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Original Paper

New Prognostic Factors in Melanoma: mRNA Tumour Markers

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Circulating tumour cells in the peripheral blood may be important for haematogenous spread of malignant disease. Monitoring these cells may therefore be of prognostic value. Reverse transcriptase-polymerase chain reaction (RT-PCR)-based assays to detect occult neoplastic cells offer the highest sensitivity for the study of tumour dissemination and minimal residual disease. This review summarises technical considerations and clinical investigations in melanoma patients of various disease stages. The clinical data are promising, but to clearly define the clinical usefulness of messenger RNA (mRNA) tumour markers, methodological issues must be resolved and the clinical value must be assessed prospectively in sufficiently large patient cohorts. © **1998 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

Despite advances in cancer treatment, disseminated disease continues to pose a major problem in clinical management. Traditional tumour markers are large molecules, mostly glycoproteins, which are overexpressed and released into the serum from tumour cells. Low levels of these proteins are usually expressed in normal tissue from which the malignant cell was derived. In selected tumours, elevated serum levels of these tumour markers are useful in monitoring disease status, but false-positive results limit their value for screening purposes.

The relationship between circulating tumour cells and development of secondary disease is not fully understood, but for cells to metastasise they must be capable of entering and surviving in peripheral blood or bone marrow. Although it is believed that less than 0.01% of circulating tumour cells successfully establish metastatic colonies [1], a method to detect the small numbers of these cells would allow evaluation of their role in the disease process. This may provide a powerful tool to predict recurrence and subsequently improve long-term clinical outcomes.

Using light microscopy, small aggregates of tumour cells in the bone marrow or peripheral blood of patients with metastatic disease can sometimes be identified. Early studies isolated tumour cells from blood and assessed the cells by cytology. However, this method has limited sensitivity and cytological artifacts cause a high rate of false-positive results [2]. Subsequent studies with monoclonal antibodies (MAbs) found occult single cells, derived from solid malignancies, in the bone marrow [3–6] and peripheral blood [7] of many patients. Modern molecular techniques substantially increase the sensitivity and specificity of occult tumour cell detection. Reverse transcription of RNA and subsequent polymerase chain reaction (RT-PCR) facilitate assessment of gene expression under many circumstances. Application of this technique for the assessment of disseminated disease allows detection of one malignant cell in up to 10^7 normal cells [8]. The technique uses tissue-specific expression of messenger RNA (mRNA) as a target for the characterisation of tumour cells. Since mRNA is only transcribed in viable cells and free mRNA is not stable in haematological tissues, the identification of a tissue-specific target mRNA in peripheral blood indicates the presence of circulating tumour cells. The potential value of RT-PCR-based assays to monitor occult haematogenous spread and residual disease has resulted in the initiation of many studies in patients with solid tumours and haematological malignancies.

TARGET GENES FOR DETECTION OF MELANOMA CELLS

Selection of suitable tissue-specific targets for RT-PCR is essential. Ideally, targets would be tumour specific, such as

the expression of a chimeric fusion product and must not be expressed in normal haematopoietic tissues. However, because frequent melanoma-specific abnormalities have not yet been identified, RT-PCR to detect circulating tumour cells using tissue-specific targets has not been used. Several specific genes, mostly those encoding for melanosomal proteins, are selectively expressed in melanocytes and melanomas. Tissue-specific expression provides several potential targets for the identification of circulating melanoma cells.

Expression of the tyrosinase gene is most widely used for detection of circulating melanoma cells. Tyrosinase, the first enzyme in melanin biosynthesis, is a mono-oxygenase that catalyses the conversion of tyrosine to dopa and dopa to dopaquinone [9]. Tyrosinase is one of the most specific markers of melanocytic differentiation and its expression is conserved in many melanotic and amelanotic primary and metastatic melanomas [10]. Since melanocytes do not exist in the circulation, it is an ideal tissue-specific target for detection of circulating melanoma cells. The gene has been cloned and sequenced [11] and the genomic organisation reveals several exons that can be used to design PCR primers that span introns [12].

Further melanocyte-specific markers of differentiation include glycoprotein (gp) 100 [13], melanoma antigen recognised by T cells (Melan-A/MART-1) [14, 15] and a family of tyrosinase-related proteins [9]. The diagnostic antibodies HMB45 and NK1 beteb recognise gp100, but it is not well suited for detection of single melanoma cells for three reasons: (1) gp100 is frequently lost during tumour progression [16, 17]; (2) the expression pattern within metastases is heterogeneous [10, 17, 18]; and (3) low levels of gp100 expression can be detected in nonmelanoma cell lines [19] as well as normal tissues (Brouwensteijn and Schrier, Department of Medical Oncology, University of Leiden, The Netherlands). The expression of tyrosinase, Melan-A/MART-1 and tyrosinase-related protein is also lost in a high percentage of metastatic melanoma lesions, as shown by investigations using recently generated specific MAbs [10, 18, 20]. Melan-A and tyrosinase have been reported to be either coexpressed (90%) or both absent (10%) in the majority of melanoma specimens. This pattern of expression is usually conserved in multiple metastases from an individual patient [19], suggesting that a panel of differentiation markers in diagnostic RT-PCR for melanoma may not offer a great advantage over amplification of tyrosinase only. One concern is that a panel of markers may increase the likelihood of false-positive results.

Melanomas also express antigens of the MAGE family. Sixty per cent of metastatic lesions are reported positive for MAGE-1 and 80% for MAGE-3 using PCR [21]. Furthermore, a tumour-specific mucin isoform (MUC18) has been evaluated as a marker gene [22]. However, low-level expression of MAGE and MUC18 genes in peripheral blood preclude their use in diagnostic PCR assays.

TECHNICAL CONSIDERATIONS

Sample preparation

Appropriate collection and processing of clinical specimens is crucial for RT-PCR success. A concern is that a melanocyte could be introduced into peripheral blood from the skin during venipuncture and lead to false-positive results. The likelihood of introducing a melanocyte into the blood sample is small, given the location and number of melanocytes. To avoid even this small chance, the first tube of blood drawn after venipuncture could be discarded and a subsequent sample used for the PCR assay.

Extraction of RNA has been described from whole blood or from leucocyte preparations, both of which have advantages and disadvantages. Working with whole blood may decrease the purity and quality of isolated RNA but it eliminates the risk of losing tumour cells. Manipulations of blood samples to separate red blood cells from leucocytes (i.e. erythrocyte lysis buffers or density gradient separation techniques) can improve the purity of RNA but may result in loss of tumour cells [19].

RNA extraction and purification

For extraction of RNA guanidinium-isothiocyanate-phenol-chlorophorm-based extraction methods as first described by Chomczynski and Sacchi [23] are most commonly used. Alternatively, commercial kits for RNA extraction based on ion exchange columns have been used with apparently equivalent results. However, the efficiency of extraction from sample to sample varies and extraction of RNA from some samples can be unsuccessful. Therefore, the quality and quantity of RNA isolated from every sample should be assessed before analysis by RT-PCR either spectrophotometrically or on a 3-[N-morpholino] propanesulfonic acids (MOPS) or tris-borate-EDTA (TBE) agarose gel.

Reverse transcription

A number of protocols and reverse transcriptases have been described for this reaction that convert isolated RNA to complementary DNA (cDNA). Most methods include an inhibitor of RNAses in the reverse transcriptase reaction to prevent degradation of RNA and thus reduction in cDNA yield. It is advisable to analyse an aliquot of RNA by RT-PCR for a ubiquitous species, such as β2-microglobulin, β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), or porphobilinogen deaminase (PBGD). This will confirm the ability to generate cDNA from target RNA. GAPDH and β-actin have the disadvantage that a product of the expected size also can be generated by PCR from genomic DNA without reverse transcription due to the presence of pseudogenes. Generally, the housekeeping genes are so abundantly expressed that they can be detected even in cDNAs of rather poor quality, which are not suited for sensitive detection of a target with a template number several orders of magnitude lower.

Primers and PCR protocols

The specificity of RT-PCR for tumour cell detection is achieved by amplification of tyrosinase by PCR. The most widely used primers have originally been developed for nested PCR by Smith and associates [12] and designated as HTYR1 to HTYR4 (Figure 1). Alternatively, the primer pair VB16 and VB17 described by Brichard and colleagues [24] have been used to produce reliable results.

For cDNA amplification, several different DNA polymerases are available. For all primers and enzymes, the optimal PCR conditions must be established. With reliable thermal cyclers, the intra-assay and interassay efficiency variations of PCR are considerably smaller than the efficiency variations of RNA extraction and cDNA synthesis. In most publications, tyrosinase has been amplified by nested PCR (two times 30 cycles). As determined from spiking

experiments, nested PCR facilitates the detection of a single melanoma cell in 10 mL of blood if the cell expresses tyrosinase mRNA [22, 25–33]. Similar sensitivity can be achieved for single-round PCR using the inner primers HTYR3 and HTYR4 with 40 to 45 cycles and subsequent detection by Southern blotting. Single-round PCR reduces the number of manipulations to be performed on a given sample and thereby reduces the risk of sporadic contamination.

Two different approaches have been used to make a semiquantitative assessment of the amount of tyrosinase mRNA in a sample. Although the level of expression may vary considerably and the quantitation of the tyrosinase RNA cannot be translated into cell numbers, such a technique may be useful to assess variation in tumour burden for a given patient over time. Curry and coworkers [33] developed a competitive PCR assay with a heterologous DNA (PCR MIMIC). Brossart and associates [34] used two PCR reactions with subsequent Southern blotting of the PCR product and standardisation to the expression of a housekeeping gene. For the first approach, several PCR reactions per sample are necessary, but it yields true quantitative PCR results. In the second approach, the PCR assay is semiquantitative, but comparison with a housekeeping gene corrects for differences in efficacy of RNA extraction and cDNA synthesis. Quantitative assay systems with internal controls are under development.

Quality control

As discussed at a meeting of the European Organization for Research and Treatment of Cancer Melanoma Cooperative Group (EORTC-MCG) in January 1996 [19], standard quality control measures must be implemented to ensure sensitivity and reliability of the assay systems that are primarily developed in-house. The urgent need for quality assurance initiatives for diagnostic PCR also has been underscored by a series of quality control studies on viral nucleic acid-based amplification assays by the European Expert Group on Viral Hepatitis (EUROPHEP). These studies found that only 16–23% of participating laboratories reported faultless results [35].

The most important issue is to avoid false-positive test results due to contamination of blood samples, tubes, or reagents in the laboratory. If the amplification of a certain gene is performed routinely over a prolonged time period, PCR products accumulate in the environment of the laboratory, substantially increasing the risk of contamination. Stringent controls to detect systematic as well as sporadic contamination are essential. The standard controls, which are routinely used to ensure accuracy of RT-PCR assays (Figure 2), are not sufficient to exclude sporadic contamination. An extended set of positive and negative controls (Figure 3) can ensure the detection of systematic and sporadic contamination as well as differences in sensitivity.

Quality control studies also should be performed to prospectively assess the homogeneity of results from different laboratories. The first prospective quality assurance study was performed among member laboratories of the EORTC-MCG. This study has been completed and the encouraging results are reported elsewhere (U. Keilholz for the EORTC-MCG).

ROLE OF RT-PCR IN MELANOMA PATIENTS

After complete resection of a primary melanoma or regional metastases, RT-PCR-based detection of circulating melanoma cells could be particularly useful to assess a patient's risk of systemic spread. Such 'molecular staging' could help elucidate the process of melanoma dissemination and ultimately guide decisions for adjuvant treatment. In patients with advanced melanoma, qualitative and semiquantitative assessment of circulating melanoma cells is of interest for studying tumour biology and for follow-up of minimal residual disease after successful treatment. Furthermore, by monitoring the expression of tissue-specific genes, antigen-negative tumour variants that develop during the course of disease or that are induced by immunotherapy could be studied. In single-site studies conducted by various research groups, stage I–IV melanoma patients have been analysed for the presence of circulating melanoma cells.

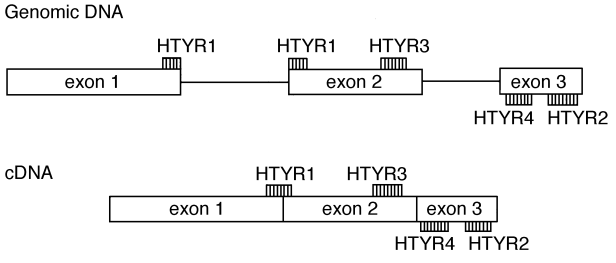

Figure 1. Primer design for amplification of human tyrosinase with nested PCR. The primers HTYR1 and HTYR2 were used during the first round of PCR. Amplification of genomic DNA is excluded because HTYR1 spans an intron. The primer pair HTYR3 and HTYR4 for the nested PCR amplify a region of the cDNA, where in the genomic DNA another intron would be amplified resulting in a larger PCR product than from correctly spliced RNA. Adapted with permission from the *Lancet* 1991 [12].

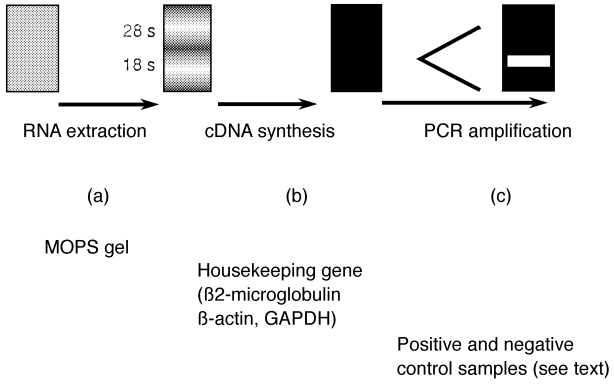

Figure 2. Standard controls for PCR assays. (a) After extraction of total RNA from a homogenised sample, the extract can be run on a MOPS agarose gel and stained with ethidium bromide. The 28s and 18s ribosomal RNA (rRNA) bands indicate the amount of intact RNA. The amount can be quantified spectrophotometrically. (b) The subsequent cDNA synthesis cannot be controlled directly. To determine whether amplifiable cDNA has been synthesised, a 'housekeeping' gene, translated in every viable cell, can be amplified by PCR and detected. If no signal is visible for the housekeeping gene, the cDNA or the RNA is of insufficient quality for reliable analysis. GAPDH = glyceraldehyde-3-phosphate dehydrogenase. (c) After PCR amplification of the target gene, the product can be run on an agarose gel and visualised by ethidium bromide staining. Simultaneously processed positive and negative control samples can exclude contamination of the reagents (but not sporadic contamination) and give an estimate of the PCR sensitivity.

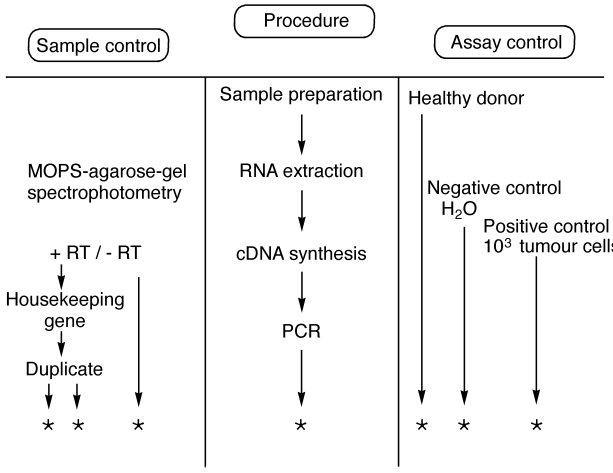

Figure 3. Extended set of controls recommended for diagnostic PCR. Contamination and low sensitivity can result from any step in the process of sample preparation, RNA extraction, cDNA synthesis, and PCR (centre), therefore all steps need to be controlled. (a) Sample control. The suitability of an RNA for analysis should be verified on a gel and quantified by spectrophotometry. The cDNA synthesis should be performed in parallel with and without reverse transcriptase (RT) to exclude systematic and sporadic contamination at the level of sample handling and RNA extraction. In the sample without RT, a positive PCR signal would indicate contamination. The cDNA of the sample with RT should be checked by amplification of a housekeeping gene. The results of the duplicate PCR for the marker gene should give identical results. (b) Assay control. At least one sample from a healthy donor should be analysed in parallel to a series of patient samples. For the PCR, a negative water control is suitable to rule out contamination of reagents and a positive control of mRNA from a blood sample spiked with a low number (10^1–10^3) of tumour cells will assess efficiency of the PCR.

Localised melanoma (UICC stages I–III)

Studies reporting the detection of circulating tumour cells in melanoma patients with disease stages I–III are listed in Table 1. The percentage of patients with evidence of occult tumour dissemination has been correlated with disease stage in most [12, 22, 25–27] but not all [28, 29] reports. Sample sizes are small and comparison of the results is difficult due to differences in patient selection. The clinical utility of the assay has been addressed by two groups and both suggest that the PCR assay is of prognostic value in melanoma. Battayani and colleagues [26] reported a significantly ($P = 0.002$) higher recurrence rate within 4 months after resection of regional lymph node metastases in patients with a positive tyrosinase signal. Furthermore, in a prospective investigation by Mellado and colleagues [27], the presence of tyrosinase transcripts in the peripheral blood of stage II and III patients was associated with significantly ($P = 0.002$) shorter disease-free survival and identified as an independent prognostic factor.

Table 1. *Detection of circulating melanoma cells in patients with disease stages I–III*

Reference	No. TYR mRNA-positive patients/*n* patients tested	
	Stage I	Stage II–III
Battayani [26]	2/10	8/18
Brossart [25]	1/10	6/17
Hoon [22]	1/6	41/47
Kunter [30]	0/16	0/16
Mellado [27]	4/17	17/39
Reinhold [29]	0/31	1/21

TYR, tyrosinase.

These results are similar to observations in other solid tumours [8]. Larger prospective investigations are now necessary to confirm the prognostic value of the PCR assay, especially in melanoma patients with disease stages I–III who are surgically rendered disease-free [36]. In addition, the value of adjuvant treatment strategies, such as interferon alfa (IFN-α), in PCR-positive and PCR-negative patients must be addressed in large controlled clinical trials to investigate the role of PCR results in guiding treatment decisions. These questions are currently being examined by the EORTC-MCG in a prospective, randomised trial studying intermediate-dose adjuvant IFN-α.

Advanced disease (UICC stage IV)

As expected, several laboratories have found PCR evidence for circulating melanoma cells in a high proportion of untreated patients with stage IV disease, either by amplification for tyrosinase mRNA [12, 25, 27] or by using a multiple-marker PCR [22] (Table 2). Other groups, including a recent publication that appeared after the meeting, have reported much lower frequencies [26, 28, 30, 31, 37]. The reason for the difference is unclear. In all reports, the PCR assays were reported to be able to detect a single melanoma cell in 10 mL of blood. Differences in patient selection, especially tumour load and treatment status, may partially explain the discrepancies. Furthermore, differences in sample processing as well as the use of various control systems to ensure sensitivity and specificity, may account for heterogeneity of results.

Despite these heterogeneous results, the findings of several studies are interesting. In one study, semiquantitative assessment revealed an association between the intensity of the PCR signal and overall tumour burden [34]. Two of the groups with a lower frequency of tumour cell detection reported that patients with stage IV melanoma and a positive PCR result were significantly ($P \leq 0.05$) more likely to experience rapid disease progression than PCR-negative patients [26, 30]. For stage IV patients in long-term complete

Table 2. *Detection of circulating melanoma cells in patients with distant metastases (stage IV)*

Reference	Sample processing	Marker gene	No. TYR mRNA-positive patients/*n* patients tested
Battayani [26]	Ficoll 1.077	Tyrosinase	25/73
Brossart [32]	Whole blood	Tyrosinase	21/21
Foss [28]	Whole blood	Tyrosinase	0/6*
Hoon [22]	Ficoll 1.077	Multiple	46/48†
Keilholz [‡]	Whole blood	Tyrosinase	30/35§
			65/81‖
Kunter [30]	Whole blood	Tyrosinase	9/32
Mellado [27]	Whole blood	Tyrosinase	33/35
Pittman [31]	Whole blood	Tyrosinase	3/24¶
Reinhold [29]	Ficoll 1.077	Tyrosinase	5/13
Smith [12]	Whole blood	Tyrosinase	4/7

TYR, tyrosinase. *Storage of blood samples without buffer at −70°C prior to processing. †Two of 39 healthy control samples tested positive. ‡U. Keilholz for the EORTC-MCG, unpublished data. §Untreated patients. ‖After treatment. ¶Including mostly patients receiving systemic treatment.

remission after treatment with IFN-α and interleukin 2 with or without resection of residual metastases, tyrosinase transcripts have been detected in most patients for over 5 years without clinical evidence of recurrence [32]. Semiquantitative assessment revealed a very low concentration of tyrosinase transcripts in the peripheral blood of these patients [34]. It is not yet known whether a rise in signal intensity could be an early indicator of relapse.

CONCLUSIONS

RT-PCR-based assays may hold promise for the detection of occult neoplastic cells and may potentially be used to predict disease recurrence. Proper sample collection and quality assurance procedures are crucial to prevent false-positive results from the assay procedure. RT-PCR-based assays have detected circulating melanoma cells in patients with disease stages I–IV. Small sample size and differences in patient selection may be at least partially responsible for inconsistencies in results. Large prospective trials are needed to confirm the diagnostic and prognostic value of these assays.

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