Molecular Staging of Malignant Melanoma

Correlation With Clinical Outcome

Steven C. Shivers, PhD; Xiangning Wang, MD; Weiguo Li, MD; Emmanuella Joseph, MD; Jane Messina, MD; L. Frank Glass, MD; Ronald DeConti, MD; C. Wayne Cruse, MD; Claudia Berman, MD; Neil A. Fenske, MD; Gary H. Lyman, MD, MPH; Douglas S. Reintgen, MD

Context.—For most solid tumors, 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 patients with melanoma who have nodal metastases.

Objective.—To determine the clinical significance of a highly sensitive molecular assay for occult nodal metastases for the staging of patients with melanoma.

Design.—A prospective cohort study of consecutive patients in which lymphatic mapping and sentinel lymph node (SLN) biopsy were performed on 114 melanoma patients with clinical stage I and stage II disease. The SLNs were bivalved, and half of each specimen was submitted for routine pathological examination. The other half was submitted for molecular detection of submicroscopic metastases using a reverse transcriptase–polymerase chain reaction (RT-PCR) assay for tyrosinase messenger RNA as a marker for the presence of melanoma cells. Patient follow-up averaged 28 months.

Setting.—A major university-based melanoma referral center at a National Cancer Institute–designated cancer center.

Patients.—A total of 114 patients with newly diagnosed cutaneous malignant melanoma who were at risk for regional nodal metastases.

Main Outcome Measure.—Melanoma recurrence and overall survival.

Results.—Twenty-three patients (20%) had pathologically positive SLNs, and all of these patients were also RT-PCR positive. Of the 91 pathologically negative patients, 44 were RT-PCR negative and 47 were RT-PCR positive. There was a recurrence rate among 14 (61%) of the 23 patients who were both pathologically and RT-PCR positive and a recurrence rate among 1 (2%) of 44 patients who were both pathologically and RT-PCR negative. For patients who were upstaged by the molecular assay (pathologically negative, RT-PCR positive), there was a recurrence rate among 6 (13%) of 47 patients. The differences in recurrence rates and overall survival between the pathologically negative, RT-PCR–negative and pathologically negative, RT-PCR–positive patient groups were statistically significant (P = .02 for disease-free survival and for overall survival). In both univariate and multivariate regression analyses, the histological and RT-PCR status of the SLNs were the best predictors of disease-free survival.

Conclusions.—The use of an RT-PCR assay for detection of submicroscopic melanoma metastases in SLNs improved the prediction of melanoma recurrence and overall survival over routine pathological examination.

JAMA. 1998;280:1410-1415

From the Cutaneous Oncology Program, H. Lee Moffitt Cancer Center and Research Institute (Drs Shivers, Wang, Li, Joseph, Messina, Glass, DeConti, Cruse, Berman, Fenske, and Reintgen), and the Departments of Surgery (Drs Shivers, Wang, Li, Joseph, Cruse, and Reintgen), Pathology and Laboratory Medicine (Drs Messina and Glass), Internal Medicine (Drs DeConti, Fenske, and Lyman), and Radiology (Dr Berman), University of South Florida, Tampa. Reprints: Douglas S. Reintgen, MD, Cutaneous Oncology Program, H. Lee Moffitt Cancer Center and Research Institute at the University of South Florida, 12902 Magnolia Dr, Tampa, FL 33612 (e-mail: doug@moffitt.usf.edu).

©1998 American Medical Association. All rights reserved.
Invasion of the regional nodes often occurs with a low volume of metastatic cells, but the question of whether micrometastatic disease is clinically important remains controversial.

Special stains that are relatively specific for melanoma, such as S-100 or HMB-45, have been available for years, but their use has never been incorporated into the routine pathological examination of lymph node specimens. This is mainly because immunohistochemical analysis of all the nodes from a complete lymph node dissection, which typically involves 10 to 30 nodes, would be prohibitively time-consuming and expensive. A major advantage of the lymphatic mapping techniques is that surgeons can now submit 1 or 2 SLNs that are most likely to contain metastatic disease, thus making a detailed pathological examination more practical. However, sensitivity of the pathological examination of regional lymph nodes is still an issue. Recently, a number of reports have described the development of highly sensitive immunohistochemical and molecular biology assays for the detection of metastatic cells in peripheral blood, bone marrow, and lymph nodes. These assays, based on special immunohistochemical stains or the reverse transcriptase–polymerase chain reaction (RT-PCR), are designed to detect markers that are commonly expressed by metastatic cells but not by other cells in the target tissue. However, the biological and clinical importance of finding submicroscopic evidence of cancer cells in tissues at risk for metastatic disease remains controversial.

This study examines the clinical significance of a molecular assay for the detection of submicroscopic metastases in SLNs from melanoma patients. The RT-PCR assay detects messenger RNA (mRNA) for the tyrosinase gene, which is uniquely expressed by normal melanocytes and a majority of melanoma cells.

METHODS

Patient Populations

Patients were recruited for the study while being evaluated for treatment for malignant melanoma by the Cutaneous Oncology Program at the H. Lee Moffitt Cancer Center and Research Institute at the University of South Florida (USF), Tampa. The study was approved by the USF Institutional Review Board.

Patients were selected based on the presence of a biopsy-proven malignant melanoma with no evidence of palpable lymph nodes or distant metastases (clinical stage I or II). One hundred fourteen consecutive patients who signed the informed consent were enrolled in the study.

Treatment Plan

Patients consented to a treatment plan consisting of wide local excision of the primary tumor and nodal staging by preoperative lymphoscintigraphy, intraoperative lymphatic mapping, and SLN biopsy. Sentinel lymph nodes were bivalved, and half of each node was evaluated for metastases by routine histological examination and the other half by RT-PCR for tyrosinase mRNA. Patients were observed for melanoma recurrence and overall survival at 6-month intervals for the first 2 years and yearly thereafter.

Surgery

To define the regional lymphatic basins at risk for metastatic disease and to identify the approximate number and locations of the SLNs, patients first underwent preoperative lymphoscintigraphy. A filtered technetium Tc 99m sulfur colloid (mean dose, 16.67 MBq [450 μCi]) was injected intradermally at 4 quadrants around the primary melanoma or previous biopsy scar. A dynamic imaging study was performed to visualize the lymphatics leading away from the primary tumor toward the regional nodal basin. Delayed images were taken to detect late drainage to a second or third basin. A handheld y probe (Neoprobe 1000, Neoprobe Corporation, Dublin, Ohio) was used to determine the approximate location of each SLN, which was marked with an intradermal tattoo.

Both radiocolloid and vital blue dye lymphatic mapping techniques were used intraoperatively to locate and identify SLNs. The operative procedures were performed 2 to 6 hours after lymphoscintigraphy so that radioactivity in the SLNs was still readily detectable and reinjection of radiocolloid was not required. The dermis around the primary melanoma site was injected with 1 mL of 1% isosulfan blue (Lymphazurin Blue, Zenith Parenterals, Rosemont, Ill) per lymphatic basin at risk (as determined by preoperative lymphoscintigraphy). If a previous biopsy scar was present at the site of the primary tumor, care was taken to inject the dye into normal skin around the scar.

Sentinel lymph node dissection was begun approximately 10 minutes after injection of the blue dye. Sentinel lymph nodes were defined by the presence of at least one of the following criteria: (1) greater than a 3:1 ratio of in vivo radioactivity compared with background, (2) greater than a 10:1 ratio of ex vivo radioactivity in the SLN compared with a neighboring non-SLN, or (3) presence of the blue dye either in the node or (4) in an afferent lymphatic leading to the node.

Wide local excision of the primary tumor was usually performed after the removal of all SLNs, except in cases where the nodal basin was close to the primary melanoma and elimination of the tumor was necessary to reduce interfering radioactivity. Wide local excision was performed with a 1-cm margin for primary tumors less than 1.0 mm in tumor thickness or with a 2-cm margin for melanomas 1.0 mm or more in thickness.

Adjuvant Therapy

Interferon α-2b has been recently approved for the adjuvant therapy of patients with resected stage III melanoma. Patients with nodal metastases identified by routine pathological examinations were taken back to the operating room for a complete node dissection and were offered adjuvant interferon α-2b therapy or other adjuvant therapy trials. No clinical decisions for further surgical and/or medical treatment were based on results of the RT-PCR assay.

Pathological Examination

Each SLN was bivalved and half of each specimen randomly underwent a routine histological examination as follows. The specimen was sectioned at 2-mm to 3-mm intervals and submitted for paraffin embedding (1-8 blocks per node). Sections of each block were taken at 3 μm (1-3 per slide) and stained with H&E, and the slides were read by a dermatopathologist.

Reverse Transcriptase–Polymerase Chain Reaction

The other half of each SLN was submitted for RT-PCR detection of tyrosinase mRNA. Figure 1 shows a schematic overview of this assay, but the technical details have been described elsewhere. Briefly, SLN specimens were sent immediately from the operating room to the laboratory, where they were trimmed of any external fat, weighed, and stored at −86°C until RNA extraction. Total cellular RNA was extracted from the entire specimen by a phenolguanidinium thiocyanate method (RNA STAT-60, Tel-Test “B” Inc, Friendswood, Tex). One microgram of purified RNA was used for the generation of tyrosinase complementary DNA (cDNA) using a specific primer, HYTR2.

The cDNA was then amplified by 2 rounds (30 cycles each) of PCR using nested primer pairs. The first round of PCR used the outer primers HYTR1 (upstream, sense) and HYTR2 (downstream, antisense) to generate a 284–
amplify the tyrosinase cDNA. The HTYR1 primer spans a boundary between exon 1 and exon 2, thus fa-

 primer (HTYR2). Two rounds of PCR using nested primer pairs (HTYR1/HTYR2 and HTYR3/HTYR4) then

mentary DNA (cDNA) fragments of tyrosinase mRNA are made by a RT step in the presence of a specific

was performed to verify the integrity of

negative specimens, a separate RT-PCR

visualization of a band at 207 bp, as long

was run with each assay. Finally, both

contamination, a blank control, which con-

pipettes was used for PCR.

To minimize contamination in the PCR

laboratory, powder-free gloves, disposable

tubes, and aerosol-resistant tips

were used throughout the procedure.

Sample preparation and RNA isolation

were performed in a fume hood, and a

set of pipettes was used for PCR.

were performed in a fume hood, and a

separate set of pipettes was used for PCR.

to rule out the possibility of carryover

contamination, a blank control, which con-

tained all assay components except RNA,

was run with each assay. Finally, both

positive (SK-Mel-28, melanoma) and nega-

tive (T47D, breast carcinoma) cell

lines were included in each assay to verify

the integrity of the assay system.

Polymerase chain reaction products

were analyzed by electrophoresis in a 2%

agarose gel containing ethidium bromide.

A 100-bp ladder (Gibco BRL, Inc, Grand

Island, NY) was included as a molecular

weight marker. After electrophoresis,

the gels were photographed using UV

transillumination and Polaroid 667 film.

Reverse transcriptase–polymerase chain

reaction positivity was defined by visualiza-

tion of a band at 207 bp, as long as

a band was not present in the blank or

negative controls. For all tyrosinase-

negative specimens, a separate RT-PCR

assay27 for the β-actin housekeeping gene

was performed to verify the integrity of the

mRNA isolated from the specimen.

### Statistical Analyses

Univariate and multivariate regression analyses were performed with prognostic factors based on the primary tumor, including tumor thickness, Clark level, ulceration, and primary site location. Age, sex, and the histological and RT-PCR status of the SLN were also included in the analyses. Mean subject ages were compared between groups using an independent sample Student t test, incorporating a pooled variance. Tumor thickness was compared between groups using the nonparametric Wilcoxon rank sum test. Categorical variables were compared between groups using either the Fisher exact test or a χ² test.

Patient survival functions were generated for overall and disease-free survival (DFS) using the product-limit method (or Kaplan-Meier method). Survival was calculated from the date of diagnosis to the date of death. Disease-free survival was calculated from the date of diagnosis to the date of first recurrence or death. Patients not experiencing an event were considered censored at the date of last contact. Statistical inference on survival functions between subgroups was based on the log-rank test (Mantel-Haenszel) for equality of the survival functions. Censored survival data were fit to Cox proportional hazards regression models. After satisfying the proportionality assumptions of the model, potential interactions were studied. Those interaction terms that failed to achieve statistical significance were not considered further in the models. Variable entry in the models proceeded in a forward stepwise fashion using an adjusted χ² statistic in variable selection. The likelihood ratio test was used to examine the hypothesis that the covariates had no influence on DFS. Regression analyses were performed for DFS only since the number of deaths in the series was limited. The adjusted hazard ratios for SLN histological and SLN PCR analyses were estimated by the exponential function of the point estimate and SE of the model coefficients. An allowed false-positive rate of 0.05 and 95% confidence intervals were used throughout the analysis.

### RESULTS

A summary of the demographics for the patient population (114 patients) is shown in Table 1. Sites of the primary tumor were equally distributed between the trunk and extremities, while 3% of the tumors were located on the head and neck. Most of the primary melanomas were Clark level 3 and 4 lesions (35% and 59%, respectively), with a mean Breslow thickness of 2.36 mm. One patient with a tumor thickness of 0.6 mm underwent an SLN biopsy and was included in the study because there was evidence of regression and the original tumor was believed to be more than 0.75 mm thick. After pathological staging of the primary tumor and SLNs, 40 patients (35%) were stage I (negative SLNs and Breslow thickness ≤1.5 mm), 51 patients (45%) were stage II (negative SLNs and Breslow thickness >1.5 mm), and 23 patients

![Figure 1.—Schematic diagram of the reverse transcriptase–polymerase chain reaction (RT-PCR) assay for tyrosinase messenger RNA (mRNA). The human tyrosinase gene is reportedly expressed only in Schwann cells, normal melanocytes, and malignant melanoma cells.34 After purification of total cellular RNA, comple-

_mentary DNA (cDNA) fragments of tyrosinase mRNA are made by a RT step in the presence of a specific

primer (HTYR2). Two rounds of PCR using nested primer pairs (HTYR1/HTYR2 and HTYR3/HTYR4) then

amplify the tyrosinase cDNA. The HTYR1 primer spans a boundary between exon 1 and exon 2, thus fa-

roring the amplification of mRNA sequences over genomic sequences. The final 207–base pair (bp) PCR2

product is detected by agarose gel electrophoresis.](image-url)
Patients were grouped according to SLN status as determined by routine histological analyses and by RT-PCR (Table 2). Twenty-three (20%) of 114 patients had a positive SLN biopsy result by routine histological analyses and all of these patients also had positive biopsy results by RT-PCR (group 3). Thus, there were no false negatives by the molecular assay. Forty-four patients (39%) had negative SLN biopsy results by both assays (group 1) while 47 patients (41%) had negative SLN biopsy results by routine histological analyses but positive biopsy results by RT-PCR (group 2).

None of the patients whose SLN biopsy results were negative both histologically and with the RT-PCR assay (group 1) has died, and only 1 patient had a recurrence of melanoma. Patients who were upstaged by the molecular assay (group 2) had an intermediate prognosis with a recurrence rate of 13%. Sixty-one percent of the patients in group 3 had a recurrence of melanoma or died from their disease. Kaplan-Meier relapse-free and overall survival curves are shown in Figures 2 and 3, respectively. Patients whose SLNs were histologically negative (groups 1 and 2) had significantly greater relapse-free (P = .02) and overall (P = .02) survival than patients with histologically positive SLNs (group 3). Furthermore, patients with histologically negative results who were upstaged by the molecular assay (group 2) had significantly lower relapse-free (P = .02) and overall (P = .02) survival than patients who were RT-PCR negative (group 1). Increasing Breslow thickness or ulceration of the primary tumor are factors that are correlated with decreased patient survival. In our patients, as the primary tumor thickness increased, the chance of finding PCR-positive material in the SLN increased. For patients with a tumor thickness less than 1.5 mm, 1.5 to 4.0 mm, or more than 4.0 mm, the chances of having an RT-PCR–positive SLN were 48%, 68%, or 77%, respectively. In addition, 70% of patients whose primary tumor was ulcerated had an RT-PCR–positive SLN, whereas only 41% of patients whose melanoma was not ulcerated were RT-PCR positive. These data represent the percentage of patients in each subgroup who were RT-PCR positive (data not shown).

The pathological stages (using routine histological analyses only) of each patient group are summarized in Table 3. For patients whose melanoma recurred, the sites of the first recurrence are summarized in Table 4. The 1 patient in group 1 who had a recurrence of melanoma experienced a local recurrence, and thus metastatic cells were not necessarily present in the SLN when the assay was performed.

Increasing Breslow thickness or ulceration of the primary tumor are factors that are correlated with decreased patient survival. In our patients, as the primary tumor thickness increased, the chance of finding PCR-positive material in the SLN increased. For patients with a tumor thickness less than 1.5 mm, 1.5 to 4.0 mm, or more than 4.0 mm, the chances of having an RT-PCR–positive SLN were 48%, 68%, or 77%, respectively. In addition, 70% of patients whose primary tumor was ulcerated had an RT-PCR–positive SLN, whereas only 41% of patients whose melanoma was not ulcerated were RT-PCR positive. These data represent the percentage of patients in each subgroup who were RT-PCR positive (data not shown).

The pathological stages (using routine histological analyses only) of each patient group are summarized in Table 3. For patients whose melanoma recurred, the sites of the first recurrence are summarized in Table 4. The 1 patient in group 1 who had a recurrence of melanoma experienced a local recurrence, and thus metastatic cells were not necessarily present in the SLN when the assay was performed.

Increasing Breslow thickness or ulceration of the primary tumor are factors that are correlated with decreased patient survival. In our patients, as the primary tumor thickness increased, the chance of finding PCR-positive material in the SLN increased. For patients with a tumor thickness less than 1.5 mm, 1.5 to 4.0 mm, or more than 4.0 mm, the chances of having an RT-PCR–positive SLN were 48%, 68%, or 77%, respectively. In addition, 70% of patients whose primary tumor was ulcerated had an RT-PCR–positive SLN, whereas only 41% of patients whose melanoma was not ulcerated were RT-PCR positive. These data represent the percentage of patients in each subgroup who were RT-PCR positive (data not shown).

The pathological stages (using routine histological analyses only) of each patient group are summarized in Table 3. For patients whose melanoma recurred, the sites of the first recurrence are summarized in Table 4. The 1 patient in group 1 who had a recurrence of melanoma experienced a local recurrence, and thus metastatic cells were not necessarily present in the SLN when the assay was performed.

Increasing Breslow thickness or ulceration of the primary tumor are factors that are correlated with decreased patient survival. In our patients, as the primary tumor thickness increased, the chance of finding PCR-positive material in the SLN increased. For patients with a tumor thickness less than 1.5 mm, 1.5 to 4.0 mm, or more than 4.0 mm, the chances of having an RT-PCR–positive SLN were 48%, 68%, or 77%, respectively. In addition, 70% of patients whose primary tumor was ulcerated had an RT-PCR–positive SLN, whereas only 41% of patients whose melanoma was not ulcerated were RT-PCR positive. These data represent the percentage of patients in each subgroup who were RT-PCR positive (data not shown).

The pathological stages (using routine histological analyses only) of each patient group are summarized in Table 3. For patients whose melanoma recurred, the sites of the first recurrence are summarized in Table 4. The 1 patient in group 1 who had a recurrence of melanoma experienced a local recurrence, and thus metastatic cells were not necessarily present in the SLN when the assay was performed.

Increasing Breslow thickness or ulceration of the primary tumor are factors that are correlated with decreased patient survival. In our patients, as the primary tumor thickness increased, the chance of finding PCR-positive material in the SLN increased. For patients with a tumor thickness less than 1.5 mm, 1.5 to 4.0 mm, or more than 4.0 mm, the chances of having an RT-PCR–positive SLN were 48%, 68%, or 77%, respectively. In addition, 70% of patients whose primary tumor was ulcerated had an RT-PCR–positive SLN, whereas only 41% of patients whose melanoma was not ulcerated were RT-PCR positive. These data represent the percentage of patients in each subgroup who were RT-PCR positive (data not shown).

The pathological stages (using routine histological analyses only) of each patient group are summarized in Table 3. For patients whose melanoma recurred, the sites of the first recurrence are summarized in Table 4. The 1 patient in group 1 who had a recurrence of melanoma experienced a local recurrence, and thus metastatic cells were not necessarily present in the SLN when the assay was performed.

Increasing Breslow thickness or ulceration of the primary tumor are factors that are correlated with decreased patient survival. In our patients, as the primary tumor thickness increased, the chance of finding PCR-positive material in the SLN increased. For patients with a tumor thickness less than 1.5 mm, 1.5 to 4.0 mm, or more than 4.0 mm, the chances of having an RT-PCR–positive SLN were 48%, 68%, or 77%, respectively. In addition, 70% of patients whose primary tumor was ulcerated had an RT-PCR–positive SLN, whereas only 41% of patients whose melanoma was not ulcerated were RT-PCR positive. These data represent the percentage of patients in each subgroup who were RT-PCR positive (data not shown).

The pathological stages (using routine histological analyses only) of each patient group are summarized in Table 3. For patients whose melanoma recurred, the sites of the first recurrence are summarized in Table 4. The 1 patient in group 1 who had a recurrence of melanoma experienced a local recurrence, and thus metastatic cells were not necessarily present in the SLN when the assay was performed.

Increasing Breslow thickness or ulceration of the primary tumor are factors that are correlated with decreased patient survival. In our patients, as the primary tumor thickness increased, the chance of finding PCR-positive material in the SLN increased. For patients with a tumor thickness less than 1.5 mm, 1.5 to 4.0 mm, or more than 4.0 mm, the chances of having an RT-PCR–positive SLN were 48%, 68%, or 77%, respectively. In addition, 70% of patients whose primary tumor was ulcerated had an RT-PCR–positive SLN, whereas only 41% of patients whose melanoma was not ulcerated were RT-PCR positive. These data represent the percentage of patients in each subgroup who were RT-PCR positive (data not shown).
COMMENT

Morton et al.,1 Balch et al.,2 and others3-7 have shown that the SLN is the node that is most likely to contain melanoma metastases. Therefore, it is rational to focus more detailed searches for metastatic cells on the SLN. We have adapted a highly sensitive molecular assay for the detection of submicroscopic melanoma metastases in SLNs. This article examines the ability of the molecular assay to provide more accurate staging information for melanoma patients at the time of their initial surgery.

It is well established that the histological detection of micrometastatic disease in lymph nodes by routine H&E staining, with or without immunohistochemistry, is clinically important. The 5-year survival of all patients with pathologically node-negative (stage I and stage II) disease is about 85%. However, the 5-year survival of patients who do not have palpable nodes but are found to have histologically positive nodes (pathological stage III) approaches 50%.31

More at issue is the clinical significance of “submicroscopic” disease that has been missed by routine histological analyses. Our initial attempts at developing more sensitive assays for occult metastases involved a tissue-culture technique, in which the regional nodes were placed into tissue culture and any malignant cells were allowed to grow out. We found that the lymph node–culture technique grew metastatic melanoma cells from 21% of patients who were histologically node negative.34 We observed a shorter DFS in culture-positive patients compared with patients who were node negative by both routine histological analyses and the cell culture assay, thus demonstrating a clinical correlation for the culture technique.35

Other indirect evidence for the clinical relevance of missed micrometastases comes from a recently reported series from our institution in collaboration with the MD Anderson Cancer Center, Houston, Tex. This study performed lymphatic mapping and SLN biopsy in patients with melanomas more than 0.76 mm in thickness. Two hundred forty-three patients with a negative SLN biopsy result were observed to define the natural history of the mapped, SLN-negative population.36 With a mean follow-up of 2 years, 15 patients (3.5%) had melanoma recurrences in the nodal basin, even though their SLN biopsy result had been negative by routine techniques (H&E). After nodal recurrence, the SLN-tissue blocks were recut for more sections and immunohistochemistry was performed. Metastatic melanoma cells were identified in 10 (66%) of the 15 patients, suggesting that two thirds (or 66%) of the nodal recurrences were due to a failed pathological examination.

Molecular staging has the potential to revolutionize the pathological examination and staging of cancer patients. Molecular assays for occult metastases have now been reported for a number of both solid tumors and hematologic malignancies based on various markers.11,20 These initial reports have invariably shown increased sensitivity when compared with routine histological analyses and correlation between stage of disease and PCR positivity. Some studies have also shown a relationship between PCR positivity and disease recurrence or survival.

The current study shows that the tyrosinase RT-PCR assay is more sensitive than routine histological examinations for the detection of SLN micrometastases. Routine histological examination by microscopy has been shown to identify 1 abnormal melanoma cell in a background of 10^6 lymphocytes. Immunohistochemistry can increase the sensitivity of the examination by allowing the identification of 1 melanoma cell in a background of 10^6 lymphocytes. Molecular assays have been reported to achieve sensitivity 2 to 3 orders of magnitude higher than routine pathological examinations.11,12,14-17

An important issue is whether the small numbers of melanoma cells detected by the molecular assay are clinically important. This article shows a statistically significant difference in both disease-free (P = .02) and overall (P = .02) survival between patients with histologically negative results with a PCR-negative SLN and those with a PCR-positive SLN.

Since the patients with histologically negative results in this study were followed up by observation only, it is interesting to compare the rate of PCR positivity in these patients with the natural history of the disease. For patients with thick melanomas (>4.0 mm), the expected rate of positive nodes after an elective lymph node dissection (by routine histological examination) would be about 25% to 40%. However, the 10-year survival of patients with thick melanomas is approximately 25%, which is more in line with our observed rate of PCR positivity (77% in patients with thick melanomas). For inter-

Table 3.—Pathological Stage*

<table>
<thead>
<tr>
<th>Pathological Stage, No. (%)</th>
<th>Histological Examination</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>Stage II</td>
<td>Stage III</td>
</tr>
<tr>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

*SLN indicates sentinel lymph node; PCR, polymerase chain reaction.

Table 4.—Site of First Recurrence*

<table>
<thead>
<tr>
<th>Site of First Recurrence, No.</th>
<th>Histological Examination</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Local</td>
<td>Intransit</td>
<td>Regional</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

*SLN indicates sentinel lymph node; PCR, polymerase chain reaction.

Table 5.—Univariate Regression Analysis*

<table>
<thead>
<tr>
<th>Variable</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor thickness</td>
<td>.09</td>
</tr>
<tr>
<td>Clark level</td>
<td>.28</td>
</tr>
<tr>
<td>Ulceration</td>
<td>.18</td>
</tr>
<tr>
<td>Primary site</td>
<td>.25</td>
</tr>
<tr>
<td>Sex</td>
<td>.30</td>
</tr>
<tr>
<td>Age</td>
<td>.94</td>
</tr>
<tr>
<td>SLN pathology</td>
<td>.04</td>
</tr>
<tr>
<td>SLN PCR</td>
<td>.04</td>
</tr>
</tbody>
</table>

*SLN indicates sentinel lymph node; PCR, polymerase chain reaction.
mediate thickness melanomas (1.5-4.0 mm), the 10-year death rate is 40%, but only 20% of these patients have positive nodes with elective lymph node dissection and routine histological examination. We found 68% of these patients have positive SLNs by the RT-PCR assay. However, the 10-year survival of patients with thin melanomas (0.75-1.0 mm) is approximately 85%, while we found a 50% rate of PCR positivity in these patients. Therefore, the prevalence of SLN-PCR positivity closely reflects the natural history of patients with thick and intermediate thickness melanomas but overestimates the risk of death for patients with thin melanomas.

One possible source of false-positive RT-PCR results is benign nevus cells, which have been observed in approximately 5% of lymph nodes examined. When present, these cells are usually found in the capsule or fibrous trabeculae of the lymph node. Although they are positive for tyrosinase, benign nevus cells can usually be distinguished morphologically from melanoma metastases by routine H&E-stained sections.

The RT-PCR assay has the potential of identifying a group of patients (those with a PCR-negative SLN) who are at a low risk of recurrence and death, and these patients can be spared further surgery and/or adjuvant therapy. The question of whether patients with negative histological results but with positive PCR results should be offered more extensive therapy (complete node dissection and/or adjuvant therapy) remains unclear. Although the chance of relapse in this patient group is still low, the presence of a significantly reduced disease-free and overall survival of patients with SLN negativity suggests that further therapeutic intervention might be beneficial. This question is being addressed by an industry-sponsored multicenter national trial, called the Sunbelt Melanoma Trial. In this trial, patients whose SLN is histologically negative but RT-PCR positive are being randomized into 1 of 3 arms: (1) observation only, (2) complete lymph node dissection, or (3) complete lymph node dissection with adjuvant interferon alfa therapy. The clinical relevance of RT-PCR positivity will be ascertained and the best treatment for submicroscopic disease will be studied. If our data are confirmed in this national trial, molecular staging should become an important part of the care of the patient with melanoma.

This study demonstrates that, when combined with surgical lymphatic mapping techniques, the RT-PCR assay for tyrosinase mRNA allows more accurate staging and identifies clinically significant disease in patients with melanoma.

Table 6.—Proportional Hazard Regression Model*  

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient</th>
<th>Hazard Ratio (95% CI)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLN histology</td>
<td>1.1032</td>
<td>3.01 (1.10-8.27)</td>
<td>.04</td>
</tr>
<tr>
<td>SLN PCR</td>
<td>2.3144</td>
<td>10.12 (1.21-84.68)</td>
<td>.03</td>
</tr>
<tr>
<td>Tumor thickness</td>
<td>0.1612</td>
<td>. . . . . . . . .</td>
<td>.27</td>
</tr>
<tr>
<td>Age</td>
<td>0.0192</td>
<td>. . . . . . . . .</td>
<td>.18</td>
</tr>
</tbody>
</table>

*CI indicates confidence interval; SLN, sentinel lymph node; and PCR, polymerase chain reaction. Ellipses indicate data not applicable.