Featured Article

Quantitative Detection of Circulating Tumor Cells in Cutaneous and Ocular Melanoma and Quality Assessment by Real-Time Reverse Transcriptase-Polymerase Chain Reaction

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Abstract

Purpose: Inconsistent reports on the detection of melanoma cells in peripheral blood by reverse transcriptase-PCR (RT-PCR) have resulted in uncertainty on the prognostic value of circulating melanoma cells.

Experimental Design: We developed real-time RT-PCR assays for quantitation of tyrosinase, MelanA/MART1, and gp100 and for porphobilinogen deaminase housekeeping gene. Melanoma tissue (n = 18), peripheral blood samples from healthy donors (n = 21), and patients with cutaneous (n = 122) and uveal (n = 64) melanoma from our institution were analyzed. For quality control, an additional 251 samples from ongoing multicenter studies were compared with in-house samples.

Results: Tyrosinase was not detected in healthy donor blood samples. For the two other markers, cutoff values had to be defined to distinct patient samples from controls. Patients with stage IV uveal and cutaneous melanoma expressed all three markers more frequently and at higher levels in peripheral blood as compared with earlier stages. The variation of expression was 4 logs and correlated with tumor load and serum lactate dehydrogenase. In 2 of 3 uveal melanoma patients, detection of circulating tumor cells preceded the development of liver metastases. The diagnostic sensitivity was optimal in blood samples containing >0.1pg/μl porphobilinogen deaminase (95.7% of in-house samples and 57.4% of multicenter samples).

Conclusions: Real-time RT-PCR is able to quantitatively define the quality of a sample and provides quantitative data for melanoma markers. Disparities in the results of previous studies may be attributable to undetected differences in sample quality. The prognostic relevance of this assay is currently under evaluation in several prospective randomized trials.

Introduction

Although the molecular monitoring of circulating tumor cells by reverse transcriptase-PCR (RT-PCR) has entered routine use to evaluate treatment outcome in patients with certain leukemias (1, 2) and lymphomas (3, 4), the application of this technique for patients with solid malignancies is still under debate. The first report on detection of tumor cell RNA in peripheral blood from solid tumor patients has been the description of tyrosinase RNA in melanoma patients (5). Subsequent investigations showed the presence of tyrosinase RNA in the peripheral blood to be associated with the stage of disease (6) and predict disease progression in patients with early as well as advanced melanoma (7, 8). Multimarker RT-PCRs have subsequently been introduced to increase the sensitivity of tumor cell detection (9). Furthermore, semiquantitative PCR assays have been developed, either based on competitive PCR (10) or Southern blot detection of the product generated by submaximal PCR (11). The level of tyrosinase gene expression detected with these semiquantitative systems in the peripheral blood of melanoma patients has been associated with tumor load (11).

However, a series of subsequent investigations by a number of groups led to conflicting reports on both: (a) the frequency of melanoma-associated gene transcripts in patients with early as well as with advanced disease; and (b) on the reliability of the method (8, 12–38). The majority of these studies investigated tyrosinase as a single marker, whereas several studies used multiple marker PCR assays, including tyrosinase, MelanA/MART1, gp100, and several others. However, there is no uniform consensus that multimarker PCRs differ in diagnostic relevance from assessment of tyrosinase only. To investigate the principal reliability of RT-PCR assays, two interlaboratory quality assurance studies have been performed. A multicenter study performed by the European Organization for Research and Treatment of Cancer (EORTC) Melanoma Group compared the results of various in-house assays on a series of samples, consisting of spiked whole blood, peripheral blood mononuclear cells, and cDNAs (39) with largely homogenous results. The second quality assurance study evaluated a commercially available kit on melanoma patient blood samples (31) with very heterogeneous results. Results from these two studies show that one major reason for disparities is heterogeneity in quality of...
cDNA generated from blood samples, as analyzed in detail in the EORTC quality assurance study.

Rigorous quality control on a per-sample basis when applying RT-PCR assays in clinical trials has therefore been the demand of the EORTC Melanoma Group (39). With conventional or semiquantitative RT-PCR, however, the determination of cDNA quality by means of detection of housekeeping genes is by far not optimal. Quality control by using detection of small amounts of plasmid or foreign cellular standards (40) admixed with the sample is also not an optimal control, because potential damage before admixing the sample with this type of standard cannot be controlled.

The recent availability of real-time PCR equipment has changed the situation. The quantitation of housekeeping gene expression should allow reliable quality control on a per-sample basis. We have developed quantitative real-time RT-PCR assays to measure housekeeping gene expression and expression of melanoma-associated RNAs. Here, we report on quantitative RNA expression of melanosomal antigens in melanoma tissue and 186 peripheral blood samples from patients with cutaneous as well as uveal melanoma and provide correlation with clinical parameters. In addition, we show the crucial role of sample quality on diagnostic sensitivity and compare our in-house samples with a second series of 251 samples collected within EORTC multicenter trials.

Materials and Methods

Patient Selection. All patients had given informed consent before the analysis. Clinical disease status was determined according to the recent American Joint Committee on Cancer guidelines (41). The patients consisted of four groups: (a) patients with primary cutaneous melanoma after resection of metastases (group 1); (b) cutaneous melanoma patients with metastases (group 2); (c) patients with primary uveal melanoma with a risk factor for developing metastases, including diffuse growth, local recurrence, largest tumor diameter > 15 mm, involvement of ciliary body, or extraocular growth (group 3); and (d) uveal melanoma patients with liver metastases (group 4). The initial samples in patients with stage III and resected stage IV cutaneous melanoma (group 1) were taken within a few weeks after surgical removal of the primary tumor or metastases. All patients with high-risk uveal melanoma (group 3) entered subsequently in an experimental adjuvant vaccine protocol with tyrosinase peptides, whereas treatment of all other patient groups was heterogeneous. In patients with metastatic disease (groups 2 and 4), samples were obtained at the occasion of outpatient clinic visits, regardless of current treatment. Follow-up samples were obtained in all patient groups on the occasion of outpatient clinic visits. Negative controls were healthy volunteers. A second series of blood samples was collected in other hospitals from patients enrolled in various EORTC trials (EORTC 18951, 18952, and 18991).

Processing of Blood Samples. Blood (10 ml) was collected in EDTA-containing tubes and processed within 2 h. Blood was centrifuged at 1600 × g for 10 min. Serum was removed, and the cell pellet was resuspended in 5 ml of guanidium thiocyanate (GTC) buffer and stored at −80 °C. EORTC samples were shipped to the central lab in GTC buffer on dry ice.

Processing of Tissue Specimens. Tissue samples were dissected under stringent sterile conditions to prevent RNA contamination and immediately frozen in liquid nitrogen. Special care was taken to prevent contact with skin to avoid contamination with skin melanocytes.

RNA Extraction and Reverse Transcription. Total RNA of peripheral blood samples was isolated by acid guanidinium thiocyanate/phenol chloroform extraction (42), including Phase Lock Gel Heavy (Eppendorf, Hamburg, Germany), and further purified by the High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany). Total RNA of cell lines and tissues was isolated by RNeasy Mini Kit, including RNase-Free DNase Set (Qiagen, Hilden, Germany). RNA integrity was checked electrophoretically and quantified spectrophotometrically. For reverse transcription, 2 μg of RNA were diluted in 15 μl of RNase-free water, incubated for 5 min at 65 °C, and placed on ice. A 7.5-μl mixture containing 2 μl of oligo-p(dT)15 primer (0.8 μg/μl), 2 μl of deoxynucleoside triphosphate (5 mm), 0.5 μl of RNAsin (40 units/μl), 1 μl of Omniscript Reverse Transcriptase (4.5 units/μl), and 2 μl of reverse transcriptase buffer (×10) was prepared and added to the diluted RNA. After incubation at 37 °C for 1 h, Omniscript Reverse Transcriptase was inactivated for 5 min at 95 °C, and cDNA was stored at −20 °C. All reverse transcription reagents, except oligo p(dT)15 primers and RNAsin (Roche Diagnostics), were purchased from Qiagen.

Quantitative Real-Time PCR. PCR conditions for the LightCycler (Roche Diagnostics) are summarized in Table 1. Each cDNA (2 μl) was diluted to a volume of 20 μl of PCR mix (LightCycler Faststart DNA Master Hybridization Probes; Roche Diagnostics), containing 0.5 pmol of primer and 0.2 pmol of probe and a final MgCl2 concentration as listed in Table 1. Primers were purchased from Metabion (Martinsried, Germany) and fluorophore probes from TIB Molbiol (Berlin, Germany) and Metabion. For amplification, an initial denaturation step at 95 °C for 10 min was used. For 55 subsequent cycles, the conditions were denaturation for 10 s at 95 °C, annealing for 12 s at a temperature provided in Table 1, and elongation for 10 s at 72 °C. This was followed by a final extension of 2 min at 72 °C. The expected size of the PCR product was confirmed by agarose gel electrophoresis.

All samples for tyrosinase and porphobilinogen deaminase (PBGD) were analyzed in duplicate. The average value of both duplicates was used as a quantitative value. If only one of the duplicates gave a positive result, the positive result was taken.

The crossing point values of this method are linear over 7 logs (40). The limit of detection was tested for tyrosinase expression in five experiments and 10 melanoma cells of the cell line SK-MEL 24 in 10 ml of blood (data not shown).

Plasmid Controls, Standard Curve. PCR products generated from tyrosinase, MelanA/MART-1, gp100, and PBGD cDNAs were cloned into the vector pCR2.1-topoisomerase (Invitrogen, Karlsruhe, Germany). Recombinant vectors, linearized with EcoRV, were serially diluted in water containing 0.4 μg/μl polyadenylic acid (Pharmacia Biotech, Freiburg, Germany). A standard curve with three dilutions of the respective plasmid in duplicates (1, 0.1, and 0.001 pg/μl) was included in each PCR
run. The quantification of serial plasmid dilutions yielded linear crossing point increases over a range of 7 logs (data not shown; Ref. 40).

Precautions. To reduce risk of contamination, RNA extraction, cDNA synthesis, thermocycling, and post-PCR steps were performed in separate laboratories. PCR mixtures were set up in a template tamer (Oncor Appligene, Heidelberg, Germany). All reagents for cDNA synthesis were prepared with RNase-free water. For all RT-PCR steps, negative controls were performed as outlined in the EORTC recommendations (43), including a reverse transcriptase-negative sample control for every sample and water control for every PCR run.

Data Analysis. With the LightCycler software (version 3), crossing points were assessed and plotted versus the concentrations of the standards. The relative sample amount was expressed as the ratio (tyrosinase, MelanA/MART-1, gp100)[µg/µL]/(PBGD)[µg/µL]. The crossing points (beginning of the PCR exponential phase) for each reaction were determined by the Second Derivative Maximum algorithm, and sample concentration was calculated using the plasmid standard curve.

To describe the variability of gene expression, all samples were considered. For comparison with clinical data, only the first sample from each patient was considered. The statistical analysis was performed using SPSS software (release 10.0). The following tests were used, where appropriate: (a) Wilcoxon’s signed rank test; (b) t test; and (c) χ² test.

Results
Quantitative Range of RT-PCR Assays
To establish the principle quantitative range and detection limit, serial dilutions of topoisomerase 2.1 standard plasmids containing the melanoma marker and PBGD sequences with 0.4 µg/µl polyadenylic acid were assayed in duplicate. The detection of four copies was possible for all markers. The dynamic range of quantitation was seven to eight orders of magnitude for all markers (data not shown).

Expression of Tyrosinase, MelanA/MART-1, and gp100 in Melanoma Tissue
The mRNA expression levels of tyrosinase, MelanA/MART-1, and gp100 varied in melanoma tissue over 3 logs with a ratio maker:PBGD ranging from 0.1 to 30 (Fig. 1). No tissue specimen in our series was completely negative for any of the markers. The median expression of MelanA/MART1 was highest, followed by tyrosinase and gp100. The two uveal melanoma tissue samples (both from liver metastases) fell in line with the 16 cutaneous melanoma samples.

Expression of Tyrosinase, MelanA/MART-1, and gp100 mRNA Transcripts in Cutaneous Melanoma Patient Blood Samples
Tyrosinase was not detectable in healthy volunteer blood samples (Fig. 2). In 5 of 50 samples (10%) from 36 clinically disease-free melanoma patients, tyrosinase mRNA expression was detectable with a ratio of TYR:PBGD < 10⁻⁴. Twenty-six of 72 samples (36%) from 47 tumor-bearing patients showed detectable tyrosinase transcript levels of ≤10⁻³ (P = 0.0011 compared with disease-free patients), which was up to one order of magnitude higher than the highest expression in stage III patients.

MelanA/MART-1 was detectable in the peripheral blood of 3 of 21 healthy volunteers (≤2.2 × 10⁻³ ratio to PBGD). Therefore, MelanA/MART-1 transcripts were arbitrarily defined...
as elevated if they exceeded the values in healthy volunteers by a factor of 2 (4.4 × 10^{-5} ratio to PBGD). In 5 (10%) of 50 samples from 36 clinically disease-free stage III melanoma patients, MelanA/MART-1 mRNA expression was above this cutoff ratio. Eighteen (25%) of 72 samples from 47 tumor-bearing patients showed elevated MelanA/MART-1 levels (P = 0.037 compared with disease-free patients). Tumor-bearing patients had up to two orders of magnitude higher transcript levels than clinically disease-free patients.

In 17 of 21 healthy volunteers, gp100 mRNA expression was detectable with a level of ≤6.6 × 10^{-4} ratio to housekeeping gene. This level was only exceeded in 2 stage IV cutaneous melanoma patients with ratios of 2.48 × 10^{-4} and 2.61 × 10^{-4}, respectively.

Expression Level of Tyrosinase, MelanA/MART-1, and gp100 mRNA Transcripts in Uveal Melanoma Patient Blood Samples

Tyrosinase transcripts were detected in 3 (12.5%) and MelanA/MART-1 in 1 (4%) of 24 samples from 21 patients with high-risk primary uveal melanoma (Fig. 3). In contrast, tyrosinase was detected in 24 (60%) and MelanA/MART-1 in 31 (77%) of 40 samples from 26 metastatic uveal melanoma patients (P = 0.0002 for tyrosinase; P < 0.0001 for MelanA/MART-1 compared with disease-free patients). gp100 mRNA was detectable above background in 1 (4%) of 24 samples from 21 patients with primary uveal melanoma and in 4 (10%) of 40 samples from 26 patients with metastases. In the positive samples, the gp100 mRNA transcript levels were only up to one order of magnitude higher as compared with healthy volunteer control blood samples.

Correlation among the Three Markers

Samples were most often positive for tyrosinase (58 of 186 samples; 31.2%), followed by MelanA/MART1 (55 of 186 samples above cutoff; 29.6%), and gp100 (7 of 186 samples above cutoff; 3.8%). When comparing tyrosinase and MelanA/MART1 on a per-sample basis, 41 (22%) of samples gave concordant positive results, and 31 samples (16.7%) gave discordant results, with approximately equivalent percentage of samples only tyrosinase positive (17 samples; 9.1%) or only MelanA/MART1 positive (14 samples; 7.5%). All discordant samples had low-level expression of the positive marker. gp100 was only expressed above cutoff in samples simultaneously positive for tyrosinase and MelanA/MART1.

Correlation with Clinical Parameters

For this analysis, only the initial sample of each patient was considered.

Metastatic Disease Patients. There is a correlation between frequency of marker-positive samples and serum lactate dehydrogenase for patients with cutaneous as well as uveal melanoma (P < 0.01; Table 2). Furthermore, the expression level of melanoma-associated markers positively correlates with serum lactate dehydrogenase (data not shown) and tumor load (Fig. 4). There was no obvious correlation between treatment and PCR results (Figs. 2 and 3).

Patients without Clinical Evidence of Metastases. All 21 patients with high-risk uveal melanoma, from whom 3 had a positive PCR test, were entered into a protocol of adjuvant tyrosinase peptide vaccination. Within a follow-up of 6 months, 3 patients developed liver metastases. Two of these 3 patients had a positive PCR test (1 patient for all three markers and 1 patient for tyrosinase only) preceding the development of liver metastases. In contrast, only 1 of 17 patients with a negative PCR result developed liver metastases.

The clinical situation as well as treatment of patients with nonmetastatic cutaneous melanoma was highly heterogeneous; therefore, no correlation with follow-up data was attempted.

PBGD Housekeeping Gene Transcript Levels As a Measure for Sample Quality

The median PBGD content in the 186 in-house samples was 0.26 pg/μl (10% percentile 0.147, 90% percentile 0.517 pg/μl; Fig. 5). A small percentage (4.3%) of the in-house samples revealed PBGD contents < 0.1 pg/μl and 1.6% of samples PBGD contents < 0.01 pg/μl. The median PBGD content in the 251 EORTC trial samples from outside hospitals was slightly lower, as compared with the in-house samples (0.129 pg/μl; P < 0.001), and the variation was considerably larger (10% percentile 0.009 pg/μl, 90% percentile 0.429 pg/μl). Almost half (42.6%) of samples from the EORTC series had PBGD expression levels < 0.1 pg/μl and 11.9% < 0.01 pg/μl.

Next, we analyzed the potential influence of PBGD concentration on the detection of tyrosinase. About a third (31.2%) of the in-house samples (various disease stages) and 5.2% of the EORTC samples from patients (without detectable metastases) were positive for tyrosinase. Taken both sample series together, 19.7% of 320 samples with a PBGD content > 0.1 pg/μl expressed tyrosinase, as compared with 8.3% of 84 samples with a PBGD content between 0.1 and 0.01 pg/μl and only 3% of 33 samples with a PBGD content < 0.01 pg/μl (P = 0.024; χ²). Among the good quality samples with PBGD content > 0.1
Fig. 2 Expression of tyrosinase, MelanA/MART-1, and gp100 in peripheral blood samples from stage III and IV cutaneous melanoma patients without (NED, no evidence of disease) or with metastases. Ratios TYR/PBGD, MelanA/MART-1/PBGD, gp100/porphobilinogen deaminase (PBGD), the relative sample amount was expressed as ratio marker (tyrosinase, MelanA/MART-1, gp100)[pg/μl]/(PBGD)[pg/μl]. The concentration was calculated using plasmid standard curves. Untreated, untreated cutaneous melanoma patients. Vaccination, current treatment with an experimental adjuvant vaccine (tyrosinase peptides). No treatment, no treatment at time of sampling. Systemic treatment, heterogeneous systemic treatment regimens. Post-treatment, ≥6 weeks after completion of systemic treatment. Healthy volunteers, healthy persons who had no evidence of any clinically detectable disease at the time of blood draw. Black bars, median. Dotted lines, cutoff values for MelanA/MART-1 and gp100.

Fig. 3 Expression of tyrosinase, MelanA/MART-1, and gp100 in blood samples from uveal melanoma patients. Ratios TYR/porphobilinogen deaminase (PBGD), MelanA/MART-1/PBGD, gp100/PBGD, the relative sample amount was expressed as ratio marker (tyrosinase, MelanA/MART-1, gp100)[pg/μl]/(PBGD)[pg/μl]. The concentration was calculated using plasmid standard curves. Untreated, untreated patients. Vaccination, patients entered on an experimental adjuvant vaccine program with tyrosinase peptides. No treatment, no treatment at time of sampling. Chemotherapy, heterogeneous chemotherapy regimens. Post-treatment, ≥6 weeks after completion of systemic treatment regimens. Healthy volunteers, healthy persons who had no evidence of any clinically detectable disease at the time of blood draw (same persons as in Fig. 2). Black bars, median. Dotted lines, cutoff values for MelanA/MART-1 and gp100.
However, the detection of tyrosinase expression was independent of PBGD content.

**Discussion**

The issue of quality control in diagnostic PCR has repetitively been addressed. Of concern are both false positive and negative findings. Although false positive findings can be minimized by using rigorous contamination controls (39), the rate of false negative results is more difficult to monitor. Previous investigations (39, 44–46) have indirectly suggested that all steps of sample processing until completion of cDNA synthesis contribute to the heterogeneity of results and that in contrast, the PCR amplification itself is a rather robust procedure. In this study, we investigated whether a range of housekeeping gene expression can be defined to label an individual sample as informative or noninformative. We chose PBGD as a low-abundance, usually unregulated constitutively expressed housekeeping gene with no known retropseudogenes. The homogeneity of housekeeping gene expression of in-house samples was remarkably uniform with the 10 and 90% percentiles only a factor of 3 apart. The samples collected within the EORTC multicenter trials were more heterogeneous in PBGD housekeeping gene content. The observed differences in housekeeping gene expression do impact on diagnostic sensitivity. Within the range of 0.1–1 pg/μl PBGD content, the likelihood for a positive result is homogenous; however, if the PBGD content is <0.1 pg/μl, only occasional samples display melanoma markers. On this basis, it can be concluded that with a PBGD content > 0.1 pg/μl, samples are uniformly informative. Considering that almost half of the samples from the EORTC series has a PBGD content of <0.1 pg/μl, it is conceivable that much of the disparities in the results among various previous studies may be attributable to undetected differences in sample quality. All samples would have been accepted as good quality samples by the definitions commonly used in studies for conventional PCR assays.

The issue of single-marker RT-PCR versus multiple marker assays is still controversial. Of the markers investigated here, gp100 appears not suitable because of high background expression in normal donors. MelanA/MART1 also has a degree of background expression; however, with definition of a clear
cutoff value, the overall sensitivity of MelanA/MART1 appears to be similar as that of tyrosinase, which, in our experience, had no detectable background expression, and in a number of patients, only tyrosinase or melanA/MART1 was found to be positive. The discrepancy between these two markers may be attributable to varying gene expression in different patients and even in the same patient over time.

Quantitative RT-PCR therefore allows not only to quantify the level of expression of melanoma markers but also assess sample quality. In our previous analysis using a semiquantitative PCR, there was a significant correlation between overall tumor load and level of tyrosinase expression (6). This correlation between tumor load and magnitude of melanoma marker expression is confirmed in the present study with quantitative PCR, although the frequency of positive samples is lower, especially in patients with lower tumor load. There is also a correlation between frequency of PCR-positive samples and serum lactate dehydrogenase as a measure for tumor growth kinetics. An analysis of a homogenous patient cohort with sufficient follow-up will be necessary to define, whether the PCR result yields useful information in addition to the known prognostic factors. Another potential application of quantitative real-time PCR for melanoma markers may be the assessment of marker expression as inclusion criterion and during follow-up in vaccine studies. This is of increasing interest as cellular and peptide vaccine approaches move into the adjuvant setting (e.g., ECOG trials 4697 and 18001).

There are only two small studies in the literature analyzing tyrosinase transcripts in peripheral blood of patients with uveal melanoma, which both failed to detect circulating melanoma cells in uveal melanoma patients (12, 13). In contrast to the previous reports, we here describe frequent and high expression of melanoma-associated RNA in the peripheral blood of patients with liver metastases from uveal melanoma. The frequent presence of circulating tumor cells indicates that despite the usual clinical presentation of metastases confined to the liver, tumor cells do have the capacity and habit to circulate in the peripheral blood. In contrast to cutaneous melanoma, the metastatic spread of uveal melanoma is primarily hematogenous, because the uvea does not have draining lymphatics. Risk factors for hematogenous spread include tumor dimensions (47) and vessel density (48). It is of interest that circulating tumor cells preceded the development of clinically detectable liver metastases in 2 of 3 patients in our analysis. Evaluation of larger patient cohorts with longer follow-up should be carried out to confirm the ability of RT-PCR assays to predict the development of liver metastases in patients with high-risk uveal melanoma to improve our understanding of the tumor biology and stratify patients within future clinical trials.

References
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