the relationship between the results of CTC genotyping and clinical outcome. However, CTC may provide real-time information regarding cancers at the whole-body level. More work is necessary to develop methods for the isolation and analysis of CTC, but there is no doubt that the information provided by CTC will improve the ability of clinicians to predict responses to molecular-targeted therapies.

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