Pathologic Assessment of Melanoma Sentinel Nodes: A Role for Molecular Analysis Using Quantitative Real-Time Reverse Transcription-PCR for MART-1 and Tyrosinase Messenger RNA

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ABSTRACT

Purpose: Molecular analysis of melanoma sentinel nodes (SN) is sensitive, but poorly specific because metastases cannot be distinguished from benign nevus inclusions (BNI). We investigated whether quantitative reverse transcription-PCR (RT-PCR) detection of MART-1 and tyrosinase mRNAs could improve this specificity and contribute to SN assessment.

Experimental Design: Two hundred twenty SNs from 95 melanoma patients analyzed by extensive immunohistopathology and real-time quantitative RT-PCR.

Results: Using histopathology, SNs and patients were allotted to three diagnostic groups: (a) metastasis positive, (b) BNI positive, and (c) melanocyte-free. Median MART-1 and tyrosinase mRNA levels in SNs were significantly different in patients with metastasis compared with patients with BNIs (P < 0.05) and patients without melanocytic lesions (P < 0.001). However, a “gray-zone” was observed where distinction, based on mRNA levels, could not be made between the three groups. For both genes, the highest mRNA level recorded in each RT-PCR-positive patient was positively correlated with Breslow’s tumor thickness. For SNs with metastases, tumor burden was significantly correlated to the mRNA level. Using the presence of a MART-1 RT-PCR signal to detect patients with metastases, a sensitivity of 100% and a negative predictive value of 100% were achieved when extensive immunohistology was used as reference.

Conclusions: Quantitative RT-PCR MART-1 and tyrosinase mRNA analysis cannot be used alone for SN diagnosis because of its poor specificity for melanoma metastasis. However, in approximately one third of cases without RT-PCR evidence of MART-1 expression, extensive histopathologic SN investigation is not necessary, thus substantially reducing the cost of SN analysis. The level of melanocyte-associated mRNA is associated with both tumor thickness and tumor burden as measured histopathologically, suggesting that this may be of prognostic value.

INTRODUCTION

Patients with intermediary thickness melanoma have an average 5-year survival rate of 80% (1). Around 30% of melanoma patients will develop regional lymph node metastases (2, 3), and once these become clinically recognizable, the 5-year survival rate decreases to 40% to 50% (4). Elective lymph node dissection may contribute to improved survival (5, 6), but the operation is associated with significant morbidity (7, 8). The sentinel node (SN) technique improves the precision of staging of early melanoma metastases with minimal morbidity (9), and it has recently been claimed that early excision of lymphatic metastases identified using this technique provides a highly significant overall survival benefit compared with delayed excision (10). However, the potential benefits of using the SN technique are dependent on the accuracy of the subsequent examination of the excised lymph node. At present, extended histopathologic examination using both serial sectioning and immunohistochemical staining is the reference standard for identifying melanoma metastases in SNs, although, for practical reasons, <1% of the submitted lymph node is actually examined with this technique. Thus, traditional methods of SN analysis are associated with a high risk of underdiagnosing melanoma metastases, particularly when these are small.

The sensitivity of histopathologic analysis of melanoma SNs can be increased by using molecular methods, such as semiquantitative reverse transcription-PCR (RT-PCR), to detect melanocyte-associated molecules in lymph node extracts as a surrogate marker for melanoma metastases. In general, such analyses can detect melanocyte-positive nodes in over 50% of SN patients compared with the ~20% found by traditional histopathology (11–17). However, given that only some 30% of melanoma patients would be expected to develop regional lymph node metastases, the clinical significance (and specificity) of a positive RT-PCR analysis is questionable. The markers most
frequently used in melanoma RT-PCR assays are MART-1 and tyrosinase mRNAs. Both molecules are associated with melanocytic differentiation, but neither is melanoma-specific and they cannot be used to discriminate between benign and malignant melanocytic cells. In this context, two important observations have recently been made. First, that the presence of melanocytic cells in SNs in the form of benign nevus inclusions (BNI) is much more common than previously assumed, with a patient incidence between 22% and 28% (18–20). Second, that tyrosinase mRNA can be regularly detected by RT-PCR in paraffin sections from SNs with histologically verified BNI, but without evidence of melanoma metastases (21). Thus, the indiscriminate use of molecular analyses to identify SN metastases is likely to result in a substantial number of “false-positive” cases comprising those patients who have BNI but not melanoma in their lymph nodes.

One approach to improve the specificity of molecular analysis may be to use quantitative RT-PCR, the assumption being that BNI would in general give lower signals compared with melanoma metastases. To investigate this hypothesis, we have previously developed and validated a single-step real-time quantitative RT-PCR technique to detect melanocytic cells in SNs (22). This technique has many advantages over classic PCR in being fast, sensitive, and reproducible even for detecting low-level transcripts. We did the current study to investigate whether melanoma metastases can be distinguished from BNI in SNs based on a quantitative RT-PCR analysis of MART-1 and tyrosinase mRNAs; (b) to compare the results of SN analysis using an extensive histopathologic examination with those obtained by quantitative RT-PCR; and (c) to compare known prognostic factors, such as Breslow’s tumor thickness and tumor burden with the MART-1 and tyrosinase mRNA levels obtained by quantitative RT-PCR. Our results show that whereas quantitative RT-PCR analysis of these marker mRNAs is too nonspecific to be used alone for SN analysis, this technique can identify a substantial number of cases in which subsequent extensive histopathologic SN evaluation is unnecessary, thus improving the efficiency of routine SN diagnosis.

MATERIALS AND METHODS

Patients and Lymph Nodes. Sentinel nodes (n = 220) were from 95 patients with primary malignant melanoma (1–4 mm) who underwent SN dissection at our hospital in the period January 2001 to December 2002. None of the patients showed clinical evidence of metastastic disease in regional lymph nodes or at distant sites. The local research ethics committee approved this prospective cohort study and written informed consent was obtained from all patients.

Sentinel Node Procedure. Lymphatic drainage patterns were identified as described (23, 24) with minor modifications. Briefly, the day before surgery, a total dose of 80 MBq of 99mTc-Technetium-labeled Nanocollloid (Nycomed Amersham, Sorin, Vercelli, Italy) was injected into the dermis surrounding the scar of the previously excised melanoma. Planar gamma pictures were taken within 2 hours to identify the draining lymphatic regions. Guided by the scintigraphy pictures and a hand-held gamma detector (C-Trak Navigator, Morgan Hill, CA), the surgeon excised the radioactive lymph nodes the following day. After excision, lymph nodes were trimmed of fat and bisected along their long axis. The gamma probe was used to confirm that there was radioactivity in both halves. One half of the excised node was immediately fixed in neutral buffered formaldehyde for histologic examination, whereas the other half was snap frozen in liquid nitrogen and stored frozen until subsequent molecular analysis. Of the 220 SNs in the main study, 75 nodes from consecutive cases were also included in a subsidiary study to determine the localization of an eventual positive RT-PCR signal. The tissue for molecular analysis from these 75 nodes was further subdivided into two parts, a central portion adjacent and parallel to the initial midline section and a peripheral portion.

Histopathology. The fixed lymph node underwent extensive histopathologic examination, with both serial sectioning and immunohistochemical staining as described (18). Briefly, 2 μm serial paraffin sections were cut at 250 μm levels throughout the entire specimen. One H&E section was stained at each level (i.e., levels 1, 2, 3, 4, etc.). At alternate levels, starting with the first (i.e., levels 1, 3, 5, 7, etc.), three additional paraffin sections were stained with primary antibodies against S-100 protein (1:10,000, MU058-UC, BioGenex, Sacramento Prohosp, Vaelrose, Denmark), gp-100 (1:50, HMB-45, M0634, DakoCytomation, Glostrup, Denmark), and Melan A (1:50, M7196, DakoCyto-

RNA Extraction and cDNA Synthesis. Total RNA was extracted from the frozen lymph nodes by an acid guanidium isothiocyanate, phenol, chloroform method as described (22) and diluted in 35 μL distilled water. The purity and the amount of total RNA were determined by UV spectrophotometry and A260 nm/A280 nm ratios. Two micrograms of total RNA were reverse transcribed using 1× PCR buffer II (Applied Biosystems, Foster City, CA) supplemented with 6.3 mmol/L MgCl2, 0.3 mmol/L of each of the four deoxyribonucleoside triphosphates (dATP, dTTP, dGTP, dCTP), 2.5 mmol/L 16mer oligo(dT) nucleotide, 20 units RNase inhibitor, and 50 units murine leukemia virus reverse transcriptase (Applied Biosystems) in a total reaction volume of 20 μL. Reverse transcription was done at 42°C for 30 minutes followed by 99°C for 5 minutes.

Real-Time PCR. Quantitative real-time PCR was done using the LightCycler system (Roche Molecular Biochemicals, Indianapolis, IN) as described (22). This system is based on continuous cycle-by-cycle monitoring of generated PCR products, by measuring the fluorescence emitted when the probes anneal to their target. The fluorescent signal correlates with the amount of product. By definition, samples within the quantification range of the calibration curve were regarded as positive. MART-1 and tyrosinase mRNAs were quantified using sequence specific intron-spanning primers and hybridizing probes. MART-1 primer sequences were sense: 5′-ATGCCAA-GAGAAAGATGCT-3′ and antisense: 5′-GGAGAACATTA-GATGTCGT-3′ corresponding to a product size of 439 bp. Tyrosinase primer sequences were sense: 5′-ACAACAGCATT-CAGTCT-3′ and antisense: 5′-CTGTACCTGGAATGATTC-3′ corresponding to a product size of 304 bp. MART-1 probes sequences were TTTCGTCTTCTCAAATACCAACAGCC-GATG-FL and LC Red640-GCAGTAAGACTCCCAGGAT-CACGTGCAG-P; tyrosinase probe sequences were TTTCGTCTTCTCAAATACCAACAGCC-GATG-FL and LC Red640-GCAGTAAGACTCCCAGGAT-CACGTGCAG-P; tyrosinase probe sequences were TTTCGTCTTCTCAAATACCAACAGCC-GATG-FL and LC Red640-GCAGTAAGACTCCCAGGAT-CACGTGCAG-P;
Two microliters of the cDNA were used as template in a reaction mixture consisting of 4 mmol/L MgCl₂, primers (MART-1, 15 pmol of each primer; tyrosinase, 2.5 pmol of each primer), probes (for both genes, 6 pmol of each probe), and 2 µL probemix (containing Taq DNA polymerase, reaction buffer, deoxyribonucleoside triphosphates; Roche Molecular Biochemicals) in a total volume of 20 µL. PCR was done in the LightCycler System (Roche) with an initial denaturation step at 95°C for 30 seconds and then 50 cycles (40 cycles for tyrosinase) with a three-step schedule: 95°C denaturation for 1 second, 55°C (60°C for tyrosinase) annealing for 15 seconds, and 72°C extension for 13 seconds. Primer specificity was verified by gel electrophoresis (2%) of the generated product. The correct identity of the MART-1 and tyrosinase PCR products was verified by direct nucleotide sequencing.

Quantification was done using a standard curve generated with each run. This curve was based on analysis of serial dilutions of SK-MEL-28 melanoma cell RNA in water and displayed a linear relationship between the log of the initial template concentration (SK-MEL-28 melanoma cells RNA) and the amount of product generated. The measuring ranges in arbitrary units for MART-1 and for tyrosinase were 0.5 to 1,000 and 5 to 10,000 SK-MEL-28 melanoma cell equivalents, respectively, where 1 arbitrary unit was defined as the amount of product generated. The measuring ranges in arbitrary units for MART-1 or tyrosinase were 0.5 to 1,000 and 5 to 10,000 SK-MEL-28 melanoma cell equivalents, respectively, where 1 arbitrary unit was defined as the amount of MART-1 or tyrosinase mRNA present in one SK-MEL-28 melanoma cell.

Tissue processing, RNA extraction, reverse transcription, and PCR assay set-up were done in separate designated rooms to prevent cross-contamination. To control for the presence of amplifiable RNA, all specimens were analyzed for β₂-microglobulin RNA as described (22). Amplifiable RNA was present in all cases. To control for false-positive results caused by amplification of contaminating DNA, RT-PCR reactions without reverse transcription enzyme were done on selected SN tissues from cases with histologically verified melanoma metastases. These were consistently negative for PCR products. Finally, to control for false-positive results due to contamination, a negative sample (without RNA) was included in every run and the run was repeated in cases with a positive signal.

Statistics. Statistical analyses were done with the GraphPad Prism 4 statistical software package (GraphPad Software, Inc., San Diego, CA). mRNA expression in the different study groups was compared using the Kruskal-Wallis test and Dunn’s multiple comparison test. The relationships between mRNA quantity on the one hand, and tumor burden and Breslow’s tumor thickness on the other, were analyzed using Spearman’s correlation test. P < 0.05 was considered significant. χ²-test was used to compare frequencies.

RESULTS

Primary Tumors. Ninety-five patients with intermediate thickness melanoma (1-4 mm) were included in the study. Clinical and pathologic data are shown in Table 1. The mean tumor thickness was 1.8 mm (median, 1.6 mm). Tumor thickness could not be determined in 13 cases either because the primary tumor had been cut through or because there was substantial loss of the tumor due to ulceration or regression.

Sentinel Nodes. A total of 220 SNs were excised and studied. At least one SN was harvested successfully in each patient. The mean number of SNs per patient was 2.3 (median 2, range 1-6). One SN was obtained in 23 patients, 2 in 41, 3 in 18, and >3 in 13 patients.

Histopathology. Histopathology found melanoma metastases in formalin-fixed, paraffin-embedded samples from 38 of 220 SNs (17%; Table 2). Twenty-seven of 95 patients (28%) had at least one SN with histologically verified melanoma metastasis. Nineteen of the patients had a single SN with metastasis, five patients had two SNs with metastases, and three patients had three SNs with metastases. The mean number of SNs with metastases was 1.4 (median 1). BNIs were identified histologically in 29 of 220 SNs (13%). The majority (88%) had BNI in just one SN. BNIs were identified in nodes from 26 of 95 patients (27%). The mean number of nevus inclusions was 1.1 (median 1). Four of the patients with histologically proven metastatic melanoma also had histologic evidence of BNI in at least one SN. In 155 of 220 SNs (71%), corresponding to 46 patients (48%), there was no histologic evidence of melanocytic cells (i.e., neither melanoma metastases nor BNI).

MART-1 and Tyrosinase Messenger RNA Expression. Using histopathology as a standard reference, the 220 SNs were divided into three diagnostic groups: (a) metastasis-positive,
Table 2  Histopathologic results

<table>
<thead>
<tr>
<th></th>
<th>SN (%)</th>
<th>Patient (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metastasis</td>
<td>38 (17%)</td>
<td>27 (28%)</td>
</tr>
<tr>
<td>BNI</td>
<td>29 (13%)</td>
<td>26 (27%)</td>
</tr>
<tr>
<td>Melanocyte-positive (metastasis and/or BNI)</td>
<td>65 (29%)</td>
<td>49 (52%)</td>
</tr>
<tr>
<td>Melanocyte free</td>
<td>155 (71%)</td>
<td>46 (48%)</td>
</tr>
</tbody>
</table>

NOTE. Extended histologic and immunohistochemical examination was done on 220 SNs from 95 patients. Cases are shown divided into four groups according to the presence or absence of melanoma metastasis and/or BNIs: The number (%) of involved SNs and patients in each of the groups is shown.

(b) BNI-positive, and (c) melanocyte-free (i.e., no evidence of either metastasis or BNI). The 95 patients were allocated to the same three groups according to whether they had one or more SNs with either metastases or BNI, or whether all their SNs were melanocyte-free on histology. Patients who had SNs with both metastases and BNI (four cases) were placed in the “metastasis” group. Table 3 gives the results of RT-PCR analysis of mRNA expression of MART-1 and tyrosinase (either alone or together) in the three diagnostic groups, according to both the number of positive SNs and the number of patients with at least one positive SN. There was no significant difference in the number of patients with MART-1 mRNA-positive SNs compared with tyrosinase mRNA-positive SNs (P > 0.5). Mean MART-1 and tyrosinase mRNA values in the 220 SNs were 78.9 (median 0) and 848 (median 8) arbitrary units per 2 µg RNA, respectively. A significant correlation between the level of MART-1 and tyrosinase mRNA was found (r = 0.60, P = 0.0001).

Quantitative RT-PCR and Histopathologic Analysis According to Lymph Node Status. Quantitative RT-PCR analysis showed significant differences in both MART-1 (P < 0.001) and tyrosinase (P < 0.001) mRNA levels comparing metastasis-positive SNs with melanocyte-free SNs (Table 4). The median mRNA values for SNs with BNI were intermediate compared with the other two groups, and differed significantly from the values for SNs with metastases (MART-1, P < 0.01 and tyrosinase, P < 0.001). For MART-1, but not tyrosinase, the median mRNA value for SNs with BNI also significantly differed from that for melanocyte-free SNs (P < 0.05). However, a clear overlap existed between mRNA values for the three groups (Fig. 1). For both markers, mRNA values were below the detection limit in five SNs with histopathologically proven metastasis. In seven SNs with histopathologically proven metastases, the mRNA expression levels of one or other of the two markers was below the detection limit (data not shown).

The relation between tumor/melanocyte burden and marker mRNA levels was evaluated according to lymph node status. The SN specimen subjected to histologic examination was sectioned entirely at 250 µm levels. Tumor burden was expressed as the number of levels with tumor relative to the total number of levels examined in the particular lymph node specimen. For metastasis-positive SNs, a significant correlation between tumor burden and MART-1 or tyrosinase mRNA levels was observed (MART-1, r = 0.57; tyrosinase, r = 0.52, P < 0.0007). In the group of SNs with BNI, no significant correlation between either MART-1 or tyrosinase mRNA expression and melanocyte burden was found.

To determine whether a positive PCR signal originated preferentially in a particular part of the lymph node, the frozen halves from 75 SNs were further subdivided into two parts, each of which then underwent separate molecular analysis. A positive RT-PCR signal for MART-1 was obtained in a total of 36 of these SNs: 16 from cases with histologic evidence of metastasis, 7 with BNI, and 13 without melanocytic cells on histology. In 12 of 16 (75%) of the metastasis SNs, a positive PCR signal was obtained in both central and peripheral parts of the node, suggesting wide distribution of metastases. In contrast, only 3 of 7 (43%) of the BNI SNs and 4 of 13 (31%) of the melanocyte-free SNs were positive in both parts.

Quantitative RT-PCR and Histopathologic Analysis According to Patient Status. The MART-1 and tyrosinase mRNA values from the SN with the highest level were used to analyze data according to patient status (Fig. 2; Table 4). A significant difference in mRNA levels in patients with metastasis-positive SNs compared with patients with melanocyte-free SNs was found for both MART-1 (P < 0.001) and tyrosinase (P < 0.001). Histopathology of the lymph node from the patient in the melanocytic-free group with the highest mRNA values for both MART-1 and tyrosinase revealed a cluster of Melan A (equivalent to MART-1) positive cells on immunohistochemical staining. Although this was suspicious, the morphology was very poor and there was no clear evidence of malignancy according to the stringent criteria used by us (18) in this analysis and the Melan A–positive cells in this case were classified as an artifact.

MART-1 and tyrosinase mRNA levels in SNs from patients with BNI-positive SNs were intermediate and significantly differed from the mRNA levels in patients with metastasis-positive SNs (P < 0.05; P < 0.01). However, even when

Table 3  RT-PCR marker analysis according to both lymph node (n = 220) and patient (n = 95) status

<table>
<thead>
<tr>
<th></th>
<th>MART-1 mRNA positive</th>
<th>Tyrosinase mRNA positive</th>
<th>Both markers positive*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SN</td>
<td>Patient</td>
<td>SN</td>
</tr>
<tr>
<td>Metastasis</td>
<td>33/38 (87%)</td>
<td>27/27 (100%)</td>
<td>33/38 (87%)</td>
</tr>
<tr>
<td>BNI</td>
<td>18/27 (67%)</td>
<td>18/22 (82%)</td>
<td>13/27 (48%)</td>
</tr>
<tr>
<td>Melanocyte free</td>
<td>44/155 (28%)</td>
<td>19/46 (41%)</td>
<td>83/155 (54%)</td>
</tr>
<tr>
<td>Total</td>
<td>95/220 (43%)</td>
<td>64/95 (67%)</td>
<td>129/220 (59%)</td>
</tr>
</tbody>
</table>

*Expression of both MART-1 and tyrosinase mRNA in the same SN.
†Number of SNs positive for marker/total number of SNs.
‡Number of patients with at least one marker positive SN/total number of patients.
calculating data in terms of the SN with the highest mRNA value per patient, an overlap in the range 0 to \(100\) arbitrary units per 2 \(\mu\)g RNA for MART-1 and 0 to 600 arbitrary units per 2 \(\mu\)g RNA for tyrosinase was found comparing the three diagnostic groups.

**Breslow’s Tumor Thickness.** Breslow’s thickness could be assessed in tumors from 23 of 27 patients with histologically verified metastases and from 59 of 68 of patients without metastases. Median tumor thickness in patients with histologic metastases was significantly higher (2.04 mm) compared with that in patients without metastases (1.4 mm; \(P < 0.005\)). For 82 of 95 patients with measurable tumor thickness, the relationship between Breslow’s tumor thickness and mRNA quantity (highest expressive SN per patient) revealed a weak but significant correlation for both MART-1 (\(r = 0.25, P = 0.02\)) and tyrosinase (\(r = 0.23, P = 0.04\)).

**Quantitative Reverse Transcription-PCR as a Screening Tool.** Using histology as a reference standard, and regarding a positive RT-PCR signal as an indicator for metastasis, the sensitivity and specificity for MART-1 used as a single marker were 87% and 66%, respectively, at the lymph node level. The predictive value of a positive RT-PCR result for MART-1 (PV(pos)) was 35% and the predictive value of a negative result (PV(neg)) was 96%. The false-negative rate was 13% (Table 5). At the patient level, the sensitivity for the detection of histologically verified metastases was 100% and the specificity 46%. The predictive value of a positive RT-PCR result (PV(pos)) indicating

### Table 4

<table>
<thead>
<tr>
<th>Histologic status</th>
<th>MART-1</th>
<th>Tyrosinase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (arb.u.)</td>
<td>Range (arb.u.)</td>
</tr>
<tr>
<td>Lymph nodes ((n = 220))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metastases ((n = 38))</td>
<td>9.2</td>
<td>0-4,486</td>
</tr>
<tr>
<td>BNI ((n = 27))</td>
<td>1.2</td>
<td>0-18.4</td>
</tr>
<tr>
<td>Histology negative ((n = 155))</td>
<td>0</td>
<td>0-1,262</td>
</tr>
<tr>
<td>Patients ((n = 95))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metastases ((n = 27))</td>
<td>98.5</td>
<td>0.2-4,486</td>
</tr>
<tr>
<td>BNI ((n = 22))</td>
<td>3.2</td>
<td>0-18.4</td>
</tr>
<tr>
<td>Histology negative ((n = 46))</td>
<td>0</td>
<td>0-94.6</td>
</tr>
</tbody>
</table>

**NOTE.** Median values and range for MART-1 and tyrosinase mRNA expressed as arbitrary units of melanoma cells (as defined in Materials and Methods).

Abbreviation: arb.u., arbitrary units.

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**Fig. 1** Quantitative RT-PCR analysis of MART-1 and tyrosinase mRNA in 220 SNs. SNs are grouped according to histologic status. Circles, SNs; horizontal lines, medians. The median is zero for MART-1 in SNs free of melanocytic cells (Free) and for tyrosinase in SN with BNI.
the presence of metastasis was 42%, whereas the predictive value of a negative result (PVneg) indicating the absence of histologically verifiable metastases was 100%. The false-negative rate was thus 0%.

**DISCUSSION**

We used our previously developed single-step real-time RT-PCR technique (22) to quantify MART-1 and tyrosinase mRNAs in SNs from patients with primary malignant melanoma. The present large study of 220 SNs from 95 patients confirms this technique to be both convenient and reliable in use. Previous RT-PCR-based molecular studies of melanoma SNs have most often used tyrosinase mRNA as a target marker for detecting melanoma cells (12, 15, 17, 25), the percentage of patients with positive SNs varying from 33% (26) to 88% (14). Although fewer studies have investigated the expression of MART-1 mRNA in melanoma SNs (13, 14, 27–29), these have found a similar wide variation in expression, with positive cases ranging from 24% (14) to 81% (28). A third melanocyte-associated marker (melanoma inhibitory activity) has been put forward as a more specific marker of melanoma. However, a recent study has shown the widespread presence of low levels of melanoma inhibitory activity mRNA in nonmelanoma cells, casting doubt on its suitability as a specific screening marker (30). The fraction of patients with tyrosinase-positive (73%) and MART-1-positive (67%) SNs found in our study is comparable with the upper range of previously published studies, all of which used a nested PCR technique (13, 15, 17, 31). Thus, although our technique is based on a single-step PCR method, it seems to be highly sensitive.

However, whereas the high sensitivity of RT-PCR analysis may be advantageous in increasing the detection of micrometastases in SNs, this comes at the price of low specificity. The high proportion of patients with RT-PCR marker–positive melanoma SNs found in this and in previous molecular studies correlates poorly with clinical data that indicate that only ~30% of patients can be expected to develop regional lymph node metastases (2, 3). Thus, the prevailing assumption that a positive melanocyte RT-PCR signal in SN analysis can be used as a direct

**Table 5** Concordance between malignant histology and RT-PCR for MART-1: Analysis according to lymph node and patient status

<table>
<thead>
<tr>
<th>Histology</th>
<th>PCR Positive*</th>
<th>PCR Negative1</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lymph nodes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>33</td>
<td>62</td>
<td>95</td>
</tr>
<tr>
<td>Negative</td>
<td>5</td>
<td>120</td>
<td>125</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>38</td>
<td>182</td>
<td>220</td>
</tr>
<tr>
<td><strong>Patients</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>27</td>
<td>37</td>
<td>64</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>31</td>
<td>31</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>27</td>
<td>68</td>
<td>95</td>
</tr>
</tbody>
</table>

*Histology positive: SNs contain metastasis.

1Histology negative: SNs contain BNI or are without melanocytic cells.

**Fig. 2** Quantitative RT-PCR analysis of MART-1 and tyrosinase mRNA in SNs from 95 patients. Each patient is represented by one SN (the highest expressive). Horizontal line, median. For MART-1 patients with SN without melanocytic cells, the median was zero.
surrogate for the histopathologic identification of melanoma metastasis is unjustified. Accordingly, attention has recently focused on the importance of nonneoplastic cellular sources of melanocyte mRNA (including BNI) as a potential source of false-positive results in the molecular analysis of melanoma SNs (32). SN BNI have been shown to be rather common, especially in melanoma patients, with a patient incidence up to 28% and a nodal (SN) incidence up to 13.4% (18, 19). In a paraffin section study, Starz et al. (21) confirmed, in a number of SNs, that BNI consistently gave a positive PCR signal for tyrosinase mRNA.

In cases with histologically proven metastases, we found a positive RT-PCR signal for MART-1 and tyrosinase in the lymph node half submitted for molecular analysis in 87% and 87% of SNs, respectively (100% and 93% of the patients). Analysis of SNs with histologically identified BNI also found high values for these two markers in 67% and 48% of SNs, respectively (82% and 68% of patients), whereas the corresponding figures for cases without any histologic evidence of melanocytes were 28% and 54% of SNs, respectively (41% and 63% of patients). The number of histologically “unexplained” cases, defined as RT-PCR-positive/histopathology-negative patients, is reported to be around 50% (11, 17, 31, 15). By using an extended histopathologic protocol, we identified melanocytes (melanoma metastasis and/or BNI) in 29% of SNs and in 52% of patients (Table 2), thus reducing the fraction of “unexplained” cases in our study to only 20%. Similarly, Cook et al. (20) also reported a better correlation between histopathology and RT-PCR results when using an extensive histopathologic protocol and by defining histopathologically positive results to include both metastasis and BNI. Nonetheless, both in that study and in our present study, some 20% of the RT-PCR-positive results cannot be accounted for by histopathology. There are various possible explanations for this discrepancy. Even in an extensive histopathologic protocol, only a small fraction of the entire SN is actually examined microscopically, and both small metastases and BNI may be overlooked. Indeed, in our series, histology of the lymph node from the patient in the melanocyte-free group with the highest mRNA values for both MART-1 and tyrosinase revealed a cluster of Melan A–positive cells on immunohistochemical staining. Although this was suspicious, this case did not meet the stringent criteria for metastasis we used in this study (18) and the Melan A stain was classified as a false-positive artifact. It is possible, and perhaps likely, that this interpretation was wrong, thus explaining the high RT-PCR levels found. In addition, intranodal nerve cells may show nonspecific RT-PCR expression of tyrosinase (33). If occult nonneoplastic sources of melanocyte mRNA account for a substantial proportion of the positive RT-PCR results in SNs, this might explain the improved survival seen in patients with histologically negative, but RT-PCR positive, SNs (15, 17, 31, 34) compared with patients with SNs positive by both histopathology and RT-PCR.

Because SN metastases express more than one melanocyte marker (13, 31, 35), we hypothesized that metastases and BNI might be distinguishable if we adopted a more rigorous definition of a PCR-positive sample (i.e., one that required positivity for both tyrosinase and MART-1 mRNAs). However, whereas most SNs with metastases were positive for both markers (82%, corresponding to 93% of the patients), this was also true for a substantial number of SNs with BNI (44%, corresponding to 64% of the patients) and of SNs without melanocytes (21%, corresponding to 36% of the patients), and this approach provided no better distinction between the three groups.

Thus, qualitative RT-PCR assessment of current melanocytic markers was unable to distinguish SNs carrying metastases from those with BNI. Because the latter occur in SNs from melanoma patients at a much higher frequency than previously expected, the specificity of qualitative RT-PCR analysis is too low for use in a clinical setting. In an alternative approach, we used quantitative molecular analysis to try to improve the distinction between different types of melanocyte marker–positive SNs. We found significant differences in MART-1 mRNA values comparing the three histopathologically defined groups of SNs (i.e., those with melanoma metastasis, those with BNI, and those without evidence of melanocytes), these groups showing high, intermediate, and low values, respectively (Figs. 1 and 2). A similar pattern was seen for tyrosinase mRNA values, except that the median values in SNs with BNI and in SNs without melanocytes were not statistically different (Figs. 1 and 2). Although quantitative RT-PCR improved the specificity of the molecular analysis compared with qualitative RT-PCR, there was still a substantial overlap in mRNA values between the three histologically defined lymph node groups in the approximate range of 1 to 100 arbitrary units per 2 μg RNA for MART-1 and 1 to 600 arbitrary units per 2 μg RNA for tyrosinase. Nonetheless, in ~30% of the patients with melanoma metastases, SN analysis revealed MART-1 and tyrosinase mRNA values greater than the highest levels found in either nodes with BNI or in melanocyte-negative nodes, suggesting that a substantial proportion of SNs with metastatic disease can be identified using this molecular technique.

Sentinel nodes with histologically proven metastases showed a much higher variation in mRNA expression (MART-1 >10^{-2}-fold and tyrosinase >10^{-4}-fold) compared with the other types of SN (MART-1 <10^{-2}-fold and tyrosinase <6 <10^{-5}-fold) Moreover, there was an up to 25-fold intranodifferentiation variation in mRNA for MART-1 and tyrosinase in patients with more than one metastatic SN. This variation may be explained by differences in the “melanocyte burden” in a SN. This explanation is supported by the weak but significant correlation found between MART-1 and tyrosinase mRNA values and the tumor burden, estimated in SNs with histologically proven metastasis as the fraction of histologic slides with melanoma. A positive correlation between “melanocyte burden” and mRNA values was not found for SNs with histologically proven BNI. The large variation in MART-1 and tyrosinase mRNA values in SNs with histologically proven metastases may also reflect differences in the ability of tumor cells to express these markers. It has been shown in stage IV melanoma patients that low values of MART-1 or tyrosinase mRNA may indicate dedifferentiation of the tumor with loss or down-regulation of antigen expression and that this is associated with decreased survival (35–37). However, this may not necessarily apply for early-stage disease, such as SN micrometastases (35, 37).

Breslow’s tumor thickness is the strongest prognostic factor for primary malignant melanoma and was positively correlated to the presence of SN metastases in our study. Similar findings have been reported before (38, 39). The relationship between
Breslow’s tumor thickness and MART-1 and tyrosinase mRNA levels has never before been addressed. We found a weak but significant correlation between primary tumor thickness and mRNA values for both genes. Clinical follow-up in our patients is not yet sufficiently long to show whether these mRNA levels have any independent prognostic impact. However, the clear positive relationship found comparing mRNA levels with known prognostic factors, such as Breslow’s thickness and tumor burden, suggests that this may be the case.

Previous molecular studies have used different sampling protocols in both frozen and paraffin-embedded SNs (13, 21). Given our rather better false-negative RT-PCR rates for MART-1 and tyrosinase in patients of 0% (13% at the SN level) and 7% (13% at the SN level), respectively, neither of these previously published sampling techniques seems to be superior to the one we have used with regard to minimizing sampling error.

Although neither qualitative nor quantitative RT-PCR analysis of MART-1 and tyrosinase mRNAs could be used alone as a definitive diagnostic tool in SNs, we believe that the combination of molecular with (immuno)histopathologic analysis has an important role to play in SN management. An extended histopathologic protocol for assessing SNs, such as that used in our study which combines extensive serial sectioning with multiple immunohistochemical stains, has been shown to significantly increase the number of nodes with metastases detected (18). However, a protocol such as this places a heavy workload on the pathology department, which would be incompatible with the daily routine in most laboratories. Furthermore, in many cases, there would be no additional benefit from an extensive histologic examination, either because the SNs contain no melanocytes or because they harbor large tumors easily detected by routine histology. Quantitative RT-PCR for MART-1 mRNA seems to be a highly sensitive method for detecting melanocytic lesions in SNs, being positive in 87% of the SNs and in 100% of the patients with histologically proven metastasis. Thus, a negative MART mRNA result had a predictive value for the absence of melanoma metastasis (PVneg) of 96% at the SN level and 100% at the patient level. This suggests that RT-PCR can be used as a screening tool, with only SNs from MART-1 mRNA-positive patients being submitted for extended histopathologic examination. In our study, this would have spared at least one third (31) of the patients—corresponding to 26% (58 of 220) of the SNs—from undergoing a costly and time-consuming extended histologic examination.

To perform both RT-PCR and histology in our cases, the SN was divided in two. Because only histopathology is able to discriminate between BNI and metastasis, this must remain the reference on which the final diagnosis depends. Although there is a theoretical risk of missing a correct diagnosis of melanoma, if the metastasis is only located in that half of the SN used for RT-PCR analysis, our data suggest that this is not a substantial problem. In 36 RT-PCR-positive SNs, the frozen lymph node tissue was examined in two portions. In those SNs that had histologically proven metastasis, most (12 of 16; 75%) had a positive RT-PCR signal in both parts. This supports our previous observation, based on histologic study, that metastases are often widely spread throughout the SN and are, therefore, less likely to cause sampling errors, whereas BNI are more limited in their distribution (18). Thus, the smaller the size and the more focal the nature of the target lesion, the greater is the risk of false-negative results occurring as a result of either sampling or methodologic limitations.

In conclusion, quantitative RT-PCR analysis for tyrosinase and MART-1 expression can contribute to the diagnosis of melanoma SNs compared with traditional RT-PCR methods. However, this molecular technique still cannot be used alone to identify patients with melanoma metastasis because of the considerable overlap in melanocyte marker mRNA levels compared with groups with nonmalignant SNs. However, a negative RT-PCR MART-1 analysis effectively excludes the possibility that even extensive histopathology will detect melanoma metastasis in SNs from a particular patient. This makes it a useful screening tool that can help avoid the need to perform costly and time-consuming extended histologic examination in about one third of cases. Follow-up studies on our patients will be necessary to define the precise role of molecular staging in the assessment of melanoma SNs, and in particular the possible prognostic relevance of the quantitative level of melanocyte-associated mRNAs.

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Pathologic Assessment of Melanoma Sentinel Nodes: A Role for Molecular Analysis Using Quantitative Real-Time Reverse Transcription-PCR for MART-1 and Tyrosinase Messenger RNA

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