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$\beta 3$ integrin immunohistochemistry as a method to predict sentinel lymph node status in patients with primary cutaneous melanoma

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Abstract

Background—Integrins are heterodimeric proteins composed of noncovalently linked α and β subunits which are essential for a wide range of normal physiology and also play prominent roles in cancer. Here we tested whether integrin expression in diagnostic skin biopsies is associated with sentinel lymph node (SLN) metastasis.

Methods—We utilized a cohort of 854 consecutive patients with primary cutaneous melanoma to quantify the expression of β integrin subunits by reverse transcriptase quantitative PCR (RT-qPCR). In addition, we quantified the expression of $\beta 3$ integrin by immunohistochemistry (IHC) in a subset of 271 patients by H score. Outcome of interest was SLN biopsy metastasis within 90 days of melanoma diagnosis. Logistic regression analyses were used to develop models for the likelihood of SLN metastasis from molecular, clinical, and histologic variables.

Results— $\beta 3$ integrin expression quantified by IHC or RT-qPCR was associated with SLN metastasis. $\beta 1$, $\beta 5$, $\beta 6$, and $\beta 8$ integrin expression was not associated with SLN metastasis. The incremental gain in performance of a predictive model which included $\beta 3$ integrin expression as quantified by IHC in combination with established clinicopathologic variables (Breslow depth and patient age) was limited.

Conclusions— $\beta 3$ integrin is the principal integrin subunit associated with sentinel lymph node biopsy (SLNb) metastasis in primary cutaneous melanoma. However, $\beta 3$ integrin H score does not significantly improve models for the likelihood of SLN metastasis over Breslow depth and patient age.

Introduction

Established clinicopathologic variables such as Breslow depth or patient age are limited in their ability to identify primary cutaneous melanoma patients at risk of metastasis.¹ We and others have observed that the expression of certain tumor molecular biomarkers is associated

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Conflict of interest: Dr. Meves reports a financial interest in a molecular test being developed by SkylineDx in the area of melanoma. Mayo Clinic has reviewed this relationship and taken appropriate steps to protect the scientific integrity of the research.

with tumor cell metastasis.^{2–4} Specifically, we have shown that the expression of integrin cell adhesion receptors is associated with the nodal metastasis of melanoma and may be quantified to identify patients in need of a sentinel lymph node biopsy (SLNb).⁵ Integrins are heterodimeric proteins composed of noncovalently linked α and β subunits which are essential for a wide range of normal physiology and also play prominent roles in cancer.⁶ The mammalian genome encodes for 18 α and 8 β integrin subunits which combine to form 24 unique α/β heterodimers.⁷ When integrins bind to components of the extracellular matrix such as fibronectin, their cytoplasmic tail recruits and locally enriches a myriad of cell surface receptors, scaffolding proteins, and enzymes which stimulate fibrinolysis, angiogenesis, and tumor immune evasion.^{6,8,9} Here we assembled a large cohort of patients diagnosed with primary cutaneous melanoma to identify integrin subunits associated with nodal metastasis. We then tested whether immunohistochemistry (IHC) can be used to quantify integrin expression in biopsy tissue and identify patients at risk of nodal metastasis.

Materials and Methods

Patient cohort

Our cohort consisted of 854 melanoma patients who had an SLNb performed within 90 days of their diagnosis, that is, a time interval shown to not affect SLNb positivity rates.¹⁰ Patients with primary cutaneous melanoma who presented at Mayo Clinic tertiary care centers in Minnesota, Arizona, or Florida between 2004 and 2018 with known SLNb status were retrospectively identified by electronic searches of pathology reports. Seven hundred and fifty-four of the 854 patients in this cohort were included in a previously published cohort specifically for their SLNb status outcome.⁵ All specimens were analyzed by reverse transcription quantitative PCR (RT-qPCR) between February 2018 and October 2018. A subset of 271 patients with sufficient tissue for research was tested by RT-qPCR and IHC. Clinicopathologic features of this subset were comparable to the 854 patient cohort (Table 1). Not all tissues could be tested by IHC because tissue available for research is limited. Primary melanoma tissue blocks are often derived from shave or punch biopsies¹¹ which are easily exhausted. In addition, extra turnaround time sometimes prevented us from running the IHC assay.

Outcome of interest was SLNb status. Because of the standardized nature of the SLNb procedure, SLNb status is a very well-defined endpoint. SLNb status also is a highly validated marker of risk of disease progression.¹ Eligibility was determined based on histopathology data derived from patient medical records and established by two or more board certified Mayo Clinic dermatopathologists. Inclusion was determined by the AJCC 7th edition-based institutional practice guidelines of the Mayo Clinic for recommending SLNb, which are based on Breslow thickness, ulceration, mitoses, and age. Patients were eligible for this study if they met one of the following three conditions: Breslow thickness of greater than or equal to 1.0 mm; Breslow thickness of 0.75–0.99 mm and the presence of ulceration, mitoses, and/or age less than 40 years; or Breslow thickness of 0.50–0.74 mm and the presence of at least two of the following: ulceration, mitoses, and age less than 40 years.

Exclusion criteria were: M1 disease within 90 days of primary diagnosis; insufficient primary tumor diagnostic biopsy tissue; inadequate RNA harvested; and, for Minnesota,

denial of access to medical records for research purposes (per Minnesota State law). The human investigations performed in this study were completed after approval by the Mayo Clinic Institutional Review Board and in accordance with the requirements of the Department of Health and Human Services, where appropriate.

Gene expression by quantitative PCR

Quantitative PCR was performed as previously described.⁵ RNA purification was from FFPE tissue (Qiagen, Hilden, Germany). Quantitative RT-PCR was done using the BioMark HD System and dynamic array integrated fluid circuits (Fluidigm, South San Francisco, CA, USA). All cDNA was preamplified (TaqMan Preamp Master Mix, Applied Biosystems, Beverly, MA, USA). Array-based quantitative PCR was with the help of the TaqMan Gene Expression Master Mix (Applied Biosystems). After thermal cycling, raw cycle threshold (Ct) data were checked for linear amplification. Gene expression was corrected by the mean of housekeeping genes (RLP0, RLP8, and β -actin) using the Ct method.

Immunohistochemistry (IHC)

β 3 integrin was detected using the BOND ready-to-use primary antibody CD61 (clone 2f2) from Leica Biosystems (PA0308) using the BOND-MAX autostainer platform in combination with a BOND Polymer Refine Red Detection system (DS9390). Heat-induced antigen retrieval was by BOND EDTA-based pH 9.0 epitope retrieval solution 2 (AR9640) applied for 20 minutes. All stains were digitized with a Leica Aperio ScanScope system. Tumor images were analyzed by the Aperio membrane v9 algorithm and quantified based on the histo or H score as previously described.¹²

Statistical methods

Statistical analysis was performed using the SAS version 9.4 software package (SAS Institute, Inc.; Cary, NC, USA) or the XLSTAT version 2020.1.3 software package (Addinsoft, Inc; New York, NY, USA). Data were summarized using standard descriptive statistics. Gene expression levels of molecular markers by qPCR analysis were compared between the SLNb negative and positive groups using the two-sample t-test. The association of each molecular marker with SLNb positivity was evaluated in a separate multivariable logistic regression model adjusted for patient age and Breslow depth. The overall predictive ability of each model was summarized by the area under curve (AUC). AUC estimates were compared between models using the DeLong, DeLong, and Clarke-Pearson nonparametric method for comparing the AUC for correlated ROC curves. Comparisons between the patients included versus not included in the IHC analysis were evaluated using the two-sample t-test for age and the chi-square test for all categorical variables. The Cochran–Armitage test for trend was used to evaluate the relationship between protein expression by IHC and SLNb positivity. All calculated *P* values were two-sided, and *P* values less than 0.05 were considered statistically significant.

Results

We utilized a cohort of 854 patients with primary cutaneous melanoma who underwent SLNb within 90 days of diagnosis at Mayo Clinic between 2014 and 2018. Among the 854

patients, 203 (23.8%) had a positive SLNb (Table 1). Clinicopathologic features were abstracted and gene expression quantified by RT-qPCR in diagnostic biopsy tissue. Diagnostic biopsy tissue was formalin-fixed and paraffin-embedded primary cutaneous melanoma tissue that was directly obtained from patients as part of patient care during skin checks.

To identify integrin subunits associated with nodal metastasis in primary cutaneous melanoma, we determined the expression of all β integrin subunits ($\beta 1$, $\beta 3$, $\beta 5$, $\beta 6$, $\beta 8$ integrin) expressed outside of leukocytes ($\beta 2$ and $\beta 7$ integrin) and epithelial cells ($\beta 4$ integrin).⁷ We also determined the expression of $\alpha 5$ integrin, which interacts with $\beta 1$ integrin to form the $\alpha 5\beta 1$ fibronectin receptor expressed in melanoma but not in precursor lesions.¹³ Based on univariate analysis, we found that only $\beta 3$ integrin was associated with positive SLNb whereas no such association was observed for $\alpha 5$, $\beta 1$, $\beta 5$, $\beta 6$, and $\beta 8$ integrin (Table 2). $\beta 3$ integrin was still significantly associated with SLNb metastasis in a multivariable logistic regression model adjusted for Breslow depth and patient age (Table 2).

We next determined whether $\beta 3$ integrin could be quantified by IHC. In contrast to RT-qPCR, IHC is easily performed using automated protocols. We utilized a subset of 271 patients from the 854 patient cohort with sufficient tissue available for research to evaluate $\beta 3$ integrin expression by IHC. Tumor characteristics of the 271 patients were comparable to the 583 patients not included (Table 1). Sixty-three (23.2%) of the 271 patients had a positive SLNb (Table 3). Stained slides were digitized and scored by a computer algorithm (Fig. 1). As expected $\beta 3$ integrin expression as quantified by IHC or RT-qPCR was correlated, that is, tissues with higher IHC scores meaning higher protein expression on average also demonstrated lower Ct values meaning higher gene expression (Fig. 2). Univariate analysis showed that $\beta 3$ integrin expression by IHC was associated with a positive SLNb in the 271 patient cohort as was $\beta 3$ integrin expression by RT-qPCR and Breslow depth, the latter two variables serving as positive controls (Table 4).

To assess the incremental gain provided by the $\beta 3$ integrin IHC score in predicting SLNb positivity, we assessed the area under the curve of a receiver operating characteristic. When comparing a model that considered the two clinicopathologic risk factors Breslow depth and age alone versus with $\beta 3$ integrin IHC score differences were very small; clinical risk factor model: AUC, 0.77, 95% CI 0.71–0.83; versus clinical risk factors + $\beta 3$ integrin IHC score: AUC, 0.78; 95% CI 0.72–0.84; $P = 0.5$ (Fig. 3).

Discussion

Our data suggest that $\beta 3$ integrin, which interacts with αv integrin to form the $\alpha v\beta 3$ integrin fibronectin receptor,⁷ is the principal integrin associated with SLNb metastasis. Previous research has shown that $\beta 3$ integrin plays a key role in cancer cell metastasis¹⁴ and is useful for melanoma risk stratification.^{4,5,15} Here, we tested whether quantification of $\beta 3$ integrin expression by IHC in primary diagnostic biopsy tissue is useful for predicting SLNb positivity within 90 days of diagnosis. We found that a $\beta 3$ integrin IHC score was associated with nodal metastasis in univariate and multivariate models. However, when the IHC score was combined with the well-known predictive variables of Breslow depth and patient age,

the incremental performance of our predictive model was limited and unlikely to result in practical significance. Analysis of digitized $\beta 3$ integrin IHC slides relied on the well-established H score concept¹² and was automated by a commercially available, reliable, and widely used software package. More contemporary and advanced machine learning approaches might have generated better results.^{16–18}

Aggressive melanoma easily metastasizes, including to SLN, and positive SLNb identifies patients in need of adjuvant therapy. However, most melanoma does not metastasize to SLN, and the removal of negative SLN has no discernible therapeutic effect.¹ There is a need for improved melanoma risk stratification methods to, for example, identify patients who are so low risk that they can safely forgo SLNb.^{4,5} IHC has been tried extensively to quantify molecular biomarkers in an effort to improve melanoma risk stratification.¹⁹ Single color nonmultiplexed IHC as used in this study is relatively easy to perform. Unfortunately, IHC is highly labor intensive, antibody dependent, and difficult to standardize and quantify. In contrast RT-qPCR is highly sensitive, specific, easily multiplexed, and amendable to high-throughput workflows. It is therefore not surprising that the recently introduced molecular-based melanoma risk stratification tools are based on RT-qPCR.^{5,20,21} For example, $\beta 3$ integrin is one of eight biomarkers used for melanoma risk stratification in a commercially available gene expression profile which identifies patients who are so low risk that they may safely forgo SLN biopsy.^{5,22}

In conclusion, $\beta 3$ integrin is the principal integrin subunit associated with SLNb metastasis in primary melanoma. However, determining $\beta 3$ integrin expression by H score does not significantly improve models for the likelihood of SLN metastasis over Breslow depth and patient age.

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Primary Diagnostic Melanoma Biopsy

Figure 1. Semiquantitative scoring of digitized $\beta 3$ integrin IHC slides. Diagnostic biopsy tissue from a prospectively designed archival cohort was stained for $\beta 3$ integrin (CD61) using monoclonal antibody 2f2 and a red chromogen detection kit. Glass slides were then digitized. H score was calculated by a computer algorithm

Figure 2.

Boxplot depicting the distribution of RT-qPCR data for $\beta 3$ integrin expression for each of the $\beta 3$ integrin IHC categories. RT-qPCR data (y -axis) are shown as cycle threshold (Ct) values. Categorical IHC data (x -axis) is based on a histo (H) score which was calculated by a computer algorithm

Figure 3. Receiver operative characteristic (ROC) curves for discriminating between SLNb positive and negative patients. ITGB3, $\beta 3$ integrin; H Score, histo score

Table 1

Patient and tumor characteristics of cohorts used for RT-qPCR and IHC analysis

	Included in ITGB3 IHC analysis		RT-qPCR cohort (N = 854)	P value ^a
	No (N = 583)	Yes (N = 271)		
Male Gender, n (%)	367 (63.0%)	168 (62.0%)	535 (62.6%)	0.91
Age at primary biopsy (years), Mean (SD)	59.4 (16.3)	59.5 (16.8)	59.5 (16.5)	0.96
Clark level, n (%)	% of 576	% of 268	% of 844	0.05
II	6 (1.0%)	1 (0.4%)	7 (0.8%)	
III	121 (21.0%)	48 (17.9%)	169 (20.0%)	
IV	423 (73.4%)	215 (80.2%)	638 (75.6%)	
V	26 (4.5%)	4 (1.5%)	30 (3.6%)	
Breslow depth (mm)				
0.19–1	140 (24.0%)	56 (20.7%)	196 (23.0%)	0.63
1.01–2	271 (46.5%)	135 (49.8%)	406 (47.5%)	
2.01–4	150 (25.7%)	72 (26.6%)	222 (26.0%)	
>4	22 (3.8%)	8 (3.0%)	30 (3.5%)	
Mitotic rate, n (%)	% of 578	% of 270	% of 848	0.03
Absent	80 (13.8%)	21 (7.8%)	101 (11.9%)	
1–6	395 (68.3%)	190 (70.4%)	585 (69.0%)	
>6	103 (17.8%)	59 (21.9%)	162 (19.1%)	
Histologic type, n (%)	103 (17.8%)	59 (21.9%)	162 (19.1%)	–
Superficial spreading	308 (52.8%)	144 (53.1%)	452 (52.9%)	
Nodular	114 (19.6%)	59 (21.8%)	173 (20.3%)	
Desmoplastic	15 (2.6%)	7 (2.6%)	22 (2.6%)	
Lentigo maligna	28 (4.8%)	7 (2.6%)	35 (4.1%)	
Acral lentiginous	14 (2.4%)	6 (2.2%)	20 (2.3%)	
Spindled	9 (1.5%)	7 (2.6%)	16 (1.9%)	
Dermal	3 (0.5%)	0 (0.0%)	3 (0.4%)	
Spitzoid	2 (0.3%)	4 (1.5%)	6 (0.7%)	
Nevoid	7 (1.2%)	5 (1.8%)	12 (1.4%)	
Unclassifiable	46 (7.9%)	15 (5.5%)	61 (7.1%)	

	Included in ITGB3 IHC analysis		RT-qPCR cohort (N = 854)	P value ^a
	No (N = 583)	Yes (N = 271)		
Other	5 (0.9%)	0 (0.0%)	5 (0.6%)	0.47
Mixed	7 (1.2%)	3 (1.1%)	10 (1.2%)	
Not documented	25 (4.3%)	14 (5.2%)	39 (4.6%)	
Biopsy location, n (%)				
Head/neck	120 (20.6%)	46 (17.0%)	166 (19.4%)	0.22
Trunk	204 (35.0%)	89 (32.8%)	293 (34.3%)	
Upper extremities	129 (22.1%)	63 (23.2%)	192 (22.5%)	
Lower extremities	81 (13.9%)	48 (17.7%)	129 (15.1%)	
Acral	49 (8.4%)	25 (9.2%)	74 (8.7%)	0.07
Ulceration, n (%)	% of 577	% of 271	% of 848	
Absent	451 (78.2%)	201 (74.2%)	652 (76.9%)	
Present	126 (21.8%)	70 (25.8%)	196 (23.1%)	
Regression, n (%)	% of 471	% of 243	% of 714	0.95
Absent	427 (90.6%)	230 (94.7%)	657 (92.0%)	
Present	44 (9.3%)	13 (5.4%)	57 (8.0%)	
Tumor infiltrating lymphocytes, n (%)	% of 496	% of 254	% of 750	
Absent	121 (24.4%)	65 (25.6%)	186 (24.8%)	0.63
Nonbrisk	287 (57.9%)	144 (56.7%)	431 (57.5%)	
Brisk	88 (17.7%)	45 (17.7%)	133 (17.7%)	
Angiolymphatic invasion, n (%)	% of 481	% of 243	% of 724	
Absent	448 (93.1%)	229 (94.2%)	677 (93.5%)	1.00
Present	33 (6.9%)	14 (5.8%)	47 (6.5%)	
Microsatellitosis, n (%)	% of 444	% of 238	% of 682	
Absent	440 (99.1%)	236 (99.2%)	676 (99.1%)	
Present	4 (0.9%)	2 (0.8%)	6 (0.9%)	0.87
Positive SLN biopsy, n (%)	140 (24.0%)	63 (23.2%)	203 (23.8%)	

^aComparisons between patients included versus not included in the $\beta 3$ integrin IHC analysis were evaluated using the two-sample *t*-test for age and the chi-square test for all categorical variables.

Table 2

Ct Gene expression levels stratified by sentinel lymph node biopsy outcome

Molecular markers: Protein (gene name)	SLNb negative (N = 651)	SLNb positive (N = 203)	P value unadjusted analysis ^a	P value adjusted analysis ^b
α5 integrin (ITGA5)				
Mean (SD)	7.1 (0.9)	7.0 (1.1)	0.30	0.59
Median (IQR)	7.0 (6.6, 7.6)	7.1 (6.4, 7.6)		
Range	7.3 (4.0-11.8)	8.9 (4.0-13.0)		
β1 integrin (ITGB1)				
Mean (SD)	3.6 (0.7)	3.5 (0.7)	0.37	0.54
Median (IQR)	3.6 (3.2, 4.0)	3.6 (3.1, 4.0)		
Range	5.3 (1.2-6.4)	3.8 (1.6-5.4)		
β3 integrin (ITGB3)				
Mean (SD)	6.9 (1.4)	5.9 (1.7)	<0.001	<0.001
Median (IQR)	7.0 (6.1, 7.8)	5.9 (4.7, 7.1)		
Range	7.7 (2.5-10.1)	9.1 (1.1-10.2)		
β5 integrin (ITGB5)				
Mean (SD)	3.9 (0.6)	3.9 (0.6)	0.83	0.33
Median (IQR)	3.9 (3.5, 4.3)	3.9 (3.5, 4.3)		
Range	4.6 (1.8-6.4)	3.9 (2.1, 6.1)		
β6 integrin (ITGB6)				
Mean (SD)	8.3 (1.3)	8.2 (1.5)	0.34	0.19
Median (IQR)	8.2 (7.5, 9.0)	8.2 (7.3, 9.2)		
Range	13.8 (2.5-16.3)	11.8 (0.6, 12.3)		
β8 integrin (ITGB8)				
Mean (SD)	6.3 (1.2)	6.2 (1.4)	0.29	0.99
Median (IQR)	6.3 (5.8, 6.9)	6.3 (5.4, 7.0)		
Range	9.3 (1.3-10.6)	8.3 (1.8-10.1)		

^aComparisons between the SLNb negative and positive groups were evaluated using the two-sample t-test.

^bThe association of each molecular marker with SLNb positivity was evaluated in a separate multivariable logistic regression model adjusted for patient age and Breslow depth.

Table 3
Characteristics of the IHC patient cohort stratified by sentinel lymph node biopsy outcome

	SLNb negative (N = 208)	SLNb positive (N = 63)	P value ^a
Male gender, n (%)	126/208 (60.6%)	42/63 (66.7%)	0.38
Age at primary biopsy (years), Mean (SD)	61.2 (16.0)	53.8 (18.1)	0.002
Biopsy location, n (%)			
Head and neck	37/208 (17.8%)	9/63 (14.3%)	0.13
Trunk	63/208 (30.3%)	26/63 (41.3%)	
Upper extremity	55/208 (26.4%)	8/63 (12.7%)	
Lower extremity	36/208 (17.3%)	12/63 (19.1%)	
Acral	17/208 (8.2%)	8/63 (12.7%)	
Breslow thickness (mm), n (%)			
0.50–1	54/208 (26.0%)	2/63 (3.2%)	<0.001
1.01–2	108/208 (51.9%)	27/63 (42.9%)	
2.01–4	42/208 (20.2%)	30/63 (47.6%)	
>4	4/208 (1.9%)	4/63 (6.3%)	
Clark level, n (%)			
II	1/206 (0.5%)	0/62 (0.0%)	0.009
III	45/206 (21.8%)	3/62 (4.8%)	
IV	158/206 (76.7%)	57/62 (90.5%)	
V	2/206 (1.0%)	2/62 (3.2%)	
Mitotic rate type, n (%)			
Absent	19/208 (9.1%)	2/63 (3.2%)	<0.001
1–6	156/208 (75.0%)	34/63 (54.0%)	
>6	33/208 (15.9%)	27/63 (42.9%)	
Ulceration, n (%)	46/208 (22.1%)	24 (38.1%)	<0.001
Angiolymphatic invasion, n (%)	3/188 (1.6%)	11/55 (20.0%)	<0.001
Regression, n (%)	10/187 (5.3%)	3/56 (5.4%)	1.00
Tumor invading lymphocytes, n (%)			
Absent	54/198 (25.5%)	11/56 (19.6%)	0.29
Nonbrisk	107/198(54.9%)	37/56 (66.1%)	

	SLNb negative (N = 208)	SLNb positive (N = 63)	P value ^a
Brisk	37/198 (19.6%)	8/56 (14.3%)	
Microsatellitosis, n (%)	0/182 (0.0%)	2/56 (3.6%)	0.06
Histologic type, n (%)			
Superficial spreading	109/208 (52.4%)	35/63 (55.6%)	–
Nodular	44/208 (21.2%)	15/63 (23.8%)	
Desmoplastic	7/208 (3.4%)	0/63 (0.0%)	
Lentigo maligna	7/208 (3.4%)	0/63 (0.0%)	
Acral lentiginous	2/208 (1.0%)	4/63 (6.3%)	
Spindled	7/208 (3.4%)	0/63 (0.0%)	
Spitzoid	3/208 (1.4%)	1/63 (1.6%)	
Nevoid	5/208 (2.4%)	0/63 (0.0%)	
Unclassified	12/208 (5.8%)	3/63 (4.8%)	
Mixed	2/208 (1.0%)	1/63 (1.6%)	
Not documented	10/208 (4.8%)	4/63 (6.3%)	

SLNb, sentinel lymph node biopsy.

^aComparisons of the SLSNb negative and positive patients were performed using the chi-square test for categorical variables, the two-sample *t*-test for patient age.

Table 4

Assessment of a relationship between protein expression by IHC score and SLNb positivity

	SLNb positivity <i>N</i> (%)	<i>P</i> value ^{<i>a</i>}
β3 integrin IHC (H score)		
0 (<i>N</i> = 175)	30 (17.1%)	<0.001 (<i>Z</i> = −3.54)
1+ (<i>N</i> = 77)	24 (31.2%)	
2+ or 3+ (<i>N</i> = 19)	9 (47.4%)	
β3 integrin RT-qPCR (Ct)		
<5.66 (<i>N</i> = 80)	36 (45.0%)	<0.001 (<i>Z</i> = −5.49)
5.66 (<i>N</i> = 191)	27 (14.1%)	
Breslow depth (mm)		
1 (<i>N</i> = 56)	2 (3.6%)	<0.001 (<i>Z</i> = −5.41)
1.01–2 (<i>N</i> = 135)	27 (20.0%)	
>2 (<i>N</i> = 80)	34 (42.5%)	

^a*P*value is based on a two-sided Cochran–Armitage test for trend.