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Molecular Upstaging Based on Paraffin-embedded Sentinel Lymph Nodes:

Ten-Year Follow-up Confirms Prognostic Utility in Melanoma Patients

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Abstract

Objective—To determine the long-term clinical significance of molecular upstaging in histopathology-negative, paraffin-embedded (PE) sentinel lymph nodes (SLNs) from melanoma patients.

Background—Histopathologic evaluation can miss clinically relevant melanoma micrometastases in SLNs. This longitudinal correlative study is the first 10-year prognostic evaluation of a multimarker quantitative real-time reverse transcriptase–polymerase chain reaction (qRT) assay for PE melanoma-draining SLNs.

Methods—The SLN sections (n = 214) were assessed by qRT assay for 4 established messenger RNA biomarkers: *MART-1, MAGE-A3, GalNAc-T,* and *PAX3.*

Results—The qRT assay upstaged 48 of 161 histopathology-negative (hematoxylin-eosin and immunohistochemistry) SLN specimens. At a median follow-up of 11.3 years for the entire cohort, estimated rates of 10-year overall survival (OS) and melanoma-specific survival (MSS) were 82% and 94%, respectively, for histopathology-negative/qRT-negative patients; 56% and 61%, respectively, for histopathology-positive patients; and 52% and 60%, respectively, for histopathology-negative/qRT-positive patients ($P < 0.001$ for OS, $P < 0.001$ for MSS). In a multivariate analysis of known melanoma prognostic factors, qRT positivity was significant ($P < 0.05$) for disease-free survival (hazard ratio [HR], 4.3; 95% confidence interval (CI), 2.3–7.8), distant disease-free survival (HR, 6.6; 95% CI, 2.9–14.6), MSS (HR, 6.2; 95% CI, 2.6–14.4), and OS (HR, 2.8; 95% CI, 1.6–4.9).

Conclusion—The multimarker qRT assay has prognostic significance for molecular upstaging of PE melanoma-draining SLNs. Molecular upstaging of histopathology-negative SLNs confers a prognosis similar to that associated with SLN micrometastasis, and the number of positive qRT biomarkers is correlated to disease outcome.

The undisputed prognostic significance of lymph node involvement in melanoma[1,2] led to the development of sentinel lymphadenectomy (SLND), pioneered by our group.[3] This

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minimally invasive technique selectively samples the first nodes on the most likely path of lymphatic spread from a primary cutaneous melanoma.[4,5] Excised sentinel lymph nodes (SLNs) are subjected to pathologic ultrastaging, during which multiple fine sections of each SLN are examined by both hematoxylin and eosin (H&E) and melanoma-specific immunohistochemistry (IHC).[4] The SLND is generally considered the standard of care for the staging of clinically localized melanoma,[6,7] and management based on SLND can affect clinical outcome.[8]

However, current histopathologic analysis of the SLN has limitations. The IHC staining of serial sections from the SLN can detect metastases missed by standard H&E staining of bivalved sections, but it still has a 15% to 25% rate of false negatives because of sampling error.[9] Moreover, melanoma will recur in up to 30% of patients who have no histopathologic evidence of tumor in the SLN specimen.[10–12]

For the last decade, our group has been developing and improving quantitative molecular techniques to complement and supplement histopathologic assessment of the SLN. In 2004, we reported that the detection of tumor-related messenger RNA (mRNA) by quantitative real-time reverse transcriptase–polymerase chain reaction (qRT) assay of paraffin-embedded (PE) SLNs can identify clinically relevant metastases that are missed by histopathology.[13] In the present study, we report 10-year follow-up data and further analysis for the same cohort, with particular attention to patients with histopathology-negative SLNs. This is the first longitudinal study of survival based on qRT detection of PE SLN metastasis, and the first long-term data to confirm the prognostic significance of histopathology-negative, qRT-positive SLNs in patients with clinically localized melanoma.

METHODS

Patients and Tumors

As described in our initial report,[13] PE SLN tissue was obtained from consecutive patients undergoing surgery for early-stage, clinically localized melanoma at the John Wayne Cancer Institute between March 1992 and December 1999. Clinicopathologic data collected for this study included patient sex and age, American Joint Committee on Cancer stage of melanoma at presentation, and primary tumor characteristics (anatomic site, Clark level, Breslow thickness, and ulceration status). Of the 281 patients who underwent SLND for primary melanoma during the study period, 226 patients were eligible for inclusion on the basis of the availability of PE blocks, long-term follow-up, signed informed consent, and IHC analysis. The final selection of patients was performed by database management personnel, in a manner independent of the investigators and biostatisticians, as previously described.[13] Informed human subjects/institutional review board consent was received for the use of all specimens in this study.

All patients with early-stage, clinically node-negative cutaneous melanoma underwent preoperative lymphoscintigraphy to identify the draining lymph node basin. After intraoperative mapping of the SLNs with isosulfan blue dye (Lymphazurin, Hirsch Industries Inc, Richmond, Va) and radioisotope (99m technetium sulfur colloid), SLND was performed.[3–5] Complete lymph node dissection (CLND) of the regional basin was performed if SLN metastasis was detected by histopathology.[14] Patients with histopathology-negative, qRT-positive SLNs did not undergo CLND.

Patients were observed by conducting serial clinical and diagnostic examinations. Melanoma recurrences were treated with surgery and/or best available medical therapy. All patient data were recorded in the John Wayne Cancer Institute melanoma database under the control of the database coordinator.

Histopathologic Examination and RNA Isolation

After a portion of each SLN was removed for frozen-section evaluation, the remaining bisected node was formalin-fixed and was PE. For histopathologic analysis, 4-μm sections were cut from each PE block at 2 different levels separated by 40 μm, according to our standard technique in the early-SLN era. Each section was stained with H&E and antibodies to HMB-45, MART-1 and/or S-100P.[4,14]

For molecular analysis, 10 serial sections (each 10 μmin thickness) were cut from the remaining SLN.[15] Sections were cut with a new sterile microtome blade, deparaffinized, and digested with proteinase K before extraction of RNA with a modified protocol for the Paraffin Block RNA Isolation Kit (Ambion, Austin, TX), as previously described.[15] RNA was quantified by ultraviolet photospectrometry and RiboGreen assay (Invitrogen, Carlsbad, CA). Eleven patients with poor quality, undetectable RNA from PE SLNs were excluded from the study.[13]

Molecular Biomarker qRT Assay

Primer and probe sequences were designed as previously described.[16] Assays were optimized by using PE primary and metastatic melanomas, PE histopathology-negative lymph nodes, and metastatic melanoma cell lines as controls, with *GAPDH* expression as an internal reference for mRNA integrity. Polymerase chain reaction (PCR) conditions and annealing temperatures for each biomarker were assessed by receiver operating characteristic curve analysis.[13] These initial studies used an independent set of 71 PE nodal tissues from 71 patients; 39 histopathology-verified tumor-negative lymphoid tissues from patients with breast cancer and benign disease, and 32 histopathology-verified metastatic melanomas.[13] Comparisons were made of PE SLN with frozen SLN to validate equivalent qRT efficiency on both specimen types.

All assays incorporated positive controls (established melanoma lines, PE metastatic melanomas), negative controls (normal or tumor-free lymph nodes), and reagent controls (reagents without complementary DNA or RNA). Each assay was performed in triplicate for verification and the mean copy number was used for analysis. The mRNA copy number was calculated using Real-Time Detection Software.[13]

Biostatistical Analysis

The *t* test was used for continuous variables and the χ^2 test was used for categorical variables. Kappa analysis and Spearman correlation coefficient analysis were used to compare mRNA copy numbers. Overall qRT positivity was defined by at least 1 positive biomarker. Cox proportional hazard (Cox-PH) models were used for multivariate analysis. Backward stepwise model-selection procedures were used to find the optimal set of predictors for overall survival (OS), melanoma-specific survival (MSS), disease-free survival (DFS), and distant DFS (DDFS). These clinical end points were measured from the time of SLND. *Recurrence* was defined as either distant or locoregional. The cumulative survival rates for patient groups were calculated by using Kaplan-Meier methods. The subject categories based on SLN status were compared with the log-rank test. For the histopathology-negative SLN subjects, Cox-PH models were constructed for each clinical end point by using Breslow thickness and ulceration of the primary melanoma and individual and overall qRT biomarker status of the SLN. All *P* values were assessed as 2-sided and were significant at 0.05.

RESULTS

Demographics and Baseline Characteristics

The study cohort comprised 214 patients: 126 men and 88 women, with a mean age of 50.9 years.[13] Melanomas were located primarily on the extremity, followed by the trunk and then head and neck. Ulceration was present in 39 (19%) of 208 primary lesions (ulceration values were missing for 6 subjects).

The SLN specimens were histopathology positive in 53 patients and qRT positive in 47 (89%) of these 53 patients. The SLN specimens were histopathology negative in 161 patients and qRT positive in 48 (30%) of these 161 patients. Mean thickness of the primary melanoma was 2.9 mm versus 1.9 mm for patients with histopathology-positive versus histopathology-negative SLNs ($P < 0.001$) and 1.9 mm versus 2.0 mm for patients with qRT-positive versus qRT-negative SLNs (not significant). Of the 208 patients with known ulceration status, 156 had histopathology-negative SLN specimens, 25 (16%) of which were associated with ulcerated primary melanomas.

Melanoma Recurrence

During an overall median follow-up of 11.3 years, melanoma recurred in 76 (36%) patients: 28 (53%) of 53 patients with histopathology-positive SLNs and 48 (30%) of 161 patients with histopathology-negative SLNs. The median time to recurrence at any site was 72.5 months for patients with histopathology-positive SLNs, 63.3 months for patients with histopathology-negative/qRT-positive SLNs, and not available (too few events) for patients with histopathology-negative/qRT-negative SLNs; corresponding means were 73, 63, and 144 months, respectively. Of the 48 patients who had histopathology-negative SLNs and recurrence, 27 (56%) had qRT-positive SLNs. Four (8%) recurrences in the histopathology-positive group were associated with qRT-negative SLNs. The median number of positive qRT biomarkers was greater in the recurrence group (1 vs 0) (Table 1).

Recurrence was significantly associated with Breslow thickness (mean ± standard deviation of 2.3 ± 2.0 mm in the recurrence group vs 1.8 ± 1.7 mm in the no-recurrence group; $P = 0.03$) but not with Clark level. Of the 156 patients who had histopathology-negative SLNs and known ulceration data, 9 (20%) of 44 with recurrence had an ulcerated primary-melanoma, compared with 16 (14%) of 112 with no recurrence. However, ulceration was not significantly associated with DFS.

Table 2 shows the correlation between first observed site of recurrence and tumor status of the SLN specimen. Rates of locoregional and distant recurrence were similar between patients with histopathology-negative/qRT-positive SLNs and patients with histopathology-positive SLNs. In patients with histopathology-negative SLNs, the likelihood of locoregional and distant recurrence increased with the number of positive qRT biomarkers (Table 1). No individual biomarker correlated with first recurrence at a distant versus a locoregional site. Twenty-one (10%) patients with recurrence had SLNs that were negative by histopathology and qRT. Of these 21 recurrences, 13 (62%) were locoregional and 8 (38%) were distant. Two distant recurrences occurred in the brain; other first sites of distant recurrence were lung, vertebra, pancreas, adrenal gland, rectum, and an unspecified site.

Survival

Estimated 10-year OS rate was 82.3% for patients with histopathology-negative/qRT-negative SLNs, 56% for patients with histopathology-positive SLNs, and 51.9% for patients with histopathology-negative/qRT-positive SLNs (Fig. 1A). Similarly, estimated 10-year MSS rate was 93.5% for patients with histopathology-negative/qRT-negative SLNs, 60.8%

for patients with histopathology-positive SLNs, and 60.4% for patients with histopathology-negative/qRT-positive SLNs (Fig. 1B). Although OS and MSS differences across all 3 groups reached significance ($P < 0.001$), differences between histopathology-positive and histopathology-negative/qRT-positive groups were not significant. As the number of positive qRT biomarkers increased, OS and MSS decreased ($P < 0.001$) (Figs. 1C, D).

The same trends were found in recurrence patterns. Estimated 10-year DFS rate was 83.6% for patients with histopathology-negative/qRT-negative SLNs, 48.1% for patients with histopathology-positive SLNs, and 44% for patients with histopathology-negative/qRT-positive SLNs (Fig. 2A). Similarly, estimated 10-year DDFS rate was 93.6% for patients with histopathology-negative/qRT-negative SLNs, 67.2% for patients with histopathology-positive SLNs, and 56.5% for patients with histopathology-negative/qRT-positive SLNs (Fig. 2B). Although DFS and DDFS differences across all 3 groups reached significance ($P < 0.001$), differences between histopathology-positive and histopathology-negative/qRT-positive groups were not significant. As the number of positive qRT biomarkers increased, DFS and DDFS decreased ($P < 0.001$) (Figs. 2C, D).

Multivariate Analysis

A Cox-PH model was used to evaluate the correlation of standard prognostic factors, rate of recurrence, and OS in patients with SLNs positive for 1 or more qRT biomarkers. Hazard ratio was calculated for each individual biomarker and for overall qRT positivity. As shown by the hazard ratios in Table 3, qRT positivity was a strong prognostic factor for DFS ($P < 0.05$), DDFS ($P < 0.05$), MSS ($P < 0.05$), and OS ($P < 0.05$). Among individual biomarkers, *GalNAc-T* positivity had the strongest correlation with DFS ($P < 0.05$) and *MAGE-A3* positivity had the strongest correlation with DDFS ($P < 0.05$). *MART-1* positivity had the strongest correlation with OS ($P < 0.001$) and MSS ($P < 0.001$).

DISCUSSION

In a recently reported collaborative blinded study with the Sydney Cancer Centre, we confirmed that qRT assay could identify occult metastasis in SLNs that were negative by initial histopathology: qRT assessment of 74 archived PE SLNs from 33 patients upstaged melanoma in 4 patients (12%).[10] That study also found that although both conventional histopathologic assessment and qRT assay depend on accurate surgical and nuclear medicine techniques, qRT assessment of SLN sections has greater sensitivity. But do qRT-detected tumor cells have clinical relevance?

In this long-term follow-up study, we demonstrate that patients with qRT-positive SLNs had significantly worse OS, MSS, DFS, and DDFS than patients with histopathology-negative/qRT-negative SLNs. Since our 2004 report of this cohort, the OS and DDFS curves for qRT-positive and histopathology-positive patients have separated: by about 7 years, qRT-positive patients had a worse OS; by about 8 years, they also had a worse DDFS. Interestingly, the pattern of recurrence associated with histopathology-negative/qRT-positive SLNs was comparable with the pattern of recurrence that we previously reported for patients with a falsely negative SLN.[17]

Although our findings suggest that molecular detection of melanoma metastasis in the SLN predicts an increased risk of distant as well as locoregional recurrence, they must be qualified by the reminder that all patients with histopathology-positive SLNs underwent immediate CLND, whereas patients with histopathology-negative/qRT-positive SLNs received treatment only for recurrence. Prospective analysis of the prognostic significance of qRT-positive SLNs is underway in the second Multicenter Selective Lymphadenectomy Trial. This randomized international phase III trial is the first study to stratify patients for

treatment (CLND or nodal ultrasono-graphic observation) on the basis of the molecular status of the SLN specimen. Results not only will determine whether CLND is necessary in all patients with SLN involvement, but also will establish the clinical utility of nodal upstaging on the basis of detection of molecular biomarkers.

The qRT biomarker panel that was used in this study is currently being applied in second Multicenter Selective Lymphadenectomy Trial. The panel comprises comprised biomarkers with a high level of clinical utility for disease outcome in melanoma patients. This selection criterion eliminated tyrosinase and gp100, because these 2 well-known proteins of the melanogenesis pathway[14,18–20] have unacceptably high false-positive rates in the regional lymph nodes.[21–23] Of the 4 biomarkers used in this study, *MART1* is a melanoma-associated antigen commonly expressed by melanomas but absent in nonmelanoma malignancies and normal non-cancer lymph nodes[13,20,24] Likewise, *MAGE-A3* is a melanoma-associated antigen not produced in normal tissues except male germline cells and placenta.[25] *GalNAc-T, PAX3,* and *MAGE-A3* are not detected in nevi or differentiated melanocytes. Importantly, these biomarkers are from nonoverlapping malignant pathways.[13,18,23,26–28] For example, *GalNAc-T,* a key enzyme for biosynthesis of tumor-associated gangliosides GM2 and GD2,[29,30] is a biomarker for aggressive melanoma progression, as shown previously by our group.[31] *PAX3* a transcription factor involved in melanoma proliferation and is resistance to apoptosis, migration, melanogenesis regulation, and differentiation.[32,33] *PAX3* is activated during fetal-tissue development; it is suggested to block terminal differentiation and thereby maintain the stem cell function of melanoblasts.[34,35] *PAX3* expression was a significant hazard for disease recurrence in this study (Table 1).

Several large studies have found no link between molecular biomarkers and prognosis in patients with clinically localized melanoma.[24,25,36] The Sunbelt Melanoma Trial, a multicenter prospective randomized study of melanoma staging and adjuvant therapy, reported no difference in DFS, DDFS, or OS between reverse transcriptase-PCR (RT-PCR)–positive and -negative patients followed up for a median of 30 months.[37] However, this trial included *TYR* as a weighted molecular biomarker for positivity; also, it used a nonquantitative gel-based assay, and it did not standardize SLN pathology sampling. Both histopathology- and RT-PCR–positive patients were randomized to different adjuvant treatment arms with α-interferon, which would have diluted differences in outcomes. In an unusual result, Hilari et al[21] recently reported low specificity for any melanoma biomarker (*TYR, MART1, SSX2, MAGE-A3, PAX3, GalNAc-T, PLAB,* and *L1CAM*) and no association with disease recurrence. However, significant low recurrence rates and a high percentage of thin melanomas in that study would have biased its interpretation. Several of the biomarkers used have been shown to be of clinical utility by our group and other groups.[21]

A recent meta-analysis review by Mocellin et al[23] examined 22 large studies of PCR upstaging of the SLN. Three of the studies used quantitative RT-PCR assays; most of the remaining studies used standard or nested gel-based RT-PCR. These traditional RT-PCR assays have limited sensitivity. Only 12 studies included at least 2 biomarkers in the RT-PCR analysis, and 11 of the 12 studies used tyrosinase. The heterogeneity of technique complicated the analysis but did not prevent some useful conclusions about RT-PCR as a prognostic factor. Overall, the RT-PCR positivity rate of 51.1% was significantly higher than the histopathology positivity rate of 20.3%, a finding that is in agreement with the data presented in our current report. The RT-PCR was positive in 42.3% of histopathology-negative SLNs, and the false-negative rate for RT-PCR was only 4.9%. The disease-recurrence rate was significantly higher when RT-PCR results were positive versus negative (16.8% vs 8.7%). A negative predictive value of 91.3% indicates a useful prognostic factor. These data suggest that molecular staging of the SLN yields valuable, informative prognostic findings despite variability in molecular assays used.

The data presented in this report are limited by the era in which the PE specimens were generated (1992–1999), as would be the case for any study of long-term outcomes based on retrospective analysis of specimens. Since the introduction of SLND for melanoma in 1992,[3] histologic evaluation of the SLN has become more efficient and more detailed in some centers.[4] Use of the current standard of SLN ultra-staging may have increased the yield of histopathology-positive SLNs in our study, decreasing the number of histopathology-negative/qRT-positive SLNs and influencing the survival curves. However, it would not have decreased the number of qRT-positive SLNs and likely would not alter the results of the multivariate analysis. As recently reported by our group, the multiple disciplines involved in SLN detection and staging increase the potential for variations and errors but do not diminish the ability of molecular staging to detect occult micrometastases missed by conventional techniques.[10]

Another consideration is the evolution of melanoma staging, which has become more accurate and sophisticated in recent years. The presence of ulceration may predict a higher likelihood of recurrence even when the SLN is histologically negative. Thus, our study's inclusion of ulcerated lesions may account for its high rate of recurrence; the 30% recurrence rate is higher than expected at 10 years of follow-up. However, the 19% ulceration rate is in line with previously reported rates of ulceration for primary-tumor depths of 1 to 2 mm.[38] Again, changes in state-of-the-art diagnosis and treatment are inevitable limitations faced by any longitudinal prognostic biomarker study.

Our study of molecular upstaging for histopathology-negative SLNs from American Joint Committee on Cancer stage I/II patients has the longest follow-up for molecular staging of regional lymph nodes in any solid cancer. The study is also the first to demonstrate the significant prognostic utility of molecular upstaging on the basis of PE tissues. The data confirm a prognostic role for multimarker qRT assessment of SLN PE tissue from patients with clinically localized melanoma and support molecular analysis as part of the comprehensive prognostic assessment.

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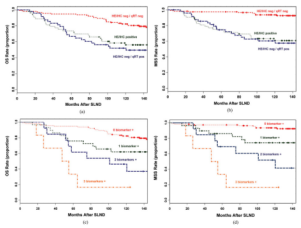

FIGURE 1.

(A) Overall survival for all patients according to the histopathologic (H&E/IHC) and molecular (qRT) status of the SLN. (B) Melanoma-specific survival for all patients. (C) Overall survival for patients with histopathology-negative SLNs stratified by the number of positive qRT biomarkers. (D) Melanoma-specific survival for patients with histopathology-negative SLNs stratified by the number of positive qRT biomarkers.

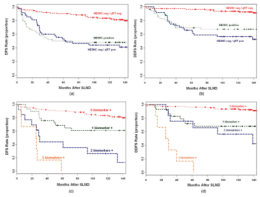

FIGURE 2.

(A) Disease-free survival for all patients according to the histopathologic (H&E/IHC) and molecular (qRT) status of the SLN. (B) Distant disease-free survival for all patients. (C) Disease-free survival for patients with histopathology-negative SLNs stratified by the number of positive qRT biomarkers. (D) Distant disease-free survival for patients with histopathology-negative SLNs stratified by the number of positive qRT biomarkers.

TABLE 1

Correlation of Risk Factors With Recurrence Status in Patients With Histopathology-negative SLN*

	No Recurrence (n = 113)	Recurrence (n = 48)	
Risk Factors	**Number of Patients (%)**	**Number of Patients (%)**	***P***
Sex			
Male	67 (59)	29 (60)	0.89
Female	46 (41)	19 (40)	
Primary site			
Head/neck	14 (12)	12 (25)	0.02
Trunk	53 (47)	12 (25)	
Extremity	46 (41)	24 (59)	
Breslow (mm)			
≤ 1.00	41 (38)	13 (23)	0.03
1.01–2.00	50 (39)	15 (34)	
2.01–4.00	12 (10)	14 (29)	
>4.00	9 (8)	4 (9)	
Clark level			
I	1 (1)	1 (2)	0.62
II	7 (6)	4 (9)	
III	50 (44)	14 (26)	
IV	49 (43)	23 (52)	
V	6 (5)	2 (5)	
Unknown	0 (0)	4 (9)	
Ulceration positive	16 (14)	9 (20)	0.48
MART-1 positive	3 (3)	7 (15)	0.004
GalNAc-T positive	7 (6)	20 (42)	<0.001
PAX3 positive	13 (12)	15 (31)	0.002
MAGE-A3 positive	1 (1)	7 (15)	<0.001
No. positive biomarkers			
0	92 (81)	21 (44)	<0.001
1	18 (16)	11 (23)	
2	3 (3)	10 (21)	
3	0 (0)	6 (12)	
4	0 (0)	0 (0)	

* n=161 patients.

TABLE 2

Correlation of First Recurrence With qRT Biomarker Status

Tumor Status of SLN	No. Recurrences/Patients	No. Locoregional Recurrences/ Patients (Stage III at Recurrence)	No. Distant Recurrences/ Patients (Stage IV at Recurrence)
Histopathology positive/qRT positive	23/47	11/47	12/47
1 biomarker	6/9	1/9	5/9
2 biomarkers	3/10	3/10	0/10
3 biomarkers	5/8*	2/8	2/8
4 biomarkers	10/20	5/20	5/20
Histopathology positive/qRT negative	4/6	1/6	3/6
Histopathology negative/qRT positive	27/48	12/48	15/48
1 biomarker	11/29	3/29	8/29
2 biomarkers	10/13	5/13	5/13
3 biomarkers	6/6	4/6	2/6
4 biomarkers	0	0	0
Histopathology negative/qRT negative	21/113	13/113	8/113

* In the histopathology-positive/qRT-positive group, 1 patient with 3 positive molecular biomarkers did not have a designated site/stage at first recurrence.

TABLE 3

Cox Proportional Hazard Models for Survival in Patients with Histopathology-negative, qRT-positive SLN

	HR (95% CI)			
	Disease-free Survival	**Distant Disease-free Survival**	**Melanoma-specific Survival**	**Overall Survival**
Modeling based on positivity of individual qRT biomarkers				
MART-1	3.6 (1.4–8.8)*	4.1 (1.4–12.1)*	4.8 (1.7–13.5)*	4.4 (1.7–11.2)*
GalNAc-T	3.7 (1.8–7.4)*	3.4 (1.4–8.1)*	4.7 (2.1–10.9)*	3.0 (1.6–5.8)*
PAX3	1.8 (0.92–3.5)	2.0 (0.9–4.4)	1.4 (0.6–3.6)	0.8 (0.4–1.8)
MAGE-A3	2.8 (1.1–6.8)*	6.1 (2.1–17.6)*	2.3 (0.8–6.8)	2.1 (0.8–5.4)
Breslow depth	1.2 (1.0–1.3)*	1.3 (1.1–1.5)*	1.2 (0.99–1.5)	1.2 (1.0–1.4)*
Ulceration	1.2 (0.5–2.4)	2.0 (0.9–4.6)	3.4 (0.96–5.8)	2.2 (1.1–4.4)*
Modeling based on positivity of combined qRT biomarkers				
Quantitative PCR positive/negative	4.3 (2.3–7.8)*	6.6 (2.9–14.6) *	6.2 (2.6–14.4)*	2.8 (1.6–4.9)*
Breslow depth	1.1 (0.98–1.3)	1.2 (1.1–1.4)*	1.2 (0.96–1.4)	1.2 (1.0–1.3)*
Ulceration	1.5 (0.7–3.1)	3.0 (1.3–6.7)*	3.3 (1.4–7.6)*	2.8 (1.5–5.3)*

*$P < 0.05$.