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Absolute quantitative PCR for detection of molecular biomarkers in melanoma patients: A preliminary report

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ABSTRACT

Background: Malignant melanoma is the most malignant tumours of skin and mucous membranes mainly due to its aggressive biological behaviour and tendency to generate early metastases. Unfortunately, the mechanisms underlying the development, progression and the expression of an aggressive melanoma phenotype still remain largely unknown.
Objectives: The purpose of this study was to determine whether a multi-panel of molecular transcripts can be predictive for risk of recurrent disease in malignant melanoma patients.
Results: Peripheral blood was collected from 31 malignant melanoma patients in follow-up for melanoma and from 30 healthy volunteers randomly selected. Each specimen was examined by qRT-PCR analysis for the expression of six markers: PAX3d, TYR, MITFm, MCAM, TGFβ2 and ABCB5. Malignant melanoma patients expressed an important number of markers, with a median value of four markers. Only PAX3d displayed a trend in terms of differences when the levels of gene expression were made in function of Breslow index. Furthermore, PAX3d showed the best diagnostic capacity among the remaining residual markers or in combination with TGFβ2 and MTIF.
Conclusions: We demonstrated the usefulness of multimarker qRT-PCR to detect circulating melanoma cells in blood and to potentially assessing patient disease status or progression, especially when PAX3d was used in combination with MTIFm and TGFβ2.

1. Introduction

Cutaneous malignant melanoma (CMM) belongs to the most malignant tumours of the skin and mucous membranes mainly due to its aggressive biological behaviour and tendency to generate early metastases [1]. In 2008, 62,480 new melanoma cases were diagnosed and about 8,420 deaths are expected in the United States due to a mortality rate of about 5% of all cancers in males and 4% in female. In the EU, the incidence of melanoma is annually 9 cases/100.000, while in Italy, a total of 3,143 newly diagnosed cases among males and 2,851 among females [2]. Unfortunately, despite the increasing availability of screening program for melanoma prevention, the sensitivity of both clinical and laboratory diagnostic methods is still low in terms of early discovering of the primary melanoma or of prediction of its metastatic spreading [1]. Currently, the major part of therapies are ineffective for treating metastatic disease, with an overall response rate of 20%–40% and a complete response rate (or cure rate) less than 5%. Assessment of primary and/or metastatic melanoma has been addressed in the new American Joint Committee on Cancer (AJCC) staging criteria. The staging system, however, does not accurately take into account the disease progression events [3]: imaging methods are usually able to detect the presence of lymph-node or visceral metastasis in advanced stages of disease when the concomitant hematogenic diffusion may occur [4]. Molecular technologies could exponentially increase the detection of nodal micrometastases or circulating melanoma cells (CMCs) in blood. For these reasons, the detection of CMCs has been proposed as a sensitive method for selecting patients at high risk of recurrence or relapse [5,6]. Although molecular monitoring of melanoma blood circulating RNA transcripts is still under debate [7–9], mainly due to the lack of standardized protocols regarding the methods applied to this type of analysis, different strategies have been proposed. Up to now, qualitative and semi-quantitative techniques have been performed by many researchers in order to assay the presence of specific melanoma markers in peripheral blood (PBL) in relationship with patients'

Abbreviations: CMM, cutaneous malignant melanoma; HC, healthy control; CMCs, circulating melanoma cells; VPP, positive predictive value; VPN, negative predictive value; IR, risk index.

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outcome [10–16]. In particular, quantitative real-time qRT-PCR assays offers the most robust, accurate and less laborious approach to molecular diagnostics, allowing rapid and reproducible quantitative analysis for detection of few tumor cells in tissues and blood. Investigators have reported the CMCs detection in blood through both single and multimarker qRT-PCR approach, but only recent studies have assessed the agreement of qRT with evidence of disease progression [3]. An interesting article recently published by Reid et al. [17], reported a novel multimarker qRT-PCR assay used to investigate the phenotype of CMCs in 230 MM patients over a period of 3 years and 9 months. This work evidenced that two markers, namely, MLANA and ABCB5, had the greatest prognostic value and that MCAM expression correlated with poor treatment outcome in patients non surgically treated.

Therefore, the purpose of this study was to determine, whether a multi-panel of molecular transcripts, assayed by qRT-PCR, was predictive for risk of recurrent disease in MM patients. In the current study, we have in part included markers reported by Reid et al., in order to confirm his data regarding PAX3d isoform, TGFβ2, MCAM, ABCB5, predominantly expressed in melanoma [18,19]; furthermore, we have added MITFm melanocyte-specific isoform [20] and TYR mRNAs in PBL samples of selected MM patients. In detail, these markers represent melanocyte, tumour progression and stem cell factors, respectively. In the present paper, we show that multimarker qRT-PCR analysis could improve the general sensitivity and specificity of molecular diagnostic methods in melanoma patients. In this regard, we tested the qRT-PCR analytical sensitivity, specificity and diagnostic performances, evaluating the relationship between molecular markers expression and some clinic-pathological patients' features.

2. Materials and methods

2.1. Patient and control selection

Thirty patients, in the first period of follow-up, soon after they received the histopathological diagnosis of cutaneous malignant melanoma, were enrolled. They were subsequently subcategorized as 24 patients with primary cutaneous melanoma and 6 with metastatic melanoma, classified in stage II ($n = 10$), stage III without metastasis ($n = 14$) and stage IV + metastatics ($n = 6$). These patients were consecutively recruited inside the Dermatological Department of Catholic University and Cristo Re Hospital of Rome and clinically evaluated and classified accordingly to the recent American Joint Committee on Cancer guidelines [21]. All patients were enrolled within the year 2014. The period of follow-up, for defining the variables of outcome, is relatively short, since some of.

For all patients, physical examination, medical history and clinical-pathological characteristics (age, tumor borden, histologic grade, lymph-node metastasis and AJCC stage) of disease at the time of blood drawing were recorded. In order to define baseline expression of mRNA markers in PBLs, we enrolled, as negative controls, thirty healthy volunteers (randomly selected among 30 consecutive individuals who attended our hospital for general routine screening) who accepted to be enrolled in this study. Obviously, none of the control subjects had history or clinical of skin cancer or was under treatment for other type of benign and malignant diseases. A focused questionnaire was administered, from a trained laboratory medicine specialist, in order to verify if the randomly selected individual meet the inclusion criteria. Both patients and healthy volunteers gave consent for the inclusion in this study, and they provided a written consent and the investigational protocol was made following the Helsinki criteria for research studies.

2.2. Recovery assay workflow: melanoma cell line and spiking in PBLs

Human melanoma cell line UACC257 cells were cultured in RPMI-1640 (Lonza, Verviers, Belgium) supplemented with 10% foetal bovine serum (Lonza), 2 mM L-glutamine, penicillin G (100 U/ml) and streptomycin (100 μg/ml), in a humidified atmosphere with 5% CO_2 at 37 °C in a T75 cm^2 flask (Greiner, Monroe, NC, USA). Cells reached at 70%–80% confluence were detached by 0.05% trypsin (Lonza), centrifuged, washed twice with phosphate-buffered saline (PBS) and immediately used to spike PBLs samples of non melanoma healthy donors. To evaluate the qRT detection limit and to mimic the in vivo condition of occult metastatic melanoma cells in blood, we performed serial dilutions of UACC257 cells (20, 10, 5 and 0 cells), and each diluted aliquot was mixed with 3 ml donor-derived PBLs prepared as a follows: 13 ml of PBLs from an unique healthy donor was collected and subdivided in 4 tubes each of 3 ml labelled as C0, C5, C10 and C20. Only the first one (C0) was not spiked with melanoma cells. All samples were subjected to RNA extraction, reverse transcription and assayed for all markers by qRT-PCR. This in vitro assay was performed three times in duplicate to validate the reproducibility and robustness of the assay system.

2.3. Sample processing, RNA extraction and cDNA synthesis

To prevent the contamination of epithelial cells through a venipuncture for each individual, first 5 ml of blood was discarded, and then 3 ml of blood was drawn into a tube with EDTA and processed. Total RNA from blood samples (spiked or not with diluted cells) was extracted and treated with DNase using QIAmp RNA blood mini kit (Qiagen, Hilden, Germany) and RNAsi-free DNAse set (Qiagen) according to manufacturer's recommendation. RNA integrity and quantification were electrophoretically determined using high-sensitivity "Experion chip" (Biorad, Hercules, CA). All RNA samples were stored at −80 °C until used. All samples were reverse-transcribed and subsequently amplified. Reverse transcription was carried out on about 500 ng of total RNA following the instructions provided by Transcriptor First Strand cDNA synthesis kit (Roche Applied Science, Indianapolis, IN). A 20 μl reaction mixture was prepared by adding 1 μl oligo$(dT)_{18}$ primer (50pmol/μl), 2 μl random hexamer primer (600pmol/μl), 2 μl dNTPs (10 mM), 0.5 μl RNasi Inhibitor (40U/μl), 0.5 μl Transcriptor Reverse Transcriptase (20U/μl), 4 μl RT buffer (5×) and ultrapure water to volume. After initial incubation at 65 °C for 10 min, the reaction was conducted at 25 °C for 10 min followed by 60 min at 50 °C and final inactivation for 5 min at 85 °C. The intact cDNAs were stored at −20 °C or immediately used for subsequent amplification reactions.

2.4. Construction and titration of standard curves

In order to generate standard curves, the cDNA of UACC257 cell line was amplified by PCR using the specific cloning primers for MCAM, ABCB5, PAX3d, TGFB2, TYR, MITFm and GAPDH transcripts. The amplified products were digested with *Hin*dIII (5′) and *Bam*HI (3′) enzymes (New England Biolabs Hitchin, UK) for TYR, MCAM, ABCB5 and GAPDH targets and with *Eco*RI (5′) and *Xho*I (3′) (Biolabs) for PAX3d, TGFB2 and MITFm targets. Subsequently, each product was ligated to a pcDNA3 + vector (Invitrogen, Carlsbad, CA) previously digested with the same enzymes, using T4 DNA Ligasi (1 μl) and T4 Buffer (10X) supplied by Biolabs. Ligated vectors were transformed into *Escherichia coli* DH5α cells (Invitrogen), and each plasmid DNA was isolated from recombinant clones using Plasmid Maxi kit (Qiagen). All recombinant clones were sequenced to verify the integrity of DNA construction in 3500Genetic Analyzer (Applied Biosystems, Foster City, CA) (data not shown). Subsequently, we performed the titration of our constructs by using a quantitative assay of dsDNA, based on real-time PCR measurement of fluorescence due to the interaction of PicoGreen dye with dsDNA. We applied a Quan-IT Pico Green assay (Invitrogen) in LightCycler 480 instrument (Roche). The averaged fluorescence values were converted into DNA amounts using a calibration curve prepared with λ-DNA standard supplied by kit. The DNA concentrations were determined and the corresponding copy numbers were calculated. Serial 10-fold dilutions from recombinant plasmids were used as standard

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