Chapter 9

MINIMAL RESIDUAL DISEASE IN MELANOMA

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Abstract

A number of specific genes encoding for melanosomal proteins are selectively expressed in melanocytes and melanomas. For detection of circulating melanoma cells, the expression of the tyrosinase gene is most widely used. Several cohorts of melanoma patients from single institutions have been analyzed by various research groups for the presence of circulating melanoma cells in all stages of disease. The percentage of patients with evidence for occult tumor dissemination has been correlated with the stage of disease in several, but not all, reports. Two prospective analyses suggest that the PCR result is of prognostic value in melanoma. Several laboratories have found PCR evidence for circulating melanoma cells in the great majority of untreated patients with Stage IV disease, other groups have reported much lower frequencies. Taken together, there is a wide range of results. Methodological differences are likely to account for this discrepancy. With the availability of true quantitative real-time reverse transcriptase (RT)-PCR systems, accurate quantification of tyrosinase transcripts over a range of 1 to 10,000 tumor cells per milliliter of blood is possible. Quantitative real-time RT-PCR systems also dramatically improve quality control, since exact quantitation of housekeeping gene mRNA facilitates determination of sample quality. Two large clinical trials are currently under way within the EORTC and in the US to adequately determine the clinical usefulness of PCR detection of minimal residual disease in melanoma.

1. INTRODUCTION

Malignant melanoma accounts for 1% to 3% of all malignant tumors (1). Once disseminated beyond the regional lymph nodes, malignant melanoma is largely incurable, with a median survival of 4–6 months (2). Early identification of melanoma patients at risk for hematogenous spread of the disease would be desirable. Therefore, polymerase chain reaction (PCR) tests to detect circulating melanoma cells have been developed.

In principle, single tumor cells in the bone marrow, for instance, can be detected by immunohistological techniques. However, this method is not sufficiently sensitive to reliably monitor early metastasis or minimal residual disease,
since only a very limited number of cells can be assayed at one time. For the detection of occult metastasis from melanoma cells, several marker genes have been used, including tyrosinase, a key enzyme in the melanin biosynthetic pathway, melanA/MART-1, a melanosomal protein of unknown function or the melanoma-associated antigen A (MAGE-A) genes.

One limitation of those reverse transcriptase (RT)-PCR assays was the inability to quantitate the transcript amount accurately. The recently developed TaqMan and Light Cycler techniques combine amplification, detection and quantification, and are (a) easy to handle, (b) very rapid, (c) reproducible and (d) suited for high throughput screening applications. The novel possibility of real-time PCR is changing this whole field of investigation in two ways. First, real-time PCR provides quantitative data on minimal residual disease, which may be more informative. Secondly, real-time PCR allows much more detailed analysis of sample quality.

2. PRINCIPAL PCR DETECTION METHODS

This chapter describes the principles of occult tumor cell detection using PCR, and summarizes the currently available clinical data from trials utilizing PCR-based techniques. RNA-based methods require active transcription of the gene of interest. Fortunately, a high transcript number from the gene of interest is usually present in a tumor cell. RNA-based detection, therefore, has the advantage of high sensitivity and of detecting primarily viable cells, although detection of unviable cells in the early stages of apoptosis is theoretically possible. The number of RNA copies of a gene in any particular tumor cell may, however, vary during the cell’s life cycle or as a result of de-differentiation.

2.1 Qualitative PCR Assay

RT-PCR is a highly sensitive method for detecting rare tumor-cell derived mRNA, allowing the diagnosis of tumor dissemination at early stages. This information may have important prognostic and therapeutic implications because residual tumor cells that are below the limit of detection using standard diagnostic techniques are nevertheless associated with increased risk for overt clinical relapse (3).

First, the RNA is extracted from the sample and the mRNA is reverse-transcribed into cDNA. The gene of interest is then amplified using primers specific for that gene. Ideally, these primers should not amplify genomic DNA, which often contaminates the cDNA preparation. Amplification of a cDNA sequence without amplification of the genomic counterpart of this sequence can be achieved if one primer is interrupted by an intron in the genomic DNA. The intron will have been deleted during RNA processing and, therefore, will not interrupt the primer sequence in the cDNA version of the gene. Alternatively, primers can be chosen that flank an intron in the genomic sequence, thereby
facilitating easy differentiation between a PCR product derived from genomic DNA and one derived from cDNA based on the size of the amplicon. If intron/exon boundaries are unknown or targeted genes are intron-less, it is necessary to treat the RNA with RNase-free DNase.

One limitation of RT-PCR assays was the inability to quantitate the transcript amount, information that would be of interest for monitoring tumor progression or assessing the response to therapy in patients who prior to treatment had PCR evidence for circulating tumor cells.

2.2 Semiquantitative PCR Assay

Previous investigations have attempted to quantify tyrosinase transcripts using either serially diluted and differently sized competitor target molecules (4, 5, 6) or Southern blot analyses with a standardization to the expression of a housekeeping gene (7). Competitive PCR with a heterologous DNA (PCR MIMIC) as an internal standard was used. The method was validated by demonstration of similar amplification efficiencies for both molecules and by accurate quantitation of an artificial fourfold difference in the level of tyrosinase mRNA. The ratio of amplified target to amplified standard (at/as ratio) was determined (6). Alternatively, our groups developed a semiquantitative assessment to detect and quantitate circulating tumor cells by comparing the amount of RNA equivalent to tyrosinase mRNA content in a defined number of SK-mel 28 cell line (7, 8, 9). This latter principle is similar to the real-time PCR assays described below.

Both of these semiquantitative assays reside on close-to-end-point quantitation of transcript amounts, which is prone to influence PCR by rate-limiting reagents and product inhibition.

2.3 Real-time PCR

Real-time RT-PCR offers for the first time the possibility to quantify templates rapidly and rather accurately using crossing points which mark the early exponential phase. This technique makes quantification much more precise and reproducible, because at the beginning of the exponential phase none of the reagents is rate limiting and only minimal inhibitory effects occur.

Using real-time PCRs, post-PCR steps are no longer necessary. PCR products are detected either by a dual labeled TaqMan probe with a reporter (FAM) and a quencher dye (TAMRA) at the 5’ and 3’- end or two Light Cycler hybridization probes labeled with a donor (Fluorescin) or acceptor fluorophore (LC Red 605/705) at the 3’ or 5’- end annealing to the target sequence in close proximity. Fluorescence signals arise by fluorescence energy transfer between donor and acceptor fluorophore (FRET) during primer annealing (Light Cycler probes) or by separating the reporter dye due to the 5’-nuclease activity of the Taq polymerase during elongation (TaqMan probe). Fluorescence signals are proportional to the
initial number of target cDNA and are plotted versus crossing points which mark the cycle number when fluorescence becomes significantly different from baseline signal.

The transcript amount is calculated from the linear regression of a standard curve received by serial dilutions of (a) marker-specific PCR products, (b) marker expressing cell lines, (c) plasmids harboring the desired marker sequence or (d) recombinant plasmid derived in vitro transcripts. The relative amount of marker transcript-derived amplicons is expressed as ratio: marker – by housekeeping-gene transcripts. Normalization is important to compensate for differences in RNA and cDNA quality (sample to sample variation). Housekeeping genes are usually used for normalization because they are expected to be expressed at a constant level among different tissues and at all stages of development unaffected by experimental treatment. With the Light Cycler technique, PCR products can also be detected by Sybr Green which binds to nascent double-stranded DNA. This results in an increase in fluorescence that falls off when DNA is denatured. Specificity is guaranteed by amplicon-dependent Tm determined by melting curves.

3. CLINICAL DATA ON MELANOMA PATIENTS

3.1 Marker Genes

A number of genes encoding for melanosomal proteins are expressed specifically in melanocytes and melanomas. Tyrosinase is the first enzyme in the melanin biosynthesis pathway. It is a monoxygenase that catalyzes the conversion of tyrosine to dopa and of dopa to dopaquinone. Tyrosinase is, therefore, one of the most specific markers of melanocytes, and it is conserved in most amelanotic melanoma metastases. Expression of the tyrosinase gene is the most widely used indicator for the detection of circulating melanoma cells. Other melanocyte-specific proteins include gp100, which is recognized by the diagnostic antibodies HMB45 and NKI-beteb (10), Melan A/MART1 (11, 12) and a family of tyrosinase-related proteins. gp100 is less suited for monitoring of melanoma, since it is known to be frequently lost during tumor progression (13, 14). The expression of Melan-A/MART1 and tyrosinase-related proteins has been less well studied, but it has been shown that their expression is also lost in a significant percentage of metastatic lesions as detected by specific monoclonal antibodies (15, 16). Melanomas also express tumor-associated genes such as the MAGE family: about 60% of metastatic lesions are positive for MAGE-1, and 80% for MAGE-3 as detected by PCR (17–20). The family of proteins encoded by these genes may, therefore, represent another useful marker for metastatic melanoma.

Smith et al. (21) reported in 1991 that circulating melanoma cells can be detected by PCR of tyrosinase mRNA. Their PCR assay is used most frequently today because of its optimal primer design. The primers, designated as HTYR1
to HTYR4, exploit the presence of two introns in the tyrosinase gene. HTYR1 spans an intron, and another intron is located between the nested primers HTYR3 and −4. This primer design virtually excludes amplification of genomic DNA.

The initial report led to a number of more detailed investigations on the presence of tyrosinase mRNA in the peripheral blood of melanoma patients (7, 9, 22–26). Non-melanoma controls were always negative for expression of tyrosinase using this technique, suggesting that normal melanocytes do not circulate in blood, and that the presence of tyrosinase mRNA can be considered to be an evidence for circulating melanoma cells.

3.2 Clinical Cross-sectional Analyses

The results reported for melanoma patients vary considerably between different laboratories (27) (Table 1 summarizes the results published until 2001). This is most obvious in Stage IV melanoma patients, where the percentage with evidence for circulating melanoma cells ranges between 0% and 100%. The most likely explanation for these discrepancies is methodological differences between laboratories. Sample processing affecting efficiency of RNA extraction and cDNA synthesis may play a major role. In particular, the use of Ficoll-Hypaq density-gradient separation prior to RNA extraction may significantly decrease the number of positive results in Stage IV patients. Quality-assurance initiatives have recently been undertaken (34) to assess and ultimately resolve the methodological differences, thus facilitating the comparison of results from different laboratories.

3.3 PCR Data and Clinical Course

3.3.1 Early Stages

The detection of circulating melanoma cells could be particularly useful in earlier stages of the disease, and could ultimately guide decisions concerning adjuvant treatment strategies. To date, however, the percentage of patients with Stage I, II, and III melanoma and the PCR evidence for circulating melanoma cells in these patients varies considerably from one report to the next. This may be not only due to differences in methodology, but also to differences in patient selection.

Several early analyses, however, have already suggested that PCR results are of prognostic value in melanoma: Battyani et al. (23) described, that after resection of regional lymph node metastases, the likelihood of recurrence within 4 months was significantly higher in patients with a positive tyrosinase signal using PCR, and that patients with Stage IV disease and a positive tyrosinase signal were significantly more likely to experience rapid disease progression within 4 months than patients tested negative in the PCR assay. Mellado et al. (28) reported, in a prospective investigation of Stage II and III melanoma, that the
The presence of tyrosinase transcripts in the peripheral blood was associated with significantly shorter disease-free survival.

Larger prospective investigations are necessary, and are still under way, in the EORTC and also in the US (Sunbelt trial) to confirm the prognostic value of the PCR assay, especially in melanoma patients with disease Stages I through III who have been rendered disease-free by surgery. These studies will also address the
value of adjuvant treatment strategies (e.g., interferon-α (IFN-α) in PCR positive and negative patients in the setting of large controlled clinical trials), and thereby investigate the possibility of guiding treatment decisions based on PCR results.

3.3.2 Stage IV

In melanoma patients with Stage IV disease who have entered long-term complete remission upon treatment with IFN-α and interleukin-2 (IL-2), with or without resection of residual metastases, tyrosinase transcripts could still be detected in most patients for a period of over 5 years without clinical evidence of recurrence. Semiquantitative assessment revealed a very low number of tyrosinase transcripts, equivalent to less than 100 SK mel 28 melanoma cells (29). It is not clarified whether a rise in signal intensity could be an early indicator for relapse. It has to be acknowledged, that the amount of tyrosinase transcripts does not allow calculation of the number of circulating tumor cells, since the expression of the marker genes has been shown to vary between tumors in different persons (30) and is also expected to vary in the tumor of the same individual. This is especially important, when testing patients undergoing vaccination with melanocyte differentiation proteins. This specific immunologic treatment may lead to a selection of tumor cells with reduced or absent expression of marker genes, as already shown on the protein level in histologic samples (31) obtained in lesions regressing after non-specific immunotherapy with IFN-α and IL-2.

3.3.3 Tissue Analysis

The value of PCR for examination of solid tissue has been investigated. Lymph node preparations from patients with Stages I or II melanoma were analyzed pathologically and by PCR in one series of experiments (32). Of 29 regional lymph node samples, 38% had pathological evidence for melanoma cells, whereas 66% including all pathologically positive nodes, were RT-PCR positive as assessed by detection of tyrosinase mRNA. The results of lymph node investigation are very encouraging, but larger studies are necessary to discern the clinical value of this procedure (3). It is important to strictly avoid contact with skin when processing tissue samples to avoid contamination of the samples with melanocytes. For peripheral blood samples, the risk of contamination with skin melanocytes is lower, and may be further reduced by discarding the first syringe of blood drawn after venipuncture and using a second syringe to draw the sample for the PCR assay.

4. CONCLUSION AND FUTURE PROSPECTS

Quantification of tumor transcripts amplified by real-time PCR is a powerful and, in principle, the most sensitive tool to monitor the course of disease, to evaluate
the response to chemotherapy in more detail and to identify patients at high risk for developing hematogenous melanoma metastasis or relapse (33, 34). If residual tumor cells are detected and the transcript amount of their tumor marker is even quantitated, clinicians could theoretically intervene early with therapy.

Prior to widespread application of these methods, it is important to first establish very rigid quality assurance systems for academic centers and, in the future, for commercial laboratories. Second, detection of molecular signals of potential occult tumor cells by PCR cannot automatically be regarded as defining tumor cell presence and competence for further metastases, or a disease stage warranting systemic treatment. To establish clinical utility and to test the prognostic value of PCR results, large prospective studies with a long follow-up period are needed and have been initiated within the EORTC Melanoma Group and the US Sunbelt trial in association with state-of-the-art clinical and pathological evaluation.

REFERENCES


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