Quantification of Melanoma Micrometastases in Sentinel Lymph Nodes Using Real-Time RT-PCR

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Detection of micrometastases in the regional tumor-draining lymph nodes is critical for staging and prognosis in melanoma patients. We applied a quantitative multiple-marker RT-PCR assay to improve the detection of occult melanoma cells in the sentinel lymph node (SLN). From 139 patients with primary cutaneous melanoma who underwent sentinel lymphadenectomy, a total of 235 SLN were assessed for Melan-A and tyrosinase expression by real-time quantitative RT-PCR. Twenty-three patients (17%) were positive by histopathology and expressed messenger RNA of one or two markers. Of the patients with histopathologically negative SLN 39 (28%) were reclassified by positive RT-PCR. Patients were examined for tumor recurrences during a median follow-up period of 29 mo. Tumor recurrences were demonstrated in eight patients (35%) with histopathologically positive SLN, in four patients (10%) with submicroscopic tumor cells detected exclusively by real-time RT-PCR, and in none of the patients negative by both methods. The differences in recurrence rates were statistically significant (p = 0.01). These data indicate that real-time quantitative RT-PCR for the detection of minimal residual melanoma in SLN improves the prediction of disease-free survival.

Key words: melan-A/quantitative PCR/regional lymph node/staging/tyrosinase


In early stages of primary cutaneous melanoma (American Joint Committee on Cancer stages I and II), Breslow’s tumor thickness was demonstrated to be the single most important prognostic factor (Morton et al, 1993). With the development of regional lymph node metastasis (American Joint Committee on Cancer stage III), the 5-y survival rate decreases 40%. At this tumor stage prognostic factors of primary melanoma no longer contribute significantly to survival prediction. The extent of nodal involvement is strongly associated with survival rates (Balch et al, 1996). Accurate nodal staging is useful for guiding adjuvant therapy (Kirkwood, 1998). The technique of sentinel lymph node (SLN) biopsy is based on the concept that it identifies the first node to receive a cutaneous afferent lymphatic from the primary tumor. If the SLN does not contain melanoma cells, then the probability that a non-sentinel node in the draining lymphatic basin contains melanoma cells is less than 1% (Morton et al, 1992). Serial sectioning and immunohistology (IH) using HMB-45 and S-100 antibodies improves the detection of occult melanoma cells, compared with conventional hematoxylin and eosin (HE) staining alone (Cochran et al, 1984; Morton et al, 1992). More recently, the technique of RT-PCR has been demonstrated to increase substantially the sensitivity of tumor cell detection (Blaheta et al, 1999). Using tyrosinase as messenger RNA (mRNA) marker SLN were analyzed by nested RT-PCR in comparison with histopathology including IH. Tumor recurrences after a median follow-up period of 19 mo were observed in 67% of patients with histopathologically positive SLN, in 25% of patients with submicroscopic tumor cells detected by RT-PCR alone, and 6% of patients negative by both methods (Blaheta et al, 2000). Histological and RT-PCR status of the SLN were demonstrated to be the only significant prognostic factors for disease-free survival. The results of two other studies equally demonstrated significant differences of tumor relapse for the three groups of patients determined by their SLN status (Shivers et al, 1998; Bostick et al, 1999).

The molecular assay based on nested PCR is designed to detect approximately three melanoma cells in 10⁷ lymph node cells (Wang et al, 1994), but the question of whether such minimal amounts of cancer cells are clinically important remains controversial. We reasoned that in particular for the patient group exclusively positive by RT-PCR, it would be instructive to perform a quantitative RT-PCR analysis of tumor-associated gene expression. It is conceivable that values of mRNA marker gene expression above a certain cut-off level may be a more powerful predictor of disease relapse than values below this cut-off. A single-step real-time RT-PCR assay has recently been established for accurate and reproducible quantitation of the melanoma-associated tyrosinase and Melan-A mRNA (Abrahamsen et al, 2004). In a prospective diagnostic study, we have developed a similar quantitative multiple marker RT-PCR that uses tyrosinase and Melan-A mRNA expression to detect occult melanoma cells in comparison with conventional IH in SLN. In this study, the prognostic impact of the presence of nodal melanoma micrometastases detected by real-time

Abbreviations: CI, confidence interval; HE, hematoxylin and eosin; IH, immunohistology; mRNA, messenger RNA; SLN, sentinel lymph node

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quantitative RT-PCR was evaluated. The question was addressed as to whether a patient group with a high risk for developing melanoma recurrence and a favorable prognostic group of patients with a low risk of relapse may be distinguished by quantitative RT-PCR.

**Results**

Real-time RT-PCR analysis of SLN A major disadvantage of nested PCR is the high risk of false-positive results because of PCR artifacts and contamination, making this technique unsuitable for clinical diagnostics. We therefore investigated whether a closed-tube real-time PCR would be a practical approach to overcome these difficulties. Best suited for our demands of fast and reliable real-time PCR, we chose the LightCycler system. Absolute mRNA levels of tyrosinase and Melan-A were determined in 235 SLN from a total of 139 patients with primary cutaneous melanoma using real-time quantitative PCR. The sample collection and preparation was modified for future automated settings. To exclude samples with low mRNA yield, a cut-off level of 12,000 transcripts of β-actin per μL cDNA was implemented. Detection of single transcripts is possible; we used, however, a concentration of 10 transcripts per 10 μL of cDNA as cut-off for the detection of the melanoma-specific parameters. Two housekeeping genes, β-actin and cyclophilin B, were quantified as internal standards (see supplement). Reproducibility and sensitivity of tumor cell detection were tested as described in the supplement.

Tyrosinase and Melan-A expression are linked We performed a quantitative comparison between Melan-A and tyrosinase expression using identical lymph node samples. There was a strict correlation between Melan-A and tyrosinase transcript numbers (Spearman's correlation coefficient ($r_s$) = 0.94; 95% confidence interval (CI): 0.89–0.98). The regression equation can be interpreted as having a slope of 1.0 within the 95% CI (see supplement), thus yielding a 2.2-fold (95% CI: 1.58–3.13) higher transcript number for Melan-A than for tyrosinase. For comparison, lymph nodes with occurrence of benign melanocytic nevus cells contained between 1.9 and 552 transcripts per μL of Melan-A and displayed ratios of Melan-A to tyrosinase expression between 2.3 and 5.2. A sample from a clinically palpable lymph node metastasis showed 25,892 Melan-A transcripts per μL and a ratio of 1.9. Nevus cells as well as the lymph node metastasis are not included.

Comparison of real-time RT-PCR with histopathology We then compared mRNA levels with the classification by histopathology. Of 25 SLN positive by HE staining, 21 were real-time PCR positive with a range of 1.2 to 73,714 Melan-A transcripts per μL (median 1466), whereas six of nine SLN positive by IH but negative by HE staining were real-time PCR positive ranging from 1.6 to 37 Melan-A transcripts per μL (median 7.6). Thirty-two SLN positive by histopathology (including IH) but real-time PCR positive ranged from 1.0 to 1336 Melan-A transcripts per μL (median 4.0, Fig 1). Melan-A transcript numbers were significantly different between the SLN groups defined by positive and negative histopathology ($p < 0.0001$).

For the population of 139 consecutive patients with primary cutaneous melanoma in which SLN biopsy was performed, the clinical and histopathologic characteristics are given in the supplement. From these 139 patients, a total of 235 SLN, ranging from 1 to 6 (median 1, mean 1.7) per patient, were investigated by standard histopathology including immunohistochemistry and by real-time quantitative RT-PCR using sets of PCR primers specific for tyrosinase as well as Melan-A. Patients were grouped according to SLN status as determined by routine histological analyses and by real-time RT-PCR (Table 1). Twenty-three (17%) of 139 patients had a positive SLN biopsy result by histopathological evaluation and a positive biopsy result by tyrosinase and or Melan-A real-time PCR (group 1). Only two patients with nodal micrometastases detected by histopathology went undetected by real-time PCR (group 0). Thirty-nine patients (28%) were identified with submicroscopic nodal metastasis using real-time PCR (group 2) whereas 75 patients (54%) had negative SLN results by both assays (group 3). During histopathological examination, special attention was paid to the presence of nodal nevocytes that may result in false-positive RT-PCR. Microfocal collections of nevus cells were identified in the capsule of eight lymph nodes from eight patients. These lymph nodes were excluded from the analysis.

Absence of correlation with Breslow's tumor thickness Patients with histopathologically proved metastatic SLN had a significantly higher Breslow's tumor thickness (Table 1, group 1, median tumor thickness 2.40 mm) as compared with patients with submicroscopic disease identified.
Real-time quantitative RT-PCR identifies patients with low risk for recurrent disease

The incidence of recurrent melanoma was evaluated for each patient group after a median follow-up for the entire patient population of 29 mo. During this period, 13 (9%) of the 139 patients developed recurrent disease (Table 1). One of these patients was positive by immune histology but had a negative PCR result. Eight of 23 (35%) patients with histopathologic proof of nodal micrometastases and positive by RT-PCR (group 1) relapsed and one of these patients died subsequently from metastatic melanoma. Four (10%) of 39 patients who were upstaged by the molecular assay (group 2) developed tumor recurrences. Finally, none of the 75 patients whose SLN biopsy results were negative by both techniques (group 3) was found to have recurrent disease.

For patients whose melanoma recurred, the sites of the first recurrence are summarized in the supplement. In general, the distribution of recurrence sites in patients who were histologically negative but RT-PCR positive (group 2) was similar to that of the patients with positive histological results (group 1). Four patients from group 1 had recurrences in the regional nodal basin, even though they underwent complete node dissection. Two patients from group 2 had recurrences regionally. They had not undergone a complete node dissection. Two patients from group 2 had recurrences regionally, even though they underwent complete node dissection.

Kaplan–Meier curves for relapse-free survival were significantly related to the status of the SLN (Fig 2). Patients whose SLN were histologically positive had significantly lower relapse-free survival than patients exclusively positive by RT-PCR (p = 0.009). Most importantly, among patients with histologically negative SLN, those who were positive by the molecular assay had significantly lower relapse-free survival than those who were RT-PCR negative (p = 0.014). Kaplan–Meier estimates after 48 mo for patients with histologically positive SLN showed a probability of relapse-free survival of 33%, for patients exclusively positive by RT-PCR of 84%, and for patients negative by both diagnostic methods of 100% (Fig 2).

Discussion

The SLN concept implies that the first draining lymph nodes are not bypassed in the draining site of a primary tumor. This has been questioned by a report that patients showed a negative sentinel node, whereas non-sentinel nodes were positive by a sensitive nested tyrosinase PCR assay (Lukowsky et al, 1999). It should be pointed out, however, that only one of these patients had a non-sentinel node that was also positive by IH. We did not directly address this question. We did observe, however, that in 16 of 21 patients with positive sentinel nodes, who were subsequently subjected to lymph node dissection, the remaining regional lymph nodes were negative by immunohistological analysis. Five patients had only low frequencies of positive non-sentinel nodes (one of four, one of nine, one of 25, two of 14, and two of 27). In the few instances with a disseminated pattern of metastasis in sentinel and non-sentinel nodes, up to three lymph nodes positive by IH had to be excised already by sentinel biopsy. We conclude that immunohistological evaluation of sentinel nodes is highly predictive of the presence of micrometastasis in the entire nodal region.

The results obtained with conventional histopathology emphasize the clinical importance of detecting occult metastatic disease using more sensitive diagnostic techniques. A highly sensitive nested RT-PCR assay for detection of tyrosinase mRNA has previously been used as a marker for screening of melanoma cells in sentinel nodes. In these studies, recurrence rates were significantly higher in patients with submicroscopic tumor cells identified by RT-PCR than in patients with negative RT-PCR (Shivers et al, 1998; Blaheta et al, 2000). It has remained unknown, however, to which extent disease-free survival is determined by minimal numbers of tumor cells just detectable by the highly sensitive nested RT-PCR assay as compared with larger RT-PCR-positive micrometastases that just have eluded immunohistological examination. Both extremes are included in patients upstaged by the molecular assay and it would

Table 1. Patient groups defined by SLN status

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<td>Path + PCR-</td>
<td>2 (1%)</td>
<td>1.90 (1.40–2.40)c</td>
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<td>1</td>
<td>Path + PCR+</td>
<td>23 (17%)</td>
<td>2.40 (1.41–5.28)</td>
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<td>3</td>
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<td>75 (54%)</td>
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*HE, hematoxylin and eosin; IH, immunohistology; SLN, sentinel lymph node.

+Path +, HE–, and/or IH+; Path–, HE–, and IH–.

Lower and upper quartiles in brackets. See supplement.

The two observed values in brackets.

Figure 2
Kaplan–Meier disease-free survival. Probability of recurrence-free survival for patients whose sentinel lymph node (SLN) were histopathologically and real-time RT-PCR positive (Path + /PCR +), for patients with histopathologically negative SLN who were reclassified by positive PCR (Path–/PCR +), and for patients with SLN negative by both assays (Path–/PCR–).

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be of great clinical relevance to determine the relative contribution of each of these to recurrent disease. We therefore subjected the SLN to a quantitative analysis of tyrosinase and Melan-A mRNA expression.

Absolute mRNA levels were determined from 139 patients using real-time quantitative PCR. Of these, 17% were positive by histopathology and real-time PCR, 28% were negative by histopathology but positive by real-time PCR, and 54% yielded negative results by both techniques. Patients with positive standard histology (HE staining) had higher median Melan-A transcript numbers than patients positive by serially sectioning and IH alone. The reliability of real-time PCR has been demonstrated by comparison with histopathology. Thus, only two of 25 patients positive by histopathology failed to be detected by real-time PCR. In both cases the corresponding SLN were negative by HE staining and positive by IH alone. In both of these SLN only single or oligocellular tumor deposits were microscopically identified. In addition, heterogeneity of tumor antigen expression in micrometastases may lead to different results. Thus, gp100 expression as determined by HMB-45 immunostaining shows intratumor heterogeneity whereas staining intensity for tyrosinase appears to be more uniform among tumor cells (Chen et al, 1995). In addition, S100 is the most sensitive marker in comparison with tyrosinase and Melan-A (de Vries et al, 2001).

To avoid false-positive detection of tyrosinase or Melan-A mRNA in melanoma patients, and fortuitous expression of these antigens in tumor-draining lymph nodes from control patients with non-melanoma malignancies, we tested—with a negative result—a lymph node from a control patient with a benign parotid tumor who had undergone surgery. In addition, non-SLN that were occasionally biopsied as negative controls tested negative by RT-PCR. One possible source of false-positive RT-PCR results, however, is the occurrence of benign melanocytic nevus cells in tumor-draining lymph nodes. We identified nevus cells in eight of 235 lymph nodes (3.4%), above the reported nodal incidence of 1.2% but similar to that in sentinel nodes (3.9%, Carson et al, 1996). These nevus cell collections were morphologically characterized by intracapsular location and immunoreactivity for S-100 but not for HMB-45. These lymph nodes were not included in our analysis.

Once patients develop regional node metastasis, histopathologic parameters of the primary tumor such as Breslow’s tumor thickness—the current basis for prognostic classification of primary melanoma—was no longer significant in predicting disease-free survival. Accordingly, we could not detect a significant correlation between transcript levels of Melan-A or tyrosinase and tumor thickness (data not shown). The clinical significance of submicroscopic tumor cells is emphasized by the observation of significantly increased recurrence rates in patients who were exclusively positive by nested RT-PCR (Shivers et al, 1998; Bostick et al, 1999; Blaheta et al, 2000). An important issue is whether the highly sensitive nested RT-PCR assay overestimates the risk of recurrent disease. Using real-time RT-PCR, we were able to detect tumor cells in the SLN from 12 of 13 patients whose melanoma recurred during a median follow-up of 29 mo. Eight patients were positive by histopathology and PCR; four patients were exclusively positive by PCR. These results were obtained with an arbitrary cut-off value of 1 Melan-A transcript per μL and among patients with histologically negative results we observed a statistically significant difference in disease-free survival (p = 0.014) between those with a PCR-negative SLN and those with a PCR-positive SLN. Our data obtained with quantitative real-time RT-PCR indicate that by decreasing the sensitivity we were able to increase the specificity of the molecular assay. Determination of the clinical impact of quantitative RT-PCR analysis of micrometastatic nodal disease will require a long-term follow-up in these patients.

Accurate staging of the SLN based on real-time RT-PCR may provide the basis for a more effective clinical follow-up schedule (Hofmann et al, 2002; Garbe et al, 2003). The intensity of clinical and technical examinations can be reduced during follow-up in the PCR-negative stage and may be intensified in the PCR-positive stage. Moreover, adjuvant therapy might improve survival for patients with PCR-positive SLN (Kirkwood, 1998), and patients with PCR-negative SLN may represent a low-risk group for disease relapse who may be spared from the side effects of post-adjuvant therapy. We conclude from these data that real-time PCR using the LightCycler technology is a precise, rapid, and reliable method for detection and quantification of SLN micrometastasis. It has the potential to more accurately reflect the risk of disease relapse and to define a subgroup of patients who may benefit from aggressive surveillance for disease recurrence and stratification into more appropriate adjuvant therapy.

Materials and Methods

Patients: SLN biopsy was performed in 139 patients with primary cutaneous melanoma (usually Breslow’s tumor thickness was >0.75 mm) from April 1999 to September 2002 at the Department of Dermatology of the University Hospital Heidelberg. None of the patients had clinical evidence of regional or metastatic disease (American Joint Committee on Cancer stages I and II). The protocol for the study was approved by the Ethics Committee of the University of Heidelberg. Patients gave their informed consent for all aspects of the investigation according to the Declaration of Helsinki principles.

SLN biopsy: SLN biopsy was performed as described previously (Kahle et al, 2000). SLN were harvested and cut in half through their longitudinal axis.

Histopathologic examination: One half of each SLN was fixed in 5% formaldehyde and sent for standard pathologic evaluation. It was embedded in paraffin and the sections were examined with HE staining. In all instances further sections were evaluated by IH by use of a monoclonal antibody to HMB-45 (Hatta et al, 1998), and antiserum to S-100 protein (Cochran et al, 1988). An SLN was defined as positive by histopathology if tumor cells were identified by HE staining and/or by immunostaining.

Sample collection for real-time PCR: From the other half of each SLN aliquots were collected in 1 mL of RNAlater (Ambion, Huntingdon, UK). After disruption of the tissue using a RiboLyser (Thermo Hybaid, Heidelberg, Germany), mRNA was isolated with the MagNA Pure LC robotic work station (Roche Applied Science, Mannheim, Germany). cDNA was synthesized using AMV-RT and oligo(dT) as primer (first strand cDNA synthesis kit, Roche Applied Science). After termination of the cDNA synthesis, the reaction mix was diluted to a final volume of 500 μL and stored at −20°C until PCR analysis.
Real-time PCR Parameter-specific primer sets optimized for the LightCycler (Roche Applied Science) were developed and kindly provided by SEARCH-LC GmbH (Heidelberg, Germany). The PCR was performed with the LightCycler-FastStart DNA Master SYBR Green I kit (Roche Applied Science) according to the protocol provided in the protocol-specific kits. cDNA input was normalized by the average expression of the two housekeeping genes β-actin and cyclophilin B. The data of two independent analyses for each sample and parameter were averaged and presented as adjusted transcripts per μL cDNA. Multiplying this number by 3000 gives an estimate of the transcripts in an average biopsy.

Nodal nevi Capsular nevocytes might be a source for tyrosinase or Melan-A mRNA detection by RT-PCR. All SLN sections were, therefore, carefully examined for the presence of benign melanocytes. Lymph node specimens with nevus cell aggregates were excluded from our analysis.

Follow-up of patients Examination findings were judged true positive when metastasis was confirmed by additional examination techniques (e.g., histopathology or other imaging techniques) or by the further course of the disease (documented growth of metastasis during the following 6 mo). Conversely, if confirmation by these criteria was lacking, findings were judged false positive.

Statistical analysis The analysis related to tumor thickness is based on a non-parametric approach using median and quartiles to describe location and scale parameters. Differences between groups were assessed with the Mann–Whitney test. Strip-plots were used to present the data graphically. Linear regression methodology was used to gauge two PCR measurements that were transformed on a log 10 base. The 95% CI for intercept and regression coefficient β were also calculated. In case of inclusion of the value 1 in the CI for β, the retransformed intercept was interpreted as the factor of change between both measurements. The approach chosen also allowed to calculate 95% CI for the factor of change. The correlation between two measurements was quantified by r and its 95% CI. The calculations were performed using SPLUS 2000 (MathSoft, Seattle, Washington) and Statxact (V.3, Cytel Software Corporation, Boston, Massachusetts).

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Supplementary Material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/JID/JID23633/JID23633sm.htm

Figure S1. Serial dilution of melanoma cells in control SLN lysates. Figure S2. Correlation of Melan-A and tyrosinase transcript numbers. Table S1. Patient characteristics Table S2. Sites of first melanoma recurrence

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