SOUTHERN SURGICAL ASSOCIATION ARTICLE

Molecular Staging of Sentinel Lymph Nodes Identifies Melanoma Patients at Increased Risk of Nodal Recurrence

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BACKGROUND: Molecular staging of sentinel lymph nodes (SLNs) may identify patients who are node-negative by standard microscopic staging but are at increased risk for regional nodal recurrence; such patients may benefit from completion lymph node dissection (CLND).

STUDY DESIGN: In a multicenter, randomized clinical trial, patients with tumor-negative SLNs by standard pathology (hematoxylin and eosin [H and E] serial sections and immunohistochemistry [IHC]) underwent reverse transcriptase polymerase chain reaction (PCR) analysis of SLNs for melanoma-specific mRNA. Microscopically negative/PCR+ patients were randomized to observation, CLND, or CLND with high-dose interferon (HDI). For this post-hoc analysis, clinicopathologic features and survival outcomes, including overall survival (OS) and disease-free survival (DFS), were compared between PCR+ patients who underwent CLND vs observation. Microscopic and molecular node-negative (PCR-) patients were included for comparison.

RESULTS: A total of 556 patients were PCR+: 180 underwent observation, and 376 underwent CLND. An additional 908 PCR- patients were observed. Median follow-up was 72 months. Disease-free survival (DFS) was significantly better for PCR+ patients who underwent CLND compared with observation ($p = 0.0218$). No statistically significant differences in OS or distant disease-free survival (DDFS) were seen. Regional lymph node recurrence-free survival (LNRFS) was improved in PCR+ patients with CLND compared to observation ($p = 0.0065$). The PCR+ patients in the observation group had the worst DFS; those with CLND had similar DFS to that in the PCR- group ($p = 0.9044$).

CONCLUSIONS: Patients with microscopically negative/PCR+ SLN have an increased risk of nodal recurrence that was mitigated by CLND. Although CLND did not affect OS, these data suggest that molecular detection of melanoma-specific mRNA in the SLN predicts a greater risk of nodal recurrence and deserves further study. (J Am Coll Surg 2016;■:1–7. © 2016 by the American College of Surgeons. Published by Elsevier Inc. All rights reserved.)

Accurate staging through sentinel lymph node (SLN) biopsy has become a crucial component of modern melanoma care. For patients with early stage melanoma, status of the regional nodal basin remains the most important predictor of survival.[1,2] Standard practice is to perform completion lymph node dissection (CLND) for patients with tumor-positive SLNs because of the possibility of metastatic disease in the remaining nonsentinel nodes.[3]

Given the prognostic significance of nodal metastases, pathologic assessment of the SLN is of paramount importance. Currently, the predominant method of SLN evaluation involves staining serial sections from each node by hematoxylin and eosin (H and E) and immunohistochemistry (IHC).[4] However, it is impractical for a pathologist to study every node in its entirety, and evaluation may consist of only 1% of submitted tissue.[5] Nodes

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Abbreviations and Acronyms

CLND = completion lymph node dissection
DDFS = distant disease-free survival
DFS = disease-free survival
H and E = hematoxylin and eosin
HDI = high-dose interferon
HR = hazard ratio
IHC = immunohistochemistry
LNRFS = lymph node recurrence-free survival
OS = overall survival
PCR = polymerase chain reaction
SLN = sentinel lymph node

may incorrectly be classified as tumor-negative when microscopic disease truly does exist (ie, a false-negative result).[6] The false-negative rate for SLN biopsy has been reported as high as 30%, although a meta-analysis of 71 studies involving more than 25,000 patients estimated the number of false-negative SLN biopsies at 12.5%.[7] The failure to identify positive nodes has clinical consequences; patients with nodal recurrence after a negative SLN biopsy have a significant decrease in overall survival.[8-10]

Molecular detection of malignant cells was introduced in order to improve the sensitivity and diagnostic accuracy of SLN biopsy. The technique of reverse transcriptase polymerase chain reaction (PCR) amplifies tumor-specific mRNA to detect malignant cells, and can identify 1 tumor cell among 10^7 normal cells.[11] The application of PCR to SLN biopsy for melanoma has been studied extensively, with mixed results.[12] Previous analysis from our group failed to show that PCR yielded any significant prognostic information at a median follow-up of 30 months.[13]

In this analysis, we re-evaluated the prognostic significance of PCR staging on disease-free (DFS) and overall survival (OS) in stage I and II melanoma patients with long-term follow-up. In addition, we evaluated the impact of CLND on survival outcomes for patients with histologically negative, but PCR positive, SLNs.

METHODS

The Sunbelt Melanoma Trial was a prospective, randomized trial involving 79 centers in North America. The central hypothesis was that adjuvant high-dose interferon α-2b (HDI) with CLND improves overall survival compared with CLND alone in patients with minimal nodal tumor burden. Ultra-staging with PCR was also used to identify a high-risk subset of patients with histologically negative SLNs that would benefit from HDI or CLND. Patients aged 18 to 71 years with invasive melanoma $\geq$ 1 mm Breslow thickness and without clinical evidence of regional or distant metastasis were eligible. Patients were enrolled between June 1997 and October 2003. The institutional review board of each institution approved the study.[14]

After enrollment and informed consent, patients underwent wide local excision of the primary melanoma with SLN biopsy, which was performed with ^{99}technetium sulfur colloid, and a hand-held gamma probe was used intraoperatively to guide SLN identification. Isosulfan blue also was used in the majority of cases. Any blue nodes, any palpably suspicious nodes, and any nodes $\geq$ 10% of the most radioactive or "hottest node" were collected as SLNs.

The SLNs were evaluated by serial sectioning (at least 5 sections per block) with H and E staining and IHC for S-100. IHC for HMB-45 was performed by some institutions, but this was not a protocol requirement. If more than 1 SLN was found, each SLN was processed identically, and a portion of each SLN (one-fourth or 2 mm^3) was snap frozen on dry ice or liquid nitrogen and stored at −80°C. A histologically positive SLN was defined as evidence of metastatic tumor cells identified by either H and E or IHC. A central pathology review committee evaluated the first 10 patients from each participating institution, as well as all tumor-positive SLN.

Patients with tumor-negative SLNs by H and E and IHC staining underwent molecular staging, and PCR was performed with specific primers for tyrosinase, MART1, MAGE3, and GP-100, followed by Southern blot detection. The Southern blot signals were analyzed by determining optical band density for samples and controls; optical densities more than 50% of the negative controls were considered positive. The a priori definition of a positive SLN PCR test was detection of tyrosinase mRNA plus at least 1 other marker. Using this definition, there were no false-positive results when 100 nodes from patients without melanoma were analyzed during initial assay validation studies.[15] Histologically negative, PCR+ patients were randomized to observation, CLND, or CLND with HDI. Histologically negative, PCR- patients were observed (Fig. 1).

In this study, we limited analysis to histologically negative patients who were molecularly staged by PCR. The patient groups were defined as PCR- patients who underwent observation (PCR-/observation); PCR+ patients who underwent observation (PCR+/observation); and PCR+ patients who underwent CLND (PCR+/CLND). Because previous analysis of the Sunbelt Melanoma Trial indicated no impact of HDI on outcome (PCR+/CLND vs PCR+/CLND + HDI: OS $p = 0.77$; DFS $p = 0.069$), patients who were PCR+ and originally randomized to either CLND or

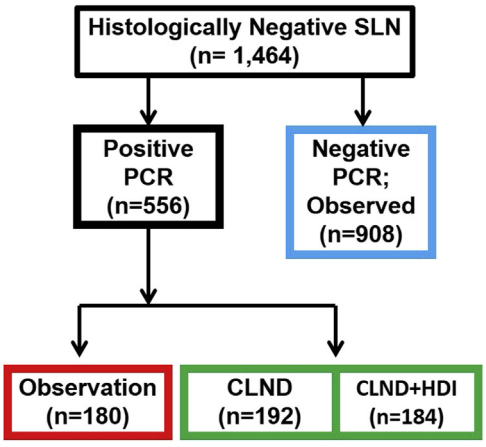

Figure 1. Schema of patients included in this study. Based on previous results that indicated no impact of adjuvant interferon, PCR+ patients who underwent CLND or CLND + HDI were considered a single PCR+/CLND group. CLND, completion lymphadenectomy; HDI, high-dose interferon; PCR, polymerase chain reaction.

CLND + HDI were considered together as a single PCR+/CLND group.[14,16] Clinicopathologic factors were compared between groups using chi-square analysis or ANOVA, where appropriate. Outcomes including OS, DFS, distant disease-free survival (DDFS), and regional lymph node recurrence-free survival (LNRFS) were compared between groups. Survival times were calculated from the date of random assignment to the event time. Survival distributions were estimated using the Kaplan-Meier method, and the log-rank test was used to assess the significance of any observed survival differences. A Cox proportional hazards model was used to identify independent predictors for DFS. Statistical analysis was performed using SAS.

Table 1. Univariate Comparison of Clinicopathologic Factors

Factor	PCR+/ observation	PCR+/ CLND	PCR-/ observation	p Value
Male, %	60.3	50.7	57.3	0.0419
Age, y	51.5	50.0	51.0	0.1327
Breslow thickness, mm	1.55	1.50	1.60	0.5526
Ulceration, %	24.2	22.5	23.1	0.9064
Lymphovascular invasion, %	4.4	8.0	5.3	0.1425
Site, %				0.2362
Trunk	43.6	46.5	49.5	
Extremity	41.9	43.1	37.6	
Head/neck	14.5	10.4	12.9	

CLND, completion lymphadenectomy; PCR, polymerase chain reaction.

RESULTS

A total of 1,464 patients were included in this study: 180 patients (12.3%) were PCR+ and underwent observation, 376 patients (25.7%) were PCR+ and underwent CLND, and 908 patients (62.0%) were PCR- and were observed (Fig. 1). Median follow-up was 72 months. Overall, the study population was 56% male, had a median age of 51 years, and had primary melanomas with a median Breslow thickness of 1.55 mm. Except for a lower proportion of males in the PCR+/CLND group, no major differences in clinicopathologic features were observed (Table 1).

On Kaplan-Meier analysis, no difference was seen in overall survival (log-rank p = 0.792). However, there was a significant difference in DFS across all 3 groups; the PCR+/observation group appeared to be an outlier with decreased DFS (log-rank p = 0.024) (Fig. 2). In

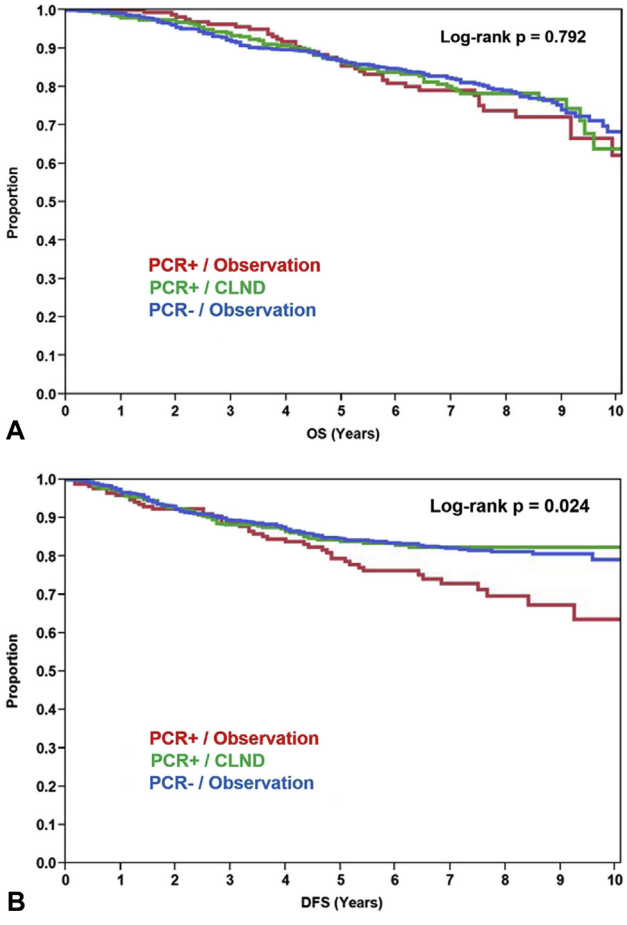

Figure 2. Overall survival and disease-free survival stratified by group: PCR+/observation, PCR+/CLND, or PCR-/observation. Although no difference is seen in OS, there is a significant difference in DFS with increased recurrence seen in PCR+/observation patients. CLND, completion lymphadenectomy; DFS, disease-free survival; OS, overall survival; PCR, polymerase chain reaction.

comparing DFS between the individual study arms, a significant difference was found between the PCR+/observation group and both the PCR-/observation (log-rank p = 0.009) and PCR+/CLND (log-rank p = 0.022) groups. However, PCR+ patients who underwent CLND had a survival curve equivalent to PCR- patients (log-rank p = 0.904) (Fig. 3).

We sought to evaluate if the worse DFS observed in the PCR+ group was related to regional nodal recurrence. Although DDFS was similar across all 3 groups (log-rank p = 0.255), recurrence in the regional lymph nodes was significantly worse in the PCR+/observation group (log-rank p = 0.026) (Fig. 4). Overall, the proportion of deaths and recurrences compared across all 3 study arms reinforces the significant difference seen in DFS and LNRFS, particularly in PCR+/observation patients (Table 2). However, these differences appear to be mitigated by CLND. In an unadjusted analysis of PCR+ patients, CLND was associated with improved DFS (hazard ratio [HR] 0.58, 95% CI 0.35 to 0.94) and LNRFS (HR 0.23, 95% CI 0.05 to 0.72), but not OS (HR 0.70, 95% CI 0.42 to 1.15), or DDFS (HR 0.64, 95% CI 0.34 to 1.14).

In order to determine independent predictors of the observed difference in DFS, a Cox proportional hazards model adjusting for multiple covariates and encompassing all patients was performed. In patients with histologically negative SLNs, a positive PCR result was independently associated with decreased DFS (HR 1.51, 95% CI 1.05 to 2.11). Furthermore, CLND significantly reduced the risk of recurrence (HR 0.57, 95% CI 0.34 to 0.93). Other factors associated with recurrence included ulceration, Breslow thickness, and the site of the primary melanoma (Table 3). Consistent with previous Sunbelt results, the use of adjuvant interferon had no significant impact on DFS (HR 1.42, 95% CI 0.82 to 2.46).

DISCUSSION

Our findings indicate that molecular staging of patients who are node-negative by standard melanoma histopathology can identify a subgroup that is at increased risk for recurrence—most notably, regional nodal recurrence. Furthermore, these patients may benefit from completion lymphadenectomy, which restores both DFS and LNRFS to a level equivalent to that observed in patients who have SLNs that are tumor-negative by both histopathology and PCR analysis.

Much has been written on molecular staging of melanoma, although its prognostic significance remains unclear. The heterogeneous results reported in the literature have not allowed any definitive conclusions.[5,11,12,17-19] Although multiple reports have suggested that PCR status significantly predicts OS or DFS, many of these studies were limited by small sample size and short follow-up times.[18,20-23] Further complicating the issue, there is no established set of markers used for PCR analysis, and the technical differences between studies add another layer of confounding variables that obscure the issue. Tyrosinase is one of the most extensively studied markers and has been shown to have some prognostic value in PCR staging of melanoma.[11] However, tyrosinase suffers from a lack of specificity and may overestimate the burden of disease in nodal tissue. For instance, benign nevus cells are a common cause of false-positive results when using tyrosinase alone. To improve diagnostic accuracy, various

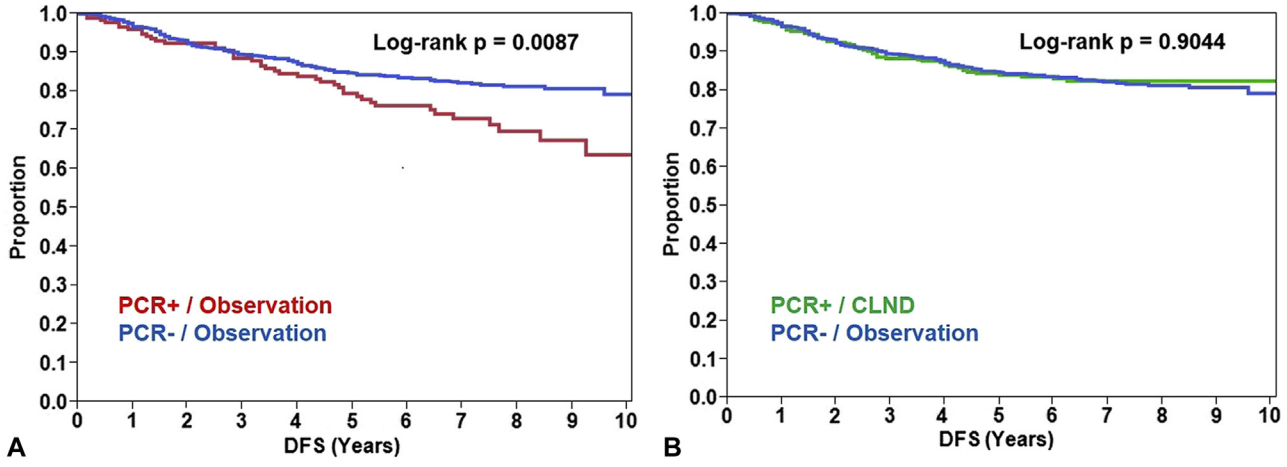

Figure 3. Impact of PCR status and CLND on DFS. (A) Compared with the PCR- control, PCR+ patients who undergo observation have a significantly decreased DFS. (B) Addition of CLND restores PCR+ patients to a survival curve that is equivalent to that of PCR- patients. CLND, completion lymphadenectomy; DFS, disease-free survival; PCR, polymerase chain reaction.

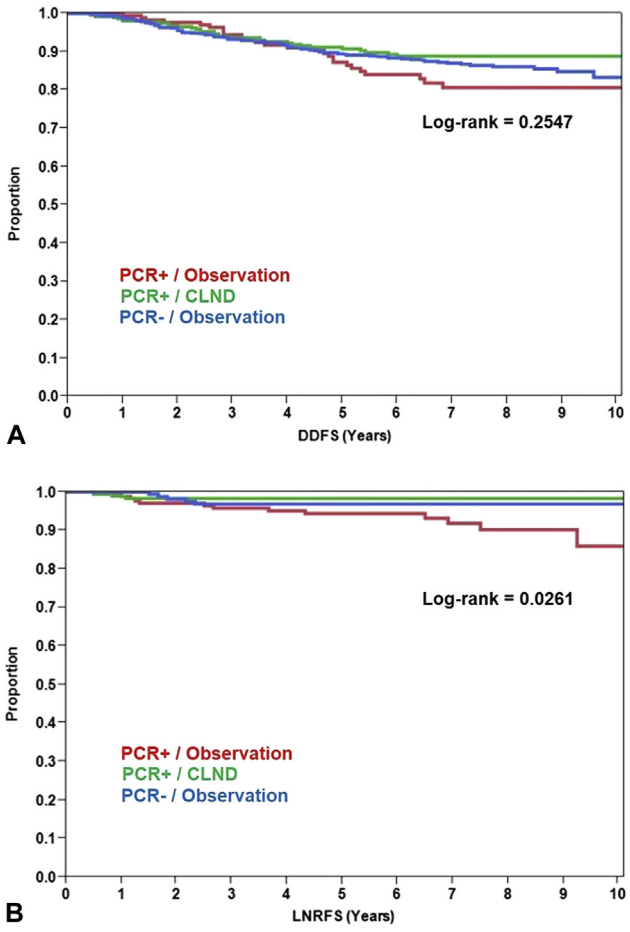

Figure 4. Patterns of recurrence. Although no difference is seen in (A) distant disease-free survival, PCR+/observation patients appear to have lower (B) lymph node recurrence-free survival. CLND, completion lymphadenectomy; DDFS, distant disease-free survival; LNRFS, lymph node recurrence-free survival; PCR, polymerase chain reaction.

combinations of different markers have been used. Beyond choosing the appropriate markers, techniques including quantitative PCR may further increase specificity.

Our group has previously published one of the largest series investigating the role of PCR in melanoma staging, which failed to demonstrate any prognostic significance for staging histologically negative SLNs with PCR.[13] Multiple markers were used for PCR analysis, and a positive result was defined as the presence of tyrosinase plus 1 additional marker (MART-1, MAGE-3, GP100). On initial validation studies, this combination showed good specificity and yielded no false positive results in a set of patients without melanoma.[15] Overall, 1,446 SLN negative patients were evaluated at a median follow-up of 30 months, and no differences were seen in OS, DFS, or DDFS.

Upon re-evaluating this study population after a median follow-up of 72 months, a delayed separation in the DFS curve of PCR+/observation patients was seen after approximately 5 years (Fig. 2). Overall, nearly one-quarter (23.9%) of PCR+ patients who underwent observation developed a recurrence. This figure is slightly higher but consistent with previous reports of recurrence among histologically negative but PCR+ patients, which include estimates up to 20%.[12,24] On multivariate analysis, a positive PCR result was an independent predictor of decreased DFS (HR 1.51, 95% CI 1.05 to 2.11). The difference in DFS appears to be driven by regional recurrences, as there was no difference observed in DDFS. Ultimately, these findings raise several questions regarding the underlying biology of melanoma progression and metastasis, as well as considerations for additional therapy such as CLND.

One hypothesis suggests that melanoma progresses in an orderly fashion; from the primary lesion, to the regional nodes, and then on to metastatic disease.[25] Multiple studies have demonstrated that the degree of micrometastatic tumor burden in the SLN corresponds to nonsentinel node involvement, DFS, and OS. Given the sensitivity of PCR, molecular ultra-staging therefore may detect trace melanoma metastases early along this continuum of disease, and identify at-risk patients deserving of close follow-up or additional therapy such as CLND. However, most previous studies of molecular staging using PCR have had short follow-up times that likely limited the detection of any delayed disease progression. In a meta-analysis of 22 studies investigating molecular staging in melanoma, only 2 studies had follow-up times of at least 60 months.[12] Nonetheless, these 2 studies failed to demonstrate any prognostic significance with

Table 2. Patterns of Recurrence

	PCR+/observation		PCR+/CLND		PCR-/observation		
Outcomes	**n**	**%**	**n**	**%**	**n**	**%**	**Chi-square p Value**
Deaths	39	21.7	64	17.0	151	16.6	0.260
Overall recurrence	43	23.9	54	14.4	133	14.7	0.0056
Regional lymph node recurrence	13	7.4	8	2.2	38	4.4	0.0179
Distant recurrence	26	14.4	33	8.8	96	10.6	0.1267

CLND, completion lymphadenectomy; PCR, polymerase chain reaction.

Table 3. Independent Predictors of Disease-Free Survival on Multivariate Analysis

All patients with histologically negative SLN (n = 1,446)		
Factor	**Hazard ratio (95% CI)**	**p Value**
PCR+	1.51 (1.05, 2.11)	0.026
CLND	0.57 (0.34, 0.93)	0.025
Breslow thickness, mm	1.23 (1.16, 1.29)	<0.0001
Ulceration	2.27 (1.74, 2.97)	<0.0001
Age, y	1.01 (1.00, 1.02)	0.066
Male sex	1.14 (0.86, 1.53)	0.366
Site		
Trunk	Reference	n/a
Extremity lesion	0.65 (0.48, 0.88)	0.006
Head/neck lesion	1.13 (0.78, 1.61)	0.499
Interferon α-2b	1.42 (0.82, 2.46)	0.207

CLND, completion lymphadenectomy; PCR, polymerase chain reaction; SLN, sentinel lymph node.

molecular staging by PCR.[17,19] More recently, Nicholl et al. evaluated molecular staging using quantitative PCR after a median follow-up of 11.3 years. In this study, node-negative patients upstaged by PCR had OS and DFS curves comparable to those seen in patients with histologically positive SLNs.[26]

In contrast to an orderly progression from primary lesion to nodal disease and then metastasis, an alternate hypothesis suggests that nodal disease in melanoma is simply a marker for the risk of systemic disease.[25] As such, some authors argue against a regional therapy such as CLND for histologically negative, but PCR+ SLNs.[24] Looking at the pattern of recurrence in our study, 26 of the 43 PCR+/observation patients experienced recurrence with systemic disease, while only 13 had recurrence confined to the regional lymph nodes. This suggests predominantly systemic spread of disease, and similar patterns were observed in the PCR+/CLND and PCR-/observation groups. The fact that most recurrences represented distant disease in all 3 groups likely contributed to the absence of an OS difference in this study.

However, although our study found no difference in OS by PCR status, a positive PCR predicted both decreased DFS and LNRFS. Although our previously published results found no prognostic significance to PCR staging, the delayed separation in DFS observed in this study may reflect a prolonged period of disease latency or just very slow progression over time. For instance, after 5 years, DFS in the PCR+/observation, PCR+/CLND, and PCR-/observation groups was 79.4%, 83.9%, and 84.6%, respectively. By 10 years, the respective DFS had become 63.6%, 82.4%, and 79.2%. These results suggest that PCR does detect real melanoma cells in the SLN, some of which will eventually turn into palpable nodal disease.

Addition of CLND appears to improve disease control and mitigate the decrease in DFS and LNRFS seen in PCR+ patients who undergo observation. Nonetheless, not all PCR+ patients will undergo progression of disease, and it remains unclear how to best apply CLND in this population. Similar to CLND in histologically positive patients, not everyone with a PCR+ result would benefit from CLND. The development of metastases requires multiple mutations, and not every melanoma cell detected by PCR will have true metastatic potential. Furthermore, there was no difference in OS associated with CLND in our study. Additional studies are warranted to investigate the role of CLND in histologically negative, PCR+ patients.

Our study represents one of the largest to address the role of PCR ultra-staging in histologically negative SLNs, and strengths include the large number of patients and randomized control trial design. However, there are still many unknowns that limit the broad clinical applicability of PCR staging or CLND in PCR+ patients. The ideal combination of markers remains to be found, and although our combination of 4 markers demonstrated good specificity in validation studies, methods such as gene-expression profiling may ultimately provide more accurate risk assessment.[27] Additionally, even though a positive PCR predicted an increased risk of nodal recurrence and decreased DFS, there was no impact on OS. Although our findings introduce the possibility of CLND to improve regional disease control, lymphadenectomy in these patients will be subject to the same controversies and questions that surround CLND for histologically positive SLNs. Randomized trials such as the Multicenter Selective Lymphadenectomy Trial II (MSLT-II) may provide further insight into the role for CLND. Additional areas for research could also include the role of alternate adjuvant therapies for PCR+ nodes. Although the Sunbelt Melanoma Trial demonstrated no benefit to HDI, advances in both targeted therapy and immunotherapy offer a new generation of pharmacologic options in the treatment of melanoma.

CONCLUSIONS

In summary, patients with microscopically negative/PCR+ SLNs have an increased risk of nodal recurrence that was mitigated by CLND. Although CLND did not affect OS, these data suggest that molecular detection of melanoma-specific mRNA in the SLN predicts a greater risk of nodal recurrence. Additional studies are warranted to clarify the role of PCR staging and CLND in patients with histologically negative SLNs.

Author Contributions

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Analysis and interpretation of data: Kimbrough, Egger, McMasters, Stromberg, Scoggins

Drafting of manuscript: Kimbrough, Egger, McMasters, Stromberg, Scoggins

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