Application of a Filtration- and Isolation-by-Size Technique for the Detection of Circulating Tumor Cells in Cutaneous Melanoma

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Analysis of circulating tumor cells (CTC) in the peripheral blood of cutaneous melanoma patients provides information on the metastatic process and potentially improves patient management. The isolation by size of epithelial tumor cells (ISET) is a direct method for CTC identification in which tumor cells are collected by filtration as a result of their large size. So far, ISET has been applied only to CTC detection from epithelial cancer patients, and the technique has never been applied to cutaneous melanoma patients. We herein investigated the presence of CTC by ISET in the peripheral blood of 140 subjects (87 with cutaneous melanomas, 10 subjects undergoing surgery for melanocytic nevi, 5 patients with non-melanoma skin tumors, and 38 healthy volunteers). The identification of the cells trapped in filters as CTC was supported by positivity for immunohistochemical markers and for tyrosinase mRNA by real-time RT-PCR. CTC were neither detected in the controls nor in the *in situ* melanoma group. In contrast, CTC were shown in 29% of patients with primary invasive melanoma and in 62.5% of metastatic melanoma patients ($P < 0.01$). CTC detection correlated with the presence of mRNA tyrosinase in blood samples, assayed by real-time RT-PCR ($P = 0.001$). CTC detection corroborated by suitable molecular characterization may assist in the identification and monitoring of more appropriate therapies in melanoma patients.

INTRODUCTION

The incidence of cutaneous melanoma is steadily increasing in the Caucasian population, faster than that of any other cancer over the past 20 years (Berwick and Wiggins, 2006; Garbe and Eigentler, 2007). Melanoma is characterized by a high tendency to develop metastasis, and given the small size of most primary lesions, the metastatic potential of melanoma is considerably greater than that of other solid tumors. Once melanoma has metastasized, the average survival is 6–9 months, with a 5-year survival rate < 40% (Shivers et al., 1998).

In most cutaneous melanomas, a subset of tumor cells detaches from the primary tumor or metastatic tumor sites, and enters the peripheral circulation. It is estimated that tumor cells circulate in the bloodstream in extremely low number, approximately one tumor cell in the background of 10^6–10^7 normal blood cells (Ross et al., 1993). Analysis of circulating tumor cells (CTC) will possibly clarify how melanoma metastasizes with potential future clinical implications (Mocellin et al., 2006; Jacob et al., 2007).

The majority of CTC detection methods available to date are based upon immuno-affinity and density gradient centrifugation enrichment procedures using antibodies against surface antigens (Paterlini-Brechot and Benali, 2007; Bertazza et al., 2008). Isolated tumor cell populations require further characterization of viability and metastatic propensity because tumor cells in the circulation may be dead or in a dormant state (Jacob et al., 2007; Paterlini-Brechot and Benali, 2007). Alternatively, real-time (RT)-PCR has been diffusely used to detect antigen mRNAs, and has been indicated as a sensitive, quantitative indirect method for CTC identification (Zieglschmid et al., 2005). In cutaneous melanoma patients, CTC have been studied only by means of real-time RT-PCR, mostly by multimarker assays including

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Abbreviations: CTC, circulating tumor cells; ISET, isolation by size of epithelial tumor cells

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tyrosinase, p97, MUC18, MelanA/MART1, MAGE3 and/or gp100/pMel-17 (Smith *et al.*, 1991; Ghossein *et al.*, 1998; Curry *et al.*, 1999; Hoon *et al.*, 2000; Proebstle *et al.*, 2000; Brownbridge *et al.*, 2001; Gogas *et al.*, 2002; Osella-Abate *et al.*, 2003; Palmieri *et al.*, 2003; Reynolds *et al.*, 2003; Mocellin *et al.*, 2004; Schmidt *et al.*, 2005; Voit *et al.*, 2005; Medic *et al.*, 2007; Visus *et al.*, 2007; Xi *et al.*, 2007). One of the main limitations of real-time RT-PCR assays is that cell integrity is lost during RNA extraction, thus preventing the analysis of cell morphology and phenotype. In addition, RT-PCR allows neither tumor cell counting, nor demonstration of tumor microemboli, which are considered to have a major role in the metastatic process. Thus, the development and optimization of new technologies to identify and characterize CTC in cutaneous melanoma are highly needed.

The isolation by size of epithelial tumor cells (ISET) is a direct method for CTC identification, in which tumor cells are collected by filtration because of their large size in comparison with peripheral blood leukocytes (Vona *et al.*, 2000, 2002). The method is sensitive, being capable of detecting one single tumor cell added to 1 ml of blood, and simple and fast, consisting in only one filtration step. Furthermore, ISET-isolated tumor cells can be subsequently recovered from the filter and evaluated by immunohistochemistry and/or molecular-genetic analyses (Vona *et al.*, 2002). So far, ISET has been applied only to CTC detection from epithelial cancer patients (Vona *et al.*, 2000, 2004; Pinzani *et al.*, 2006), and the technique has never been validated in cutaneous melanoma patients.

The purposes of this study are the following: (i) to assess whether ISET may be successfully applied to the identification of circulating melanoma cells in experimental conditions; (ii) to identify the frequency of ISET-isolated CTC in surgically treated cutaneous melanoma patients; (iii) to correlate the presence of ISET-isolated CTC with peripheral blood tyrosinase mRNA levels by real-time RT-PCR; and (iv) to correlate the presence of CTC with other conventional clinico-pathological prognostic factors.

RESULTS
Sensitivity of ISET and real time RT-PCR methods
Filtration of five artificial samples containing 1 SK-MEL-28 cell per ml of blood showed a sensitivity threshold for ISET close to one melanoma cell per milliliter of blood, as previously reported by Vona *et al.* (2000). On five samples we were able to recover 1 SK-MEL-28 cell 4 times. Similarly, 3/3 (100%) 1-cell samples produced an amplification plot by real time RT-PCR assay for the detection of tyrosinase mRNA.

Patients' cohort characteristics
The total number of patients with melanoma was 87. The mean age of patients with *in situ* melanoma ($n=17$) was 62 years (median 61, range 39–86 years). There were eight female and nine male patients. Tumor site distribution was: upper and lower extremities (five cases), trunk (11 cases) and acral (one case). These cases were all *in situ* superficial spreading melanomas. The mean age of patients with invasive malignant melanoma ($n=62$) was 61 years (median

63, range 23–94 years). Thirty-three patients (53.2%) were male, and 29 patients (46.8%) were female. The tumor site distribution was as follows: head and neck ($n=5$, 8.2%); upper and lower extremities ($n=15$, 24.2%); trunk ($n=36$, 58.1%); volar and subungueal regions ($n=4$, 6.5%), and genital area ($n=2$, 3.2%). Superficial spreading melanoma was the most common histological type ($n=48$, 77.4%), followed by nodular melanoma ($n=9$, 14.5%), acral-lentiginous melanoma ($n=3$, 4.8%) and lentigo maligna melanoma ($n=2$, 3.2%). Fourteen cases (22.6%) had a Clark's level of II, and 18 cases (29%) and 30 cases (48.4%) had levels III and IV, respectively. Thirty-seven (59.7%) patients had melanomas $\leqslant 1.0$ mm in Breslow thickness, 12 (19.4%) had lesions 1.0–2.0 mm in thickness, nine (14.5%) patients had melanomas more than 2.0 mm but less than 4.0 mm in thickness and four patients (6.4%) had melanomas >4.0 mm in thickness. Tumor mitotic rate was 0–1/mm^2 in the majority of invasive melanomas (38/60 evaluable cases). Ulceration was shown in 14 (22.6%) of invasive melanomas. Upon surgical excision, 24 patients underwent sentinel lymph node biopsy. Four patients were found sentinel lymph node biopsy positive and subsequently submitted to regional lymphadenectomy. Median and mean follow-up for primary melanomas was 22 and 28 months, respectively (range 19–32 months).

The group of metastatic melanomas ($n=8$) included patients in Stage IV disease who had developed distant metastases, the commonest sites being lung, liver, and bone. At the time of blood sampling, all metastatic patients were under adjuvant chemo-immunotherapy according to different schedules. The treatment schedule was chosen on the basis of metastatic disease extent, age, clinical conditions and co-morbidities. Median and mean follow-up for metastatic melanomas was 18 and 16.8 months, respectively (range 12–23 months).

The study series also included 10 patients submitted to surgical excision of melanocytic nevi. Upon histopathological examination, the lesions were classified as follows: common acquired melanocytic nevus ($n=1$); Spitz's nevus ($n=2$); so-called atypical, ''dysplastic nevus'' ($n=4$), common blue nevus ($n=2$), small superficial congenital nevus ($n=1$). In addition, the analysis included five patients surgically treated for basal cell carcinoma ($n=3$), seborrheic keratosis ($n=1$), and angiokeratoma ($n=1$).

Detection of circulating melanoma cells by ISET
Morphological criteria for the identification of circulating melanoma cells included: (i) cell size $\geqslant 16 \mu$m, (ii) nucleo-cytoplasmic ratio $\geqslant 50\%$, (iii) irregular nuclear shape, (iv) hyperchromatic nucleus, and (v) basophilic cytoplasm. Tumor cells were observed mostly in single units whereas microemboli or clusters of cells were rare. We were not able to identify any melanin pigment within CTC. Immunohistochemical analysis with anti-S100 protein, MART-1, or HMB-45 antibody identified circulating melanoma cells as weak to moderately stained cells (Figure 1). The melanocytic nature was confirmed in selected ISET-positive filters by assessment of tyrosinase mRNA by real time RT-PCR after direct
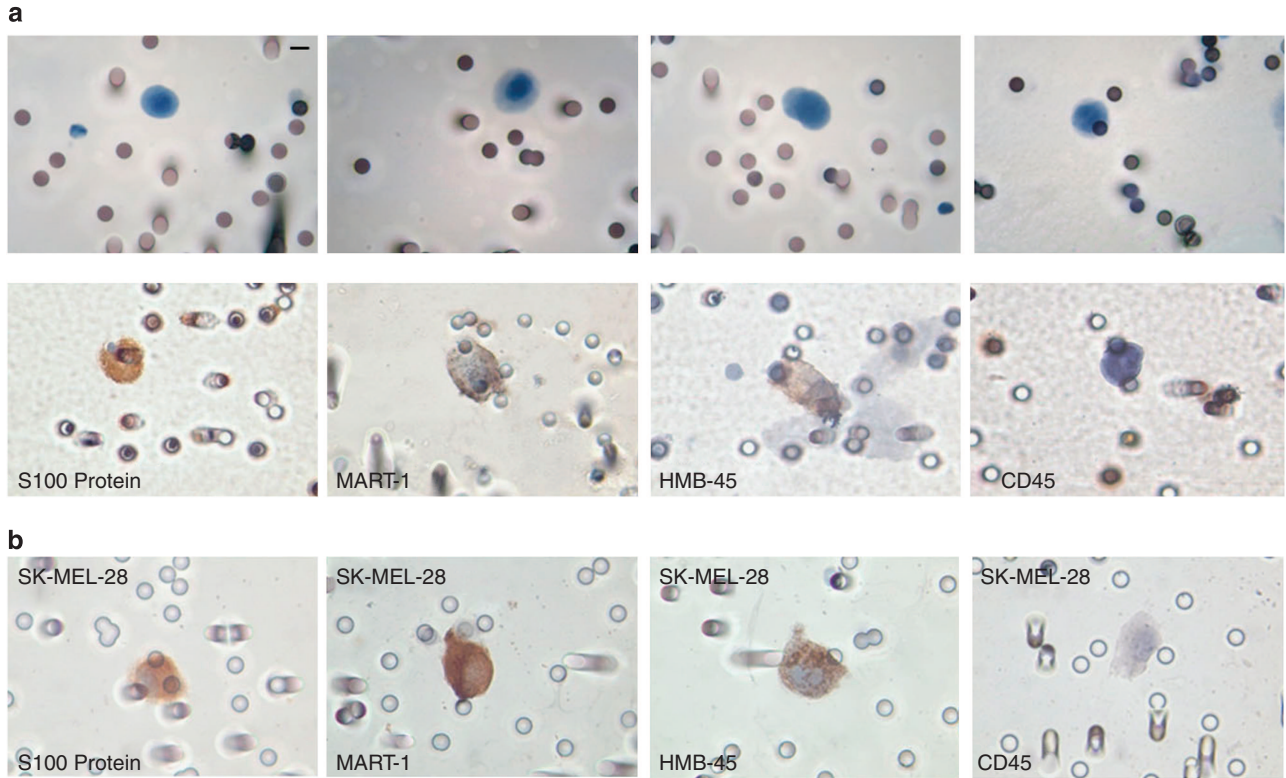
Figure 1. **Morphological and immunohistochemical identification of circulating melanoma cells.** (**a**) ISET-positive samples display isolated circulating melanoma cells. Neoplastic cells fulfill criteria for circulating tumor cells, including: (i) cell size $\geqslant 16\,\mu m$, (ii) nucleo-cytoplasmic ratio $\geqslant 50\%$, (iii) irregular nuclear shape, (iv) hyperchromatic nucleus, and (v) basophilic cytoplasm (upper row, original magnification $\times 63$, hematoxylin and eosin stain). Immunohistochemical analyses with anti-S100 protein, MART-1, HMB-45 antibodies identify circulating melanoma cells as weak to moderately stained cells. CD45 is negative (lower row, original magnification $\times 63$). (**b**) Melanoma cell lines (SK-MEL-28) immunostained in artificial samples with S100 protein, MART-1, HMB-45, and CD45. Note that the intensity of the immunostaining is stronger and more diffuse. (Original magnification $\times 63$; scale bar $10\,\mu m$).
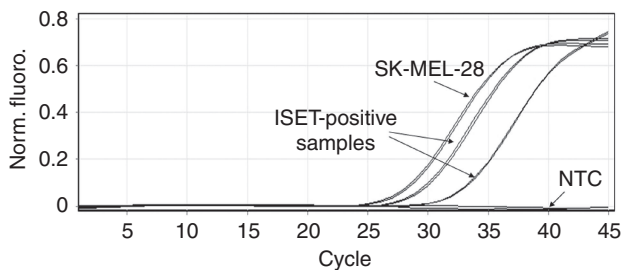
Figure 2. **Amplification plots of real-time RT-PCR assay for tyrosinase mRNA expression of SK-MEL-28 melanoma cell lines and melanoma patients' CTC isolated on ISET filters.** Evaluation of tyrosinase mRNA on selected ISET-positive filters was obtained after direct lysis of cellular material on the spot filter surface and subsequent RNA extraction.

Table 1. Detection of circulating melanoma cells by ISET according to patients' categories (n=140)

Category	Presence of CTC, n (% per category)[1]
Healthy volunteers (*n*=38)	0 (0)
Non-melanoma skin tumors (*n*=5)	0 (0)
Melanocytic nevi (*n*=10)	0 (0)
In situ melanoma (*n*=17)	0 (0)
Primary invasive melanoma (*n*=62)	18 (29)
Metastatic melanoma (*n*=8)	5 (62.5)

[1]$P<0.01$ Pearson's χ^2-test.

lysis of cellular material on the spot filter surface and subsequent RNA extraction (Figure 2). In some cases we observed morphologically doubtful cells with regular shape, slightly superior than pore in size but with a nucleo-cytoplasmic ratio $<50\%$. Most of these cells were eventually recognized as enlarged monocytes. In addition, in three cases we found large cells characterized by an abundant elongated purplish cytoplasm and lobated nuclei that were interpreted as possible immature hematopoietic cells. Leukocytes were also retained on the membrane in a low percentage, often trapped within the pore lumina. Such cells were easily identified due to their smaller size and peculiar nuclear morphology. Superposition of cells or cells and pores, nude nuclei, or excess of staining were regarded as artefacts.

Correlation of CTC with patients' characteristics and conventional prognostic factors
Table 1 summarizes the percentage of ISET-isolated CTC-positive cases according to patients' categories. CTC were not

detected in the control groups (either healthy volunteers or non-melanoma skin tumors). In addition, CTC were neither detectable among patients with melanocytic nevi nor in patients with *in situ* melanoma. Conversely, the percentage of CTC raised from 29% in the primary invasive melanoma group to 62.5% among metastatic melanoma patients ($P<0.01$). Taken together, the median CTC count of ISET-positive samples was 0.8 CTC per ml of blood (range 0.25–3.5). Among primary invasive melanomas, no significant correlation was shown between CTC positivity and main clinico-pathological parameters, including Breslow thickness, Clark's level, ulceration, histotype, anatomical site (Table 2).

Among primary and metastatic melanoma patients, 42 patients also had postoperative blood draw (15–21 days after surgery). Seven (16.7%) of these patients had positive CTC both preoperatively and postoperatively, whereas 10 (23.8%) preoperatively negative patients became postoperatively positive, with a mean number of CTC found being 0.6 (range 0.2–2.1). Finally, six (14.3%) patients had CTC only in the preoperative blood sample. Only three blood samples of a subgroup of 12 patients submitted to a third blood drawn (12–16 months after surgery) gave positive results. One of these patients was positive before surgery and never turned negative for the whole follow-up period (17 months).

When analyzing the number of CTC per ml of blood, we did not find any statistically significant difference between invasive melanoma (mean ± SE = 0.32 ± 0.09) and metastatic melanoma subjects (mean ± SE = 0.73 ± 0.33), $P=0.178$.

Expression of tyrosinase mRNA transcript in blood samples
Tyrosinase mRNA transcripts were assessed in the blood samples from most patients evaluated by ISET, including 64 patients with Stage I primary cutaneous melanoma (13 *in situ* and 51 invasive melanomas), seven patients with metastatic melanoma, 38 healthy subjects, nine patients with melanocytic nevi and five non-melanoma skin tumors. Overall, mRNA tyrosinase transcripts were found in 4 of 7 (57.1%) melanoma metastases, 12 of 51 (23.5%) of primary invasive melanomas, 3 of 13 (23.0%) of *in situ* melanomas, 0 of 9 melanocytic nevi, 0 of 5 non melanoma skin tumors, and 0 of 38 healthy subjects. Considering the whole series ($n=85$), CTC detection by ISET assay was significantly associated with mRNA tyrosinase levels in blood samples ($P=0.001$) with 77.6% concordant results (57 negative and 9 positive subjects with both methods). In addition, the percentage of tyrosinase mRNA transcripts differed according to melanoma histotype ($P=0.041$), with a higher percentage of positive cases in nodular and acral-lentiginous melanomas in comparison with superficial spreading and lentigo maligna melanoma.

When analyzing the tyrosinase mRNA quantitative results, we found a statistically significant difference between *in situ* melanoma (mean ± SE = 0.38 ± 0.32 SK-MEL-28 cell equivalents/ml blood) and invasive melanoma (mean ± SE = 4.1 ± 1.7) ($P=0.035$), but not with the metastatic melanoma subjects (mean ± SE = 19.3 ± 8.5).

Table 2. Association between circulating melanoma cells and clinical–pathological prognostic parameters in primary cutaneous invasive melanomas (*n*=62)

Parameter	Cases, *n* (%)	Presence of CTC, *n* (%)
Thickness		
≤1 mm	37 (59.7)	8 (21.6)
1.01–2.0 mm	12 (19.3)	6 (50.0)
2.01–4.0 mm	9 (14.5)	3 (33.3)
>4 mm	4 (6.5)	1 (25.0)
Level		
II	14 (22.6)	3 (21.4)
III	18 (29.0)	6 (33.3)
IV	30 (48.4)	9 (30.0)
TMR/mm² [1]		
0–1	38 (63.3)	11 (28.9)
2–5	12 (20)	4 (33.3)
>5	10 (16.7)	2 (20)
Ulceration		
Absent	48 (77.4)	12 (25.0)
Present	14 (22.6)	6 (42.9)
Anatomic site		
Head and neck	5 (8.0)	1 (20.0)
Trunk	36 (58.1)	9 (25.0)
Extremities	15 (24.2)	4 (26.7)
Acral sites	4 (6.5)	2 (50.0)
Genital regions	2 (3.2)	2 (100)
Histotype		
Superficial spreading melanoma	48 (77.4)	12 (25.0)
Nodular melanoma	9 (14.5)	4 (44.4)
Lentigo maligna melanoma	2 (3.2)	0 (0)
Acral–lentiginous melanoma	3 (4.8)	2 (66.6)

[1]Evaluated in 60 of 62 samples.

DISCUSSION
Our data show that intact circulating melanoma cells are detectable by ISET in the peripheral blood of patients with primary and metastatic melanoma and that the presence of ISET-isolated CTC correlates with peripheral blood tyrosinase mRNA levels detected by real-time RT-PCR. In this study, we developed a method to isolate tumor cells from peripheral blood of melanoma patients, and investigated in a

prospective manner whether the presence of CTC correlated with melanoma tumor progression.

As pointed out in previous studies (Vona *et al.*, 2000; Pinzani *et al.*, 2006; Paterlini-Brechot and Benali, 2007), the main advantage of the ISET procedure over other methods for CTC identification results from the possibility to morphologically identify the isolated cells. In our experience with ISET assay, general morphological criteria (large cell size, nucleo-cytoplasmic ratio, irregular nuclear shape, hyperchromatic nucleus, basophilic cytoplasm), commonly applied to the identification of epithelial cells were also valuable for the identification of melanoma cells. However, in contrast to epithelial cells, which more likely form cell aggregates and clusters, circulating melanoma cells usually appear as isolated individual cells and are recognizable as either non-pigmented large epithelioid or smaller cells with a roundish to oval shape and indented nucleus. Enlarged monocytes must be distinguished from smaller melanoma cells, and their regular shape, size slightly superior than pore diameter, and the nucleo-cytoplasmic ratio <50%, support their specific identification. Immunohistochemical analysis with S100 protein, HMB-45, MART-1 or CD45 was found to be helpful in doubtful cases to support the correct identification of melanoma cells. The intensity of immunostaining in CTC was often weak and less diffuse in comparison with melanoma cell lines analyzed under identical circumstances (filters). The difference may be due to loss of antigens' expression and therefore, more than one marker may be required to conclusively identify cells filters as CTC.

Our present analysis of 42 melanoma patients in whom both preoperative and postoperative blood draw were taken revealed that 16.7% had positive CTC both preoperatively and postoperatively, whereas 23.8% preoperatively negative patients became postoperatively positive. Preliminary investigations have suggested that intraoperative tumor manipulations at surgery for primary breast, colorectal, and prostatic cancers induce tumor cell dissemination (Weitz *et al.*, 1998; Weitz and Herfarth, 2001; Lintula *et al.*, 2004). Nevertheless, in the absence of long-term follow-up, these studies failed to provide evidence that this cell dissemination may cause secondary metastatic dissemination and affect survival (Eschwege *et al.*, 2009). Further longitudinal studies on larger series of patients are needed to clarify the clinical impact of the surgical procedures in shedding melanoma cells into the peripheral blood.

A statistically significant difference in CTC presence was found between primary invasive (18 of 62, 29%) and metastatic melanoma patients (5 of 8, 62.5%), whereas controls were negative. Thus, the false-positive rate among controls (including healthy subjects or patients with non-malignant diseases) was virtually zero, indicating a high specificity of the method. Also the quantitative evaluation of the results evidenced differences between groups. However, because of the relatively small sample size, no conclusive data can be obtained in this regard.

In melanoma patients, CTC have been investigated mostly by indirect methods, based on real-time quantitative RT-PCR assays, with controversial results on clinical significance and prognostic value in early or advanced disease stages (Smith *et al.*, 1991; Ghossein *et al.*, 1998; Curry *et al.*, 1999; Hoon *et al.*, 2000; Proebstle *et al.*, 2000; Brownbridge *et al.*, 2001; Gogas *et al.*, 2002; Osella-Abate *et al.*, 2003; Palmieri *et al.*, 2003; Reynolds *et al.*, 2003; Mocellin *et al.*, 2004; Schmidt *et al.*, 2005; Voit *et al.*, 2005; Medic *et al.*, 2007; Visus *et al.*, 2007; Xi *et al.*, 2007). Reliability of CTC detection has been questioned because of the variable results reported in patients with stage IV melanoma and the diversity in technical protocols and quality controls. Genomic instability commonly observed in metastatic tumors (Klein *et al.*, 2002), discontinuous shedding of CTC into the bloodstream, and low frequency of CTC (close to the detection limit of PCR methods), have been advocated as the most likely biological variables underlying these heterogeneous results. One of the main limitations of RT-PCR assays is that cell integrity is lost during RNA extraction, thus preventing the analysis of cell morphology and phenotype. In addition, RT-PCR does not allow counting of tumor cells and demonstration of tumor microemboli, which are considered to have a major role in the metastatic process.

In contrast with previous methods, CTC detection by ISET in melanoma seems to provide a substantial advantage for its future application in clinical practice. However, several issues remain to be answered. Initially, no direct comparison of ISET with alternative techniques on a sufficiently large cohort of patients has been performed as yet. This hampers the identification of a gold standard for the detection of CTC in melanoma. Second, in the current series for primary melanoma we were not able to show a statistically significant association between the presence of CTC and reliable prognostic factors, including tumor thickness and level, and the occurrence of ulceration. Larger studies are required also for these specific aspects, to establish the contribution of tumor thickness and other prognostic variables on the presence of circulating tumor cells and vice-versa. Third, qualitative CTC detection (presence versus absence of CTC in blood) appears to be sufficient to classify patients into different subgroups. However, it remains to be established whether quantitative CTC evaluation provides additional clinically relevant information. In the current series, no statistically significant difference in terms of number of CTC per ml of blood was detected in primary invasive versus metastatic melanoma category. Furthermore, although increased CTC numbers should be linked to a worse survival, the short follow-up of this study does not allow an assessment of the effect of ISET-isolated CTC on this key clinical outcome. In this regard, it should be underlined that an expected advantage of ISET procedure should come from its potential to characterize the isolated cells especially in patients where no tissue from the primary melanoma is available for analysis. For example, examination of the phenotypic and molecular pattern of the isolated cells might support the identification of patients who could benefit from targeted therapy. To this regard, we provided evidence that immunohistochemistry can be successfully applied to ISET-isolated CTC for their proper phenotypic identification. Our data provided evidence that the capability of ISET to detect

CTC is not restricted to epithelial cells, as the name would imply, but is also able to detect non-epithelial cells.

In conclusion, in this study ISET-isolated CTC have been identified in the bloodstream of both primary and metastatic melanoma patients. The clinical relevance of this finding is still under scrutiny and the biological fate of circulating melanoma cells must be carefully investigated. However, present results indicate the need for larger prospective studies with a number of purposes, including whether individual CTC may predict the behavior of the entire pool of occult tumor cells and drug sensitivity of the corresponding tumor tissue.

MATERIALS AND METHODS

Cell lines
The SK-MEL-28 cell line (Carey *et al.*, 1976) (ATCC, HTB-72, Bethesda, MD), derived from a human malignant melanoma was cultured in DMEM-F12 containing 10% fetal bovine serum and used for RNA extraction for real time RT-PCR standard curve and to generate artificial samples for ISET sensitivity.

Sensitivity tests
Sensitivity test was performed for both real time RT-PCR and the ISET method. Eight different artificial samples were obtained with the addition of 1 SK-MEL-28 melanoma cell to 1 ml of a blood pool by single cell micropipetting performed under the microscope. Five samples underwent ISET filtration and the remaining 1-cell samples were submitted to mRNA extraction for RT-PCR measurements.

Patients
Seventy-nine consecutive patients with primary cutaneous melanoma, eight patients with metastatic melanoma, and 38 healthy subjects were recruited for blood donation at the Dermatology Unit, Department of Critical Care Medicine and Surgery and Department of Clinical Physiopathology, University of Florence, Florence, Italy. Similarly, another 10 patients undergoing surgical excision for cutaneous melanocytic nevi and five individuals treated at the Dermatology Unit, Department of Critical Care Medicine and Surgery, University of Florence, for non-melanoma skin tumors underwent blood sampling. All patients were recruited after written consent. A medical history was taken from all patients, including details of the initial treatments and all subsequent therapeutic interventions. In surgically treated patients, the blood samples were taken preoperatively, whereas in patients with metastatic disease samples were obtained during an outpatient clinic visit regardless of current treatment. In selected patients, a second postoperative blood sample was taken, approximately 30 days (range 18–38 days) after surgery for primary melanomas or 21 days after observation for metastatic melanoma patients.

For melanoma patients, disease stage was determined according to the American Joint Committee on Cancer guidelines (UICC International Union Against Cancer, 2002). A careful pathology review of the primary melanoma, including Breslow thickness, Clark level, presence of ulceration and mitotic rate was performed. Following clinical examination, complete blood cell counts, serum biochemistry, chest radiographs and abdomen ultrasonography were performed. Patients with melanoma ≥1 mm in thickness, without distant metastases or palpable regional lymph nodes, underwent

sentinel lymph node biopsy (followed by regional lymphadenectomy in case of sentinel lymph node positivity). Other complementary examinations were performed if indicated. The study was approved by the Institutional Review Board and the Declaration of Helskinki protocols were followed.

Blood collection and isolation by size of epithelial tumor cells
Patients' blood (5–10 ml) was collected in EDTA tubes and processed within 4 hours. ISET was performed by means of an ISET Device (Metagenexl (NOTE added in Proof: Since submission of this article for publication, Metagenex name has been changed to ScreenCell.), Paris, France), consisting in an evolution of a previously described module of filtration (Vona *et al.*, 2000; Paterlini-Brechot and Benali, 2007), kindly provided by Metagenex. The instrument provides a controlled aspiration under vacuum (0.025 Bar). Filtration was carried out in a disposable filtration block, ISET Metablock (Metagenex) containing a membrane with 8 μm pores. The blocks have 10 compartments allowing processing of up to ten 1-ml blood samples from the same patient in parallel. Peripheral blood was diluted 1:10 with the ISET Metabuffer (Metagenex), and incubated for 10 minutes at room temperature. Ten milliliters of diluted solution, corresponding to 1 ml of undiluted blood, was loaded on each ISET Metablock compartment and filtered. The membrane was then washed once with phosphate-buffered saline, disassembled from the filtration module, and allowed to air-dry.

Cell staining and immunostaining
After rehydration with phosphate-buffered saline, the spots obtained on the filter (each one corresponding to 1 ml blood), were stained with hematoxylin solution S (Merck KGaA, 64271 Darmstadt, Germany), applied to the membrane for 1 minute, followed by 1% Shandon eosin Y aqueous (Thermo Electron Corporation, Thermo Fisher Scientific Inc., Waltham, MA) for 45 seconds. For immunostaining the whole Metafilter was hydrated with TBS (Tris-buffered saline pH 7.4). Having removed the white part, the Metafilter was cut into five parts each containing a pair of spots that have been washed in TBS for 1 minute. The excess TBS was removed with absorbant paper and the filters were put on the paraffin film in a humid chamber. Each spot was incubated for 5 minutes at room temperature with 70 μl of permeabilized buffer; in each immunostaining step spots were covered with a coverslip 20 × 20 mm to contain the liquid. After being washed quickly in a bath containing distilled water each pair of spots was incubated overnight with 70 μl + 70 μl of following antibodies: polyclonal anti-human S-100 protein (Dako, Glostrup, Denmark) diluted 1:100 in Antibody Diluent (Ventana Medical Systems, Tucson, AZ), monoclonal mouse anti-human melanosoma (clone HMB-45, Cell Marque Corp., Rocklin, CA) diluted 1:20 in Antibody Diluent, monoclonal mouse anti-human MART-1/Melan A (clone A103, Ventana) prediluted and monoclonal mouse anti-human CD45 (clones 2B11 + PD7/26, Dako) diluted 1:50 in antibody diluent. The filters were then washed once with TBS for 1 minute and immersed in a bath containing distilled water. Staining was achieved by treating each spot with 70 μl EnVision Detection System Peroxidase/DAB, Rabbit/Mouse (K5007, Dako) for 40 minutes at room temperature followed by 3.3′diaminobenzidine (Dako) for 10 minutes at room temperature as chromogen. Each pair of spots was then placed on a paraffin film and the nuclei were slightly counterstained with Mayer's hematoxylin for

6 minutes. Finally, the spots were rinsed with running water, dried for 10 minutes at room temperature and mounted on a slide with Faramount Aqueous mounting medium. Metafilter containing SK-MEL-28 melanoma cell lines in artificial samples have been immunostained at the same conditions, with 1 hour of incubation of the primary antibodies instead of overnight.

Real time RT-PCR for tyrosinase mRNA
Blood (2.5 ml) was collected at the same time of blood draw for ISET in ''PAXgene Blood RNA Tubes'' (PreAnalytiX GmbH, Hombrechtikon, CH), containing an RNA stabilizing reagent. Total RNA from whole blood was isolated by ''PAXgene Blood RNA kit'' (PreAnalytiX GmbH), according to the manufacturer's instructions. Evaluation of tyrosinase mRNA concentration on selected ISET-positive filters was obtained after direct lysis of cellular material on the spot filter surface and subsequent extraction by QIamp RNA micro kit (Qiagen, Hilden, Germany). The recovered material underwent the extraction protocol proposed for formalin-fixed microdissected samples. All the extracted RNA was used for reverse transcription.

RNA from blood (7.7 μl) was reverse-transcribed using a commercial kit based on random primers technique (''Taqman Reverse Transcription Reagents'', Applied Biosystems, Foster City, CA) in a final volume of 20 μl, according to the manufacturer's instructions. For real-time RT-PCR, we used a Taqman Gene Expression Assay (Hs00165976_m1, Applied Biosystems). The reaction was carried out in a 7900HT Fast Real-Time PCR System (Applied Biosystems). For each sample measurement 5 μl cDNA were used in a reaction volume of 25 μl ''2 × Taqman Universal PCR Master Mix'' (Applied Biosystems), according to the manufacturer's instructions. All samples were analyzed in duplicate. The thermal profile was the following: 95 °C for 10 minutes, then 95 °C for 15 seconds and 60 °C for 1 minute for 50 cycles. To calculate the expression of tyrosinase mRNA in each sample, we referred to an external reference curve obtained by serial dilutions of RNA from a known number of cells of the melanoma cell line SK-MEL-28. Results were expressed as number of SK-MEL-28 cell equivalents/ml blood.

Statistical analysis
Data analysis was carried out with the SPSS statistical package, version 15.0 (SPSS). Statistical differences between groups were assessed using Pearson's χ^2-test. Comparison of the mean values between groups were obtained by Student's *t*-test. Differences with $P < 0.05$ were considered statistically significant.

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