Tyrosinase mRNA levels in the blood of uveal melanoma patients: correlation with the number of circulating tumor cells and tumor progression

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We measured tyrosinase mRNA levels by real-time quantitative reverse transcription-PCR (qRT-PCR), in the blood of patients with uveal melanoma. Results were correlated with clinical data and, in a subgroup of patients, with the number of circulating tumor cells (CTC) assessed using isolation by size of epithelial tumor cells (ISET). Forty-one patients with uveal melanoma were longitudinally investigated over a period of 5 years. The standard curve of the qRT-PCR method used melanoma cell line SK-MEL-28, added to the blood of normal donors and it was calibrated on a synthetic RNA standard (1 SK-MEL-28 cell corresponding to 18 tyrosinase mRNA copies) to improve the procedural standardization to facilitate the comparison of data collected at different laboratories. Increased tyrosinase mRNA levels were found in at least one of the blood samples in 20 of 41 (49%) uveal melanoma patients (median 0.8 SK-MEL-28 cell equivalents/ml blood; range 0.1–14.4). A significant correlation was found between mRNA tyrosinase levels and tumor dimension ($P<0.01$), disease-free and overall survival ($P<0.05$). CTC were isolated by ISET in five of 16 patients (5.8, 2.33, 2.00, 1.25, and 0.75 CTC/ml of blood) and the corresponding tyrosinase mRNA levels were 2.13, 1.37, 0.83, 0.58, and 0.35 SK-MEL-28 cell equivalents/ml of blood. Tyrosinase was undetectable in 11 ISET-negative patients. Tyrosinase assay by qRT-PCR is a noninvasive method for the detection of tumor progression in uveal melanoma patients. The mRNA tyrosinase levels can be taken as an indirect parameter correlated to the number of CTC isolated from blood by ISET. *Melanoma Res* 20:303–310 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

The incidence of uveal melanoma is approximately six to seven cases/1 000 000 inhabitants [1,2] in the Caucasian population [3]. It is a slowly growing tumor, with a low mitotic index and apoptotic rate [4], often associated with an unfavorable outcome, with more than 50% of affected patients dying of the disease within 5–10 years [5].

Several clinical, histological, and genetic factors have been identified as important risk factors for local recurrence, metastasis development, and survival [6]. As most patients with uveal melanoma do not undergo surgery and are treated with radiotherapy, histological and genetic predictive factors are not usually investigated. Tumor cells of ocular melanoma disseminate through the blood stream, because of the absence of lymphatic vessels in the uveal tract. As blood-borne tumor cell dissemination is the main mechanism responsible for distant metastases, with a marked tendency to metastasize to the liver, the hematological approach may be highly desirable for detecting systemic tumor cell spread [7]. In particular, molecular markers can be useful tools for the early detection of disseminated tumour cells in cancer patients [7,8], whereas PCR-based techniques have already been applied to the diagnosis of subclinical cancer diseases, spreading and monitoring of the treatment response [9].

Several molecular markers for the detection of disseminated tumor cells in the blood of melanoma patients have been investigated [10–14] and, among them, tyrosinase (a key enzyme involved in the biosynthesis of melanin expressed by melanocytes and melanoma cells) appears to be one of the most effective, as its transcripts are generally not detected in blood samples from healthy donors [15]. Tyrosinase has been investigated mainly on cutaneous melanoma, but some studies [16–18] have also evaluated tyrosinase mRNA levels in patients with uveal melanoma. More recent reports [19] indicate that high values of circulating tyrosinase mRNA are frequently found in patients with melanoma liver metastases. Moreover, tyrosinase is an independent prognostic factor in patients with primary uveal melanoma at high risk for

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subsequent metastasis development and reduced survival probability. This assay seems to be useful for addressing adjuvant treatment studies [6].

The aim of this study was to perform a quantitative reverse transcription-PCR (qRT-PCR) method to accurately detect tyrosinase mRNA in serial blood samples of patients affected by uveal melanoma in order to indirectly quantify the number of circulating tumor cells (CTC) during follow-up. Efforts were directed toward improving the procedural standardization both in the preanalytical phase and in the assay protocol to facilitate the comparison of data collected at different laboratories and using different procedures. The method has been applied to patients affected by uveal melanoma with small, medium, and large-size tumors [20] and the tyrosinase levels were related to the main clinical parameters, including clinical outcome. In addition, in a limited number of patients we compared the results obtained by tyrosinase qRT-PCR assay with those obtained by a direct CTC detection method. For this purpose, we have chosen the isolation by size of epithelial tumor cells (ISET) technology that makes it possible to isolate, count, and characterize CTC on the basis of cell size, without the use of capture antibodies [21].

Materials and methods

Patients

Forty-one patients with uveal melanoma were consecutively examined at the Eye Clinic, Department of Oto-neuro-ophthalmology, and enrolled in the study at the Centre of Experimental and Clinical Oncology, Department of Oncology, University of Florence, Italy. Patient characteristics are reported in Table 1.

Informed consensus was obtained from all patients. The study was approved by the Institutional Review Board and complies with the Declaration of Helsinki Statements.

Peripheral blood samples were obtained before the first therapeutic intervention (three samples for each patient). All patients were treated for the primary ocular lesion with surgery or radiotherapy (11 patients with 'exenteratio orbitae' and 30 with radiotherapy). To exclude metastatic disease, patients underwent a complete clinical examination, routine biochemistry (i.e. liver enzymes and lactate dehydrogenase levels), and liver ultrasonography.

Table 1 Clinical data

No. of patients	41
Age	
Median (range)	71 (48–80)
Sex	
Female/male	20/21
Properties of uveal melanoma	
Small (basal largest tumor diameter <5.0 mm and maximum thickness <2.5 mm)	7
Medium (basal largest tumor diameter 5.0–16 mm and maximum thickness 2.5–10 mm)	13
Large (basal largest tumor diameter >16.0 mm and maximum thickness >10.0 mm)	21
Choroidal tumors	36
Ciliary body tumors	5
Extraocular growth	3

To evaluate the effectiveness of the proposed qRT-PCR assay for monitoring disease progression, blood samples were taken from each patient at about 6-month intervals.

Classification by tumor size was made according to the Collaborative Ocular Melanoma Study Group guidelines [22]. A subgroup of patients ($n = 16$) was submitted to an additional blood draw for the isolation of circulating tumor cells by the ISET method.

As negative controls, 16 patients undergoing surgery for detached retina were enrolled. Two blood samples were collected from each patient before and after the surgical intervention.

Blood collection

For gene expression study, blood (2.5 ml) was collected in 'PAXgene Blood RNA Tubes' (PreAnalytiX GmbH, Hombrechtikon, CH, Switzerland), containing an RNA-stabilizing reagent. This technology, besides protecting RNA against degradation, minimizes ex-vivo gene expression changes, thus guaranteeing a high reproducibility of qRT-PCR [23].

For ISET, 10 ml of blood was collected in EDTA tubes and processed within 4 h.

RNA extraction and cDNA synthesis

Total RNA from whole blood was isolated by 'PAXgene Blood RNA kit' (PreAnalytiX GmbH), according to the manufacturer's instructions.

RNA quality was investigated by capillary electrophoresis (Agilent Bioanalizer 2100, Agilent Technologies, Palo Alto, California, USA). RNA integrity number [24] greater than 7 were considered fully suitable to real-time RT-PCR analysis [25]. RNA quantity was evaluated by spectrophotometric measurement (Nanodrop, Thermo scientific, Wilmington, Delaware, USA).

RNA from blood (500 ng) was reverse-transcribed using a commercial kit based on random primers technique ('Taqman Reverse Transcription Reagents', Applied Biosystems, Foster City, California, USA) according to the manufacturer's instructions. The reaction was carried out in a final volume of 20 µl. The reaction mixture contained buffer 1X, $MgCl_2$ 5.5 mmol/l, dNTP 2 mmol/l, random hexamers 2.5 µmol/l, RNAse inhibitor 0.4 U/µl, and reverse transcriptase 1.25 U/µl. The reaction was performed under the following conditions: 25°C for 10 min, 48°C for 30 min, and 95°C for 2 min.

Real-time RT-PCR

To verify the amplificability of samples, we performed GAPDH qRT-PCR measurements using the predeveloped assay Human GAPDH Endogenous Control (Applied Biosystems).

To dectect the tyrosinase transcript, a Taqman Gene Expression Assay (ID: Hs00165976_m1, Applied Biosystems) was used.

Real-time PCR for both assays was performed in a 'ABI PRISM 7700 Sequence Detection System' (Applied Biosystems). For amplification, an initial denaturation step was performed at 95°C for 10 min. For 50 subsequent cycles the conditions were 95°C for 15 s and 60°C for 1 min. For each sample measurement 5 µl cDNA was used in a reaction volume of 25 µl. 2 × Taqman Universal PCR Master Mix (Applied Biosystems) was used, according to the manufacturer's instructions. All samples were analyzed in duplicate.

To calculate the expression of tyrosinase mRNA in each sample, we referred to an external reference curve generated as follows: blood from healthy donors was spiked with a given number of cells (as determined by an hemocytometer) of the melanoma cell line SK-MEL-28, to a final concentration ranging from 4000 to 0.4 cells/ml blood.

Blood was collected in PAXgene Blood RNA tubes and RNA was extracted following the same procedure used for unknown samples. A standard curve was constructed performing qRT-PCR on these samples. Each calibrator was tested in triplicate. RNA from the blood of healthy donors, with no cells added, was used as a negative control.

Results were expressed as number of SK-MEL-28 cell equivalents/ml blood.

Moreover, a synthetic cDNA was obtained by cloning the target in the expression vector pCR 2.1-TOPO (Invitrogen, Carlsbad, California, USA). The plasmid was linearized by BamHI and was used for the in-vitro production of RNA by means of the 'T7 RiboMax Express Large Scale RNA production system' kit (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions. Tyrosinase RNA standard concentration was determined by spectrophotometric measurements (Nanodrop). The RNA was reverse transcribed and a standard curve was obtained by serial dilutions of the resulting cDNA.

Correspondence between the two ways of expression was investigated.

Isolation by size of epithelial tumor cells

Blood samples from 16 uveal melanoma patients were collected simultaneously with those for qRT-PCR and submitted to ISET. ISET was carried out using the modification of an earlier described [21,26] module of filtration kindly provided by Metagenex company (Paris, France) and a disposable filtration block (ISET Metablock, Metagenex, Paris, France) containing a membrane with calibrated 8-µm-diameter pores. The module of filtration has 10 spot compartments making it possible to load and filter 10 individual samples in parallel. Peripheral blood (8–10 ml) from patients with uveal melanoma was collected on buffered EDTA, diluted 1 : 10 with the ISET Metabuffer (Metagenex), incubated for 10 min at room temperature, and filtered. Ten milliliters of diluted solution, corresponding to 1 ml of undiluted blood, was loaded into each compartment and filtered by controlled aspiration under vacuum (0.1 bar). The membrane was then washed once with phosphate-buffered saline, disassembled from the filtration module, and allowed to air-dry. The spots, each one corresponding to 1 ml of filtered blood, were then stained with hematoxylin and eosin, dried, and stored long term at −20°C. For microscopic studies, the membrane was mounted on glass slides with Faramount mounting medium (DakoCytomation Inc, California, USA).

Statistical analysis

Statistical analysis of the results was carried out using the SPSS software package, release 12.0.1 (SPSS INC, Chicago, Illinois, USA). Statistical differences between groups were assessed using the Fisher's exact test and Kruskal–Wallis test. P values of less than 0.05 were considered statistically significant. The cumulative survival probability in various subgroups was calculated using the Kaplan–Meier life tables and differences assessed with the log-rank method.

Results

Standard curve for tyrosinase mRNA quantification

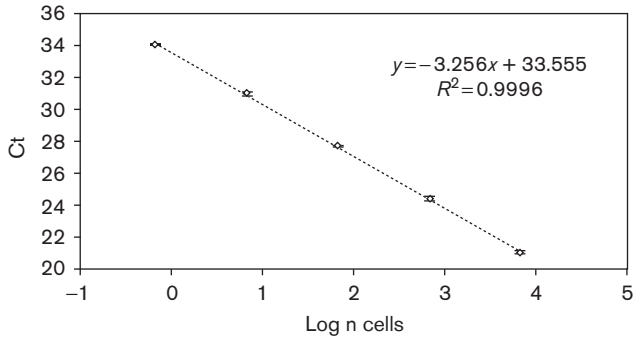
The linear regression analysis of Ct versus number of cell equivalents resulted $y = -3.15 + 40.0$; $R = 0.99$. Tyrosinase mRNA was still detectable in the sample containing the cDNA obtained from total RNA corresponding to 0.4 SK-MEL-28 cell/reaction. The calibration curve was created by plotting the concentration of each dilution of the external standard versus the value of its threshold cycle (Ct), Fig. 1.

We established a correspondence between the standard based on the use of SK-MEL-28 cell equivalents and the number of tyrosinase mRNA copies according to real-time RT-PCR results obtained by the tyrosinase RNA plasmidic standard. The number of tyrosinase mRNA copies per SK-MEL-28 cell equivalent resulted 18 and consequently the dynamic range of the standard curve varied from 72 000 to 7.2 copies of tyrosinase mRNA.

Controls

Tyrosinase mRNA expression was undetectable in all samples taken from controls ($n = 16$) except for two presurgery samples (0.08 and 0.07 SK-MEL-28 cell equivalents/ml blood) and one postsurgical sample (0.04 SK-MEL-28 cell equivalents/ml blood). Based on these data, a cut-off value was established at 0.08 SK-MEL-28 cell equivalents/ml blood. All blood samples from uveal melanoma patients showing a tyrosinase expression level above this cut-off value were considered positive.

Fig. 1



Standard curve for tyrosinase mRNA expression quantitative RT-PCR quantification.

Uveal melanoma patients: evaluation of qualitative and quantitative results

By qRT-PCR, 20 of 41 (49%) uveal melanoma patients resulted positive in at least one of the three blood samples before the first therapeutic intervention. Mean tyrosinase mRNA level evaluated by qRT-PCR in the 20 positive patients resulted 2.8 SK-MEL-28 cell equivalents/ml blood (median: 0.8; range: 0.1–14.4).

Result evaluation on the basis of tumor size

The distribution of patients as positive and negative for tyrosinase expression was significantly different among the three groups classified as small, medium, and large tumors ($P = 0.001$, Fisher's exact test), Fig. 2a.

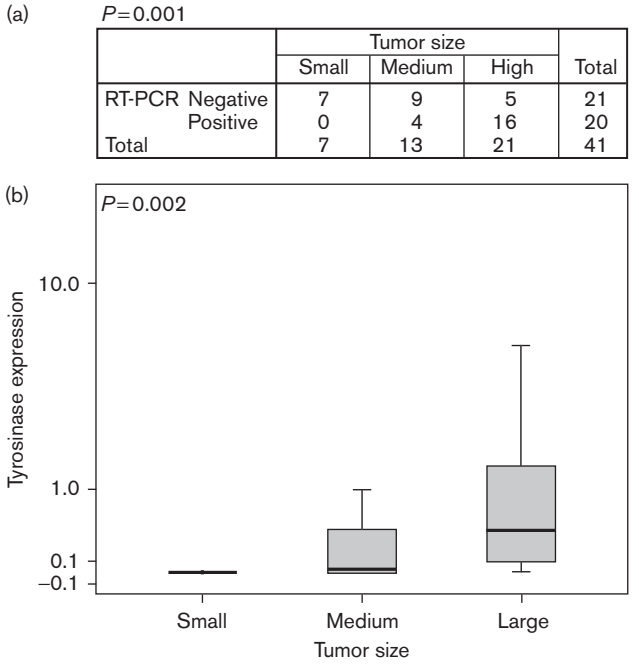
Similarly, mean values of tyrosinase mRNA levels calculated for each patient before the first therapeutic intervention were significantly different ($P < 0.01$, Kruskal–Wallis test) among patients with small ($n = 7$, undetectable values), medium ($n = 13$; median 0.02; range 0–14.4), and large melanoma ($n = 21$; median 0.3; range 0–10.9), Fig. 2b.

Longitudinal study on tyrosinase mRNA expression in uveal melanoma patients

Patients with small uveal melanoma tumors (seven of 41) showed negative results for the whole follow-up period varying from 17 to 32 months. Among the 29 of 41 patients with medium or large-size tumors, still surviving at the end of the study, 14 of 41 patients were negative for tyrosinase.

During the follow-up period, eight patients developed metastases and among them five died of disease (Table 2) In Fig. 3a–d, the longitudinal follow-up profiles based on tyrosinase quantitative levels of four representative patients who died of the disease are illustrated. Tyrosinase expression levels increased at least 1 month before the clinical detection of metastasis in all the patients who died of the disease.

Fig. 2



		Tumor size			
		Small	Medium	High	Total
RT-PCR	Negative	7	9	5	21
	Positive	0	4	16	20
Total		7	13	21	41

(a) Distribution of tyrosinase-positive and negative samples versus tumor size (small vs. medium vs. large tumors, $P = 0.001$, Fisher's exact test). (b) Tyrosinase mRNA levels in patients with small, medium, and large melanoma ($P < 0.01$, Kruskal–Wallis test).

Table 2 Longitudinal study and metastatic patients characteristics

Whole follow-up period	55 months
Metastasis development	
Number of patients	8/41
Female/male	3/6
Median time for metastasis development	21 months
Died of the disease	5/8
Metastatic sites	
Liver	7/8 patients
Lung	3/8 patients
Bones or abdominal lymph nodes	2/8 patients
Brain	1/8 patient

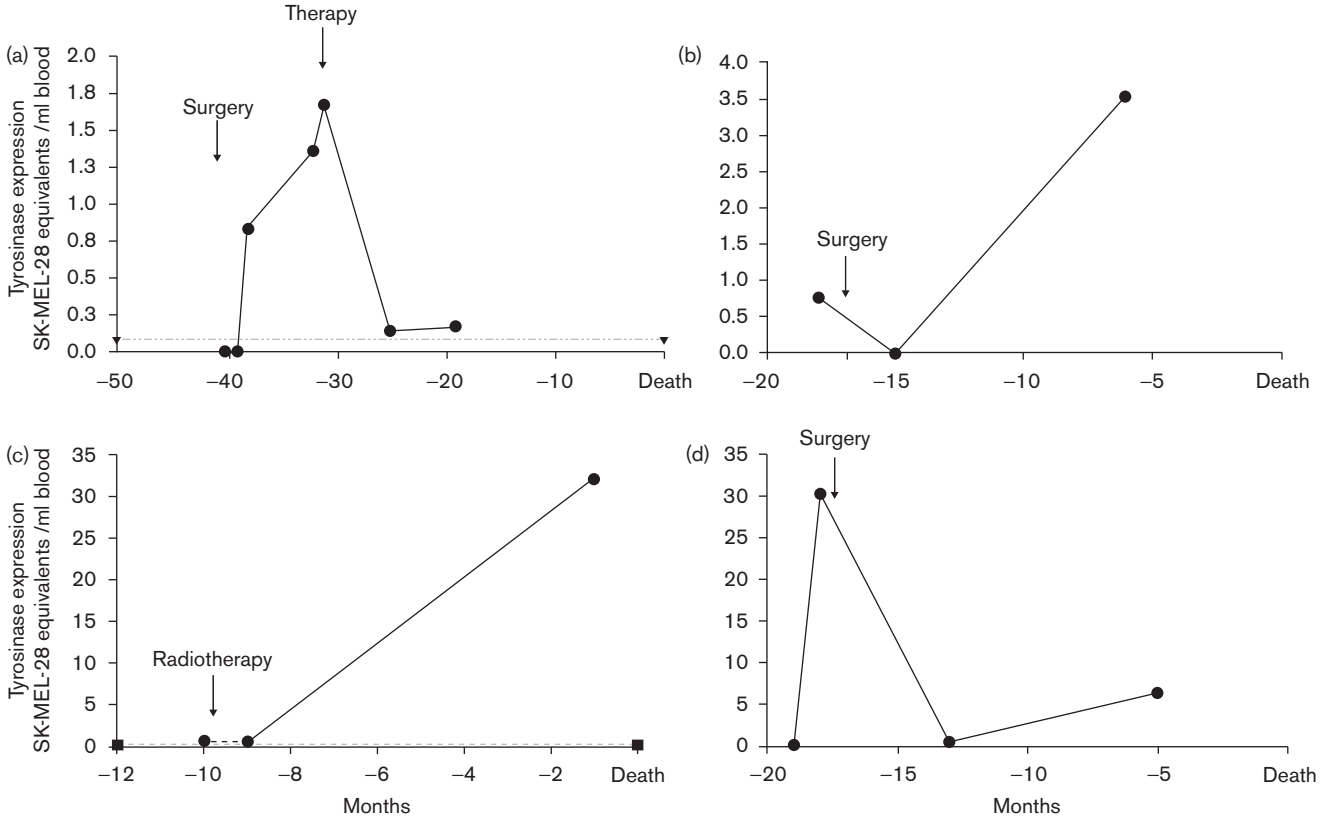
Disease-free survival and overall survival

Kaplan–Meier curves for disease-free and overall survival based on blood tyrosinase mRNA detection showed a statistically significant correlation. Five of 20 PCR-positive patients (25%) died during the first 3 years, whereas no death was reported in 21 PCR-negative patients (log-rank test $P < 0.05$, Fig. 4a). One PCR-negative patient relapsed within 11 months and 12 of 20 PCR-positive patients relapsed within 53 months (log-rank test $P < 0.05$, Fig. 4b).

ISET evaluation of uveal melanoma blood samples

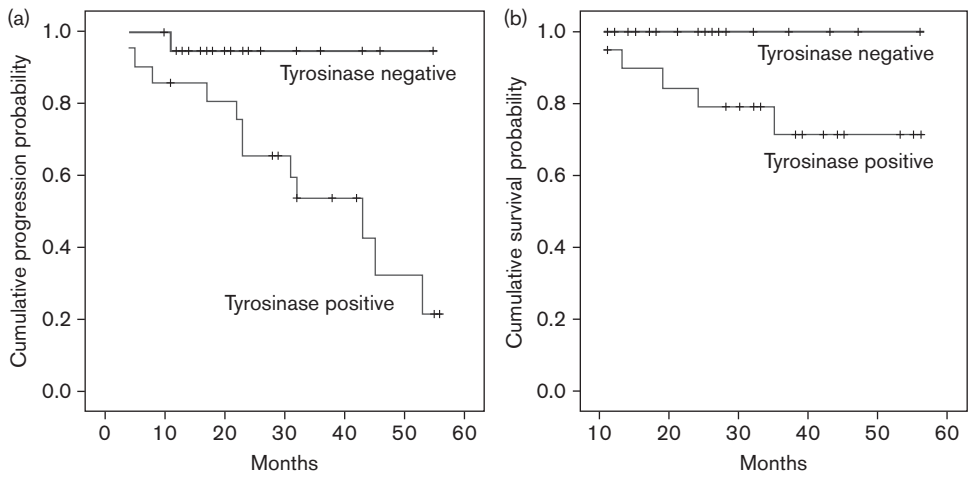
Morphological criteria for the identification of circulating melanoma cells included the following: (i) cell size ≥ 16 micron, (ii) nucleocytoplasmic ratio $\geq 50\%$, (iii) irregular nuclear shape, (iv) hyperchromatic nucleus, and

Fig. 3



(a–d) Tyrosinase mRNA expression profiles in four representative uveal melanoma patients who died of disease. Tyrosinase mRNA expression levels are reported as SK-MEL-28 cell equivalents/ml of blood; time on the *x*-axis is calculated from death. The time of surgical intervention and/or therapy has been indicated in the plot. Tyrosinase levels rise in all patients before death.

Fig. 4



Kaplan–Meier estimates for disease-free ($P < 0.05$; a) and overall survival probability ($P < 0.05$; b) according to tyrosinase mRNA expression in uveal melanoma patients.

(v) basophilic cytoplasm. Circulating melanoma cells appeared either as epithelioid or elongated cells with an abundant basophilic cytoplasm and irregular nucleus, or as smaller cells with round to oval shape and indented nucleus (Fig. 5).

In some cases, we observed doubtful cells with regular shape, slightly superior than pore in size but with a nucleocytoplasmic ratio of less than 50%. Most of these cells were eventually recognized as enlarged monocytes. Leukocytes were also retained on the membrane in a low percentage, often trapped within the pore lumina. Such cells were easily identified because of their smaller size and peculiar nuclear morphology. Superposition of cells or of cells and pores, nude nuclei, or excess of staining were regarded as artefacts.

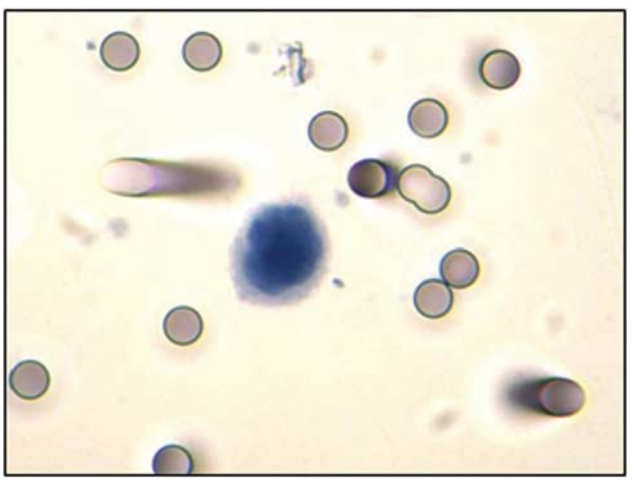
Five samples showed the presence of tumor cells on the filter. For each positive patient, CTC value was evaluated as the mean of the cells counted on up to 10 different spots. The CTC values for the five ISET positive patients were 5.8, 2.33, 2.00, 1.25, and 0.75 CTC/ml of blood, respectively.

A direct correlation was found between CTC values and tyrosinase levels (2.13, 1.37, 0.83, 0.58, and 0.35 SK-MEL-28 cell equivalents/ml of blood, respectively). The linear regression analysis of CTC/ml versus SK-MEL-28 cell equivalents/ml of blood gave the following result: $y = 0.3446x - 0.216$; $R = 0.96$. Tyrosinase levels in ISET-negative patients ($n = 11$) were undetectable.

Discussion

CTC in the peripheral blood represent a potential new prognostic factor for identifying patients at a higher risk of developing metastatic disease.

Fig. 5



Example of a circulating tumor cell isolated by ISET (isolation by size of epithelial tumor cells) from the filtered blood of a melanoma patient.

Approximately 35% of patients with uveal melanoma develop metastasis, even after successful treatment of the primary tumor [6]. To date, metastatic uveal melanoma is uniformly fatal [27] and current diagnostic tests fail to identify metastases in their earlier stages. qRT-PCR methods are the most widely used assays to identify the presence of tumor cells in the blood stream through the detection of messenger RNA specific for melanoma cells such as tyrosinase and/or Melan-A. The first study reporting the presence of CTC in the blood from uveal melanoma patients by PCR-based methods dates back to 1993 [28]. Promising results of this approach have been extensively reported on cutaneous melanoma in which tyrosinase was shown to be related to the stage of the disease [14,29] and prognosis. Only recently, a study that focused on uveal melanoma was published [6] showing the usefulness of CTC detection by qRT-PCR. The study analyzed 110 patients with uveal melanoma, selecting exclusively patients with high-risk primary tumors. The detection of tyrosinase and melan-A transcripts has shown to be an independent prognostic factor for relapse and survival [6].

This study proposes the measurement of tyrosinase expression to indirectly detect CTC in the peripheral blood of patients affected by uveal melanoma with particular attention to the analytical assessment of the qRT-PCR protocol, as the assay was specifically designed to take into account all the variables of collection and extraction procedures. Blood samples were collected in tubes specifically designed for mRNA preservation [23]. The absolute quantification of tyrosinase transcript used in this study has been calculated, as a standard, referring to the total RNA deriving from the blood of healthy donors spiked with melanoma cells (SK-MEL-28 cell line). These artificial samples are then submitted to an extraction procedure identical to that for unknown samples and results are expressed as SK-MEL-28 cell equivalents/ml of blood. As the tyrosinase expression levels of SK-MEL-28 seem to be of the same order of magnitude of the tyrosinase levels in the CTC in blood, this approach would provide the approximate number of CTC present in the patients' bloodstream.

Preliminary data obtained from a subset of patients included in this study partly support this hypothesis. In fact, comparison between the results obtained by qRT-PCR and the simultaneous enumeration of the CTC by a direct method (ISET technology [21,26]) are of the same magnitude and show a good correlation, even if the levels of mRNA of SK-MEL-28 are higher than those found in patients' CTC.

Finally, we determined the correspondence between this standard and a tyrosinase RNA plasmid with the advantage of a complete characterization of our reference material to increase the accuracy of tyrosinase mRNA detection and to allow comparisons between different clinical centers and studies.

In agreement with data already reported by other groups on melanoma [11,29], as well as on other neoplastic diseases [30,31], some patients of our control group were slightly positive; thus, we propose the value of 1.44 copies of tyrosinase mRNA (corresponding to 0.08 SK-MEL-28 cell equivalents/ml blood) as the cut-off value for positive results.

Next, the clinical utility of this test was investigated by analyzing the data of both the qualitative and quantitative results. In our study, the percentage of positive samples (at least one positive sample in a multiple sampling) was 49% and the mean CTC levels evaluated over the entire patients' population was 2.8 SK-MEL-28 cell equivalents per ml blood. Tyrosinase expression values proved to be correlated with tumor dimension, with all the small-sized tumors being negative.

Tyrosinase expression predicted overall survival and disease-free survival according to the Kaplan–Meier analysis.

Taken together, these data are consistent with the results reported in a recent publication [6] and support the clinical utility of this test. Moreover, our patient cohort is not limited to uveal melanoma with an unfavorable prognosis and provides preliminary data (limited to a subgroup of patients) on the correlation between two CTC detection methods. As far as we know, this is the first time that a comparison between a direct and indirect method for CTC isolation has been performed in uveal melanoma patients.

As uveal melanoma is generally not submitted to surgery, any additional tool to support clinical data is welcome for the management of patients and should be applied in sequential patient follow-up [27].

Our data show that tyrosinase levels correlate with metastatic disease. The prognostic relevance of tyrosinase detection in the blood is confirmed by our study showing a decrease in recurrence rate and prolongation of the disease-free interval in patients negative for blood tyrosinase.

The reliability of tyrosinase mRNA measurement as an indirect parameter for CTC detection has been confirmed by direct isolation of tumor cells by ISET. In conclusion, our data indicate that the proposed tyrosinase assay by qRT-PCR seems to be a noninvasive method suitable for monitoring the patient follow-up and to identify patients at higher risk for disease progression.

As metastasis in uveal melanoma patients seems to occur frequently, despite ocular treatment, the most accredited hypothesis is the presence of micrometastasis that occurs before primary treatment and remains dormant for prolonged period of time before emerging as clinically detectable macrometastatic disease [32]. In this context, the early detection of tyrosinase mRNA can represent a noninvasive biomarker of metastatic development and can suggest the use of pharmacological therapy.

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