RT-PCR for tyrosinase expression as a molecular marker in malignant melanoma

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Summary

As the incidence of malignant melanoma steadily increases the need for markers in detection of early metastasis and guidance of therapy has become urgent. Presence of melanoma cells in patient peripheral blood, lymph nodes or bone marrow (BM) specimens could indicate tumour dissemination, and thus a high risk of metastasis. In 1991 coupled Reverse Transcription and Polymerase Chain Reaction (RT-PCR) was firstly used to detect tyrosinase mRNA in the peripheral blood of melanoma patients. Since then numerous studies have evaluated the significance of tyrosinase expression in bone marrow specimens, lymph nodes and blood from melanoma patients and there results indicate that this method might have important clinical applications in melanoma management.

Key words: malignant melanoma, molecular markers, RT-PCR, tyrosinase expression

Introduction

During the last decades the incidence of malignant melanoma steadily increases and the need for specific markers aiming at early detection of metastasis and guidance of therapy has become urgent. Presence of melanoma cells in patient peripheral blood, lymph nodes or bone marrow specimens indicate tumour dissemination, and thus a high risk of metastasis. Detection of expression of tissue specific genes could serve as a prognostic marker for the prediction of clinical behaviour, assessment of tumour progression and improvement of patient management.

In 1991 Smith et al. [1] were the first to use coupled Reverse Transcription and Polymerase Chain Reaction (RT-PCR) in order to detect tyrosinase mRNA in the peripheral blood of melanoma patients. The tyrosinase gene is expressed only in melanocytes, melanoma cells and Schwann cells. As melanocytes and Schwann cells are not found in peripheral blood, detection of tyrosinase expression indicates presence of melanoma cells. Tyrosinase is a monooxygenase (EC 1.14.18.1) that catalyzes the first 2 reactions of the melanin biosynthesis pathway: the conversion of tyrosine to dihydroxyphenylalanin (DOPA), and subsequently the oxidation of DOPA to DOPAquinone. Since then RT-PCR has been used to identify expression of tyrosinase in the peripheral blood, bone marrow, regional lymph nodes, and subcutaneous fat resected with primary melanomas [2].

During the next years, RT-PCR-based detection for melanoma has been attempted using diverse melanoma specific markers such as MAGE-3, p97, gp100 and MUC-18. p97, gp100 and MUC-18 have been reported to show low specificity as they were detected in blood samples from non-melanoma subjects [3]. Other studies on non-melanoma tissues have supported these findings [4-6]. Moreover gp100 is known to be frequently lost during tumor progression [7], so tyrosinase has been the most extensively studied molecular marker for melanoma.
PCR is a powerful technique that enzymatically amplifies specific DNA sequences using oligonucleotide primers that flank the target sequence. In RT-PCR reverse transcription of RNA into cDNA precedes the PCR amplification. The latter method has shown high sensitivity in detecting tyrosinase expression in specimens from melanoma patients, significantly higher than that attained by other methods like immunohistochemistry [8] or nucleic acid sequence-based amplification (NASBA) [9], allowing for the detection of 1 melanoma cell in $10^6$ to $10^7$ normal cells in blood samples.

**Bone marrow**

BM aspirates were found to represent a significant reservoir for disseminated tumor cells in patients with prostate and colon cancer [10,11]. Clinical studies have demonstrated a good correlation between a positive BM PCR assay and a higher pathologic stage and metastatic spread [10]. The assay was highly specific, as no marker expression was detected in BM aspirates from control patients.

Several studies have evaluated the significance of tyrosinase expression in BM specimens from melanoma patients. RT-PCR positivity rates varied among studies. Waldmann et al. [12] reported that in patients with widespread clinical metastases, tyrosinase positivity in BM did not show to have substantial relevance to disease-free survival (DFS) or overall survival (OS). In 20 patients with advanced melanoma there was no difference in the prognosis between 8 who had detectable tyrosinase mRNA in either blood or BM and 12 patients who were negative for tyrosinase mRNA in either blood or BM.

Other authors have reported a statistically significant correlation between RT PCR positivity and a poorer outcome. Ghossein et al. [13] found that among AJCC stage II patients those who tested positive for tyrosinase mRNA in BM had a shorter median survival compared with those who tested negative, with a p value approaching statistical significance. In a study, in which 109 BM samples were assessed, coming from 119 AJCC stage II-IV patients who had been rendered surgically free of disease, tyrosinase positivity in BM was found to be an independent predictor of DFS, superior to AJJC stage, after multivariate analysis [14].

**Lymph nodes**

For melanoma, like for most solid tumours, the metastatic status of regional lymph nodes is the strongest predictor of relapse and survival. However, routine pathological examination of lymph nodes may underestimate the number of melanoma patients with nodal metastases, as only 1%, at most, of the submitted tissue is examined. Immunohistochemical staining with antibodies against S-100 protein or HMB-45 melanoma antigen, increases the yield of occult lymph node metastases [8]. Nevertheless, the number of sections that need to be examined consists a major drawback. Once nodal metastases develop, prognostic factors based on the primary tumour no longer contribute significantly to the prediction of recurrence and OS. As in this stage the extent of lymph node involvement is the main prognostic factor, it was of interest to investigate whether tyrosinase RT-PCR could be used for this purpose. Wang et al. [8] attempted the detection of lymph node micrometastases using RT-PCR for tyrosinase mRNA, and showed this technique to be superior to immunohistochemistry or morphology. Lymph node preparations from 29 patients of AJCC stage I and II undergoing elective node dissections were analyzed both by standard pathologic staining and RT-PCR. Eleven of 29 (38%) lymph node samples from 29 patients with intermediate thickness melanoma were pathologically positive. Nineteen of the 29 (66%) lymph node preparations were RT-PCR-positive, and these included all of the pathologically positive samples, so that the false-negative rate was 0%.

Sentinel lymph node (SLN) biopsy is an alternative to elective dissection or observation for managing lymph node basins in patients with cutaneous melanomas. Tyrosinase RT-PCR was demonstrated to significantly increase the detection of melanoma cells in SLNs as compared to histopathology in several studies [15-18], and constitutes a powerful tool to determine patients who are at increased risk for subsequent metastasis. Moreover, it has been shown that when combined with surgical lymphatic mapping techniques, the RT-PCR assay for tyrosinase mRNA allows more accurate staging and improves the prediction of recurrence and OS over routine pathological examination [16].

Biegliek et al. [15] reported that tyrosinase mRNA was detected by RT-PCR in 73% (19 out of 26) of SLNs from patients at risk for regional nodal metastases, including all of those with histologically positive sentinel lymph nodes, and 65% (13 out of 20) of the histologically negative specimens. Nevertheless, in this study the specificity in this case proved not to be as high as in other studies, since 11% (2 out of 18) of control nodes without melanoma were tyrosinase PCR-positive. In a study conducted by Shivers et al. [16] tyrosinase RT-PCR was used to detect metastatic melanoma in the SLNs of 114 patients. All patients tested positive by histological examination were also RT-PCR-positive. Sixty-seven percent of them relapsed after a median follow-up of 28
months. From the group of patients who tested negative by both techniques 2% relapsed, while histologically negative but RT-PCR positive patients had an intermediate relapse rate of 13%. In another study [17] 116 patients with primary melanoma were followed for a median time of 19 months. Out of the 23 (20%) patients who relapsed, 67%, 6%, and 25% were histologically positive, negative by both methods, and RT-PCR positive, respectively. The differences in recurrence rates were statistically significant (p = 0.01). In a multivariate analysis, histopathologic and RT-PCR status of the SLN were the only significant prognostic factors for predicting DFS. In a study [18] where both paraffin-embedded SLNs and peripheral blood samples from melanoma patients at various stages of disease were screened using a multimarker RT-PCR for tyrosinase and MelanA/MART1, progression of disease was significantly associated with the total number of PCR positive markers in both peripheral blood (p = 0.034) and SLN (p = 0.001) samples, indicating that RT-PCR analysis of serial sections from archival SNLs may be helpful in improving detection of occult metastases, thus improving staging of patients with melanoma. Moreover, a significant correlation between the disease stage and marker expression in both peripheral blood and SLN samples was observed.

Blood

The initial report by Smith et al. [1] led to a number of more detailed studies concerning the presence of tyrosinase in the peripheral blood of melanoma patients. Samples from healthy donors or from patients with other malignancies where always negative, (with the exception of one study [19] where among 31 control samples 2 positives where found), suggesting very high specificity.

Different research groups reported conflicting results concerning the sensitivity and clinical value of tyrosinase RT-PCR [19-27]. In patients with localized disease (AJCC stages I and II) the percentage of blood samples that test positive for tyrosinase varies greatly, ranging from 0% [21] to 45% [24]. Blood samples from untreated patients with advanced disease show remarkable differences in rates of tyrosinase positivity, which range from 0% to 44% [20] for stage III patients and from 13% [3] to 100% [28] for stage IV patients. Methodological differences in the processing of blood samples may account for these discrepancies [29,30], since different methods were used for RNA isolation and cDNA synthesis. Despite differences in sensitivity of tyrosinase RT-PCR, an important number of studies reported significant correlation between RT-PCR positivity for tyrosinase and clinical stage of disease [2,3,21, 25, 30-34].

The prognostic significance of the detection of circulating melanoma cells by RT-PCR has been evaluated by many authors. Reinhold et al. [24] detected no tyrosinase mRNA in blood samples from 26 patients with primary melanoma and 16 patients with regional lymph node metastases and concluded that tyrosinase RT-PCR in blood samples from melanoma does not help in the detection of early metastatic spread. In another study [35] comprising patients with primary melanoma it has been shown that a significantly higher percentage (p <0.05) of PCR-positive patients had tumors greater than 1.5 mm in thickness, or had ulcerated tumors, but of 16 stage II patients who developed metastases, only one was PCR-positive, leading to the conclusion that tyrosinase RT-PCR in blood samples is of limited clinical value in predicting metastasis in patients with primary melanoma.

However, several studies have reported that detection of tyrosinase in peripheral blood is able to predict DFS and OS in a statistically significant level. Detection of the tyrosinase transcript has been considered as a marker for rapid postoperative relapse after node dissection in melanoma patients with regional node metastases, for short-term relapse in high-risk disease-free patients, and for rapid and severe progression in patients with distant metastases [20]. In high-risk but apparently disease-free patients, the risk of relapse within the next 6 months was 3.82 times higher after a positive RT-PCR result than after negative test (p=0.002), and in patients with distant metastases rapid progression was 4 times more likely than slow progression or stable disease, after a positive PCR [20].

In another study [36] 340 blood samples from 225 patients with malignant melanoma where tested for the presence of circulating tumour cells by amplification of tyrosinase and MelanA/MART-1 mRNA. Positive results were obtained in 19% of patients in stage I, 31% in stage II, 29% in stage III and 52% in stage IV. Eighty-two percent and 86% of positive stage III and IV patients, respectively, relapsed within 3-20 months (median DFS was 4 months). Regardless of clinical stage, patients with a positive RT-PCR showed a 2.4 fold increased risk for relapse compared to RT-PCR negative patients.

In a study [37] conducted in long-term clinically disease-free melanoma patients (relapse-free for at least 6 months after primary tumour diagnosis), the presence of circulating melanoma cells was significantly associated with a subsequent high risk of relapse (p=0.003) and death (p= 0.001), regardless of their initial clinical stage. Proebstle et al. [30] reported a strong correlation of RT-
PCR result with DFS in stage III patients. After a median follow-up of 36 months, 87.5% (7 from 8) of tyrosinase-positive patients relapsed, while the percentage for the negative group was 38.88% (7 from 18). The difference was statistically significant (p < 0.01). While univariate analysis showed that sex and primary tumor location were associated with positive RT-PCR, multiple regression analysis revealed clinical stage and detection of tyrosinase transcripts in the peripheral blood as best prognostic factors, pointing out the usefulness of multiple regression models to identify valid prognostic factors, as frequently statistical associations are reported without considering other important factors simultaneously.

Based on these results, supporting the prognostic value of tyrosinase RT-PCR in disease-free patients at high risk for relapse, we were interested in assessing the presence of tyrosinase mRNA by RT-PCR in the peripheral blood of stage IIB and III melanoma patients on high-dose adjuvant interferon alpha-2b at multiple sequential time points from the initiation of treatment, so as to assess the value of this method as a tool for monitoring the efficacy of adjuvant therapy [38]. Four hundred eighty-eight blood samples from 60 melanoma patients were tested for tyrosinase expression. During follow-up (median 23 months) the only prognostic factor that was significantly correlated with higher risk of relapse (p = 0.03) was a positive RT-PCR result. The median DFS was 21 months for the RT-PCR positive group, while the median DFS has not been reached for the negative group. In the group of patients with sequential negative RT-PCRs there was a statistically significant difference between those who remained disease-free and those who recurred (p = 0.002). In the group of patients with positive RT-PCRs there was also a statistically significant difference between those who had a positive RT-PCR prior to adjuvant therapy and negative RT-PCRs during maintenance treatment and further follow-up and remained disease-free. Our data suggest that circulating melanoma cells are markers of high risk for relapse and shorter DFS, either detected postoperatively or during follow-up. Tyrosinase mRNA amplification by RT-PCR might be a useful tool for monitoring the efficacy of adjuvant treatment in stage IIB and III melanoma patients.

Many studies have concluded that a negative RT-PCR result does not exclude tumor progression. Reasons explaining this phenomenon might be that i) melanoma cells are shed only intermittently into the circulation [24]; ii) the lymph nodes work as a filter for tumor cells in the circulation; iii) expression levels of some genes are heterogeneous and variable, especially in patients with advanced disease; iv) the probability of detecting tumor cells in the peripheral blood depends not only on the number of cells in the circulation but also on the Poisson distribution [36].

Conclusions

Results of studies detecting expression of tyrosinase in peripheral blood, bone marrow and lymph nodes specimens from melanoma patients indicate that this method might have important clinical applications in the management of malignant melanoma. Tyrosinase RT-PCR might have an impact on predicting risk of relapse, as well as on future classifications of cutaneous melanoma [39]. Moreover, it might prove a useful tool in monitoring the effectiveness of adjuvant therapies that are associated with significant toxicity and economical cost, so as to select those patients who are more likely to benefit from such treatments.

However, its current clinical value is limited due to the variability concerning the sensitivity of the method. New approaches are being developed which are believed to further improve the reliability of the assay. One of these techniques involves immunomagnetic isolation of circulating tumour cells prior to further analysis by RT-PCR, immunohistochemistry or flow-cytometry [40], leading to enrichment of samples with tumour cells. Real time PCR methods provide a means to monitor the kinetics of the reaction, assessing its efficiency and allowing technical improvements [41]. Moreover, they facilitate exact sensitivity controls on a per sample basis, as well as exact comparison of different assay protocols [42], although quantification of the amount of tyrosinase transcripts does not allow calculation of the number of circulating tumor cells, since the transcription rate of the marker genes varies between tumor cells, as well as between individuals [43].

For an established clinical utility of the assay, larger multicenter trials under uniform optimized protocol and rigid quality assurance systems, are required.

References