Pathology & Oncology Research (2020) 26:885–891
https://doi.org/10.1007/s12253-019-00635-y

ORIGINAL ARTICLE

Immunohistochemical Staining in the Assessment of Melanoma Tumor Thickness

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Received: 3 July 2018 / Accepted: 5 March 2019 / Published online: 14 March 2019

Abstract

Vertical tumor thickness has great influence in the prognosis and staging of melanoma. The aim of this study was determination of the differences between melanoma tumor thickness in conventional hematoxylin and eosin (H&E) and immunohistochemical techniques. Thirty-six biopsy specimens were included in our study. For each sample, four adjacent tissue sections were stained with H&E, in addition S-100, Melan- A and HMB-45 staining was performed on the next serial sections. The mean thickness of tumor invasion was 2.16, 2.38, 2.22 and 2.29 mm in H&E, S-100, HMB45 and Melan-A sections evaluation, respectively. The mean difference of the Breslow thickness between H&E and S-100 and also, between H&E and Melan-A stained slides were statistically significant ($p<0.05$) while no difference was found in the tumor thickness of the H&E and HMB45 staining evaluation ($p = 0.278$). Greater tumor thickness was observed in 25 lesions (69.4%) with S-100, 20 lesions (55.5%) with Melan-A and 17 (47.2%) lesions in HMB-45 rather than H&E staining. Conclusively, it appears that H&E staining cannot prove the actual size of melanoma invasion in some cases and immunohistochemical examination can be a complementary method in this situations. Of the melanoma associated immunomarkers, the combination of S-100 and Melan-A staining may suffice to measure depth of tumor invasion.

Keywords Melanoma · Breslow thickness · Immunohistochemical staining

Introduction

Vertical tumor thickness is of great importance in the prognosis and staging of melanoma skin cancer [1]. As tumor cells invade the deeper parts of the skin, the risk of distant metastasis is considerably raised [2]. Excision of early skin melanoma in radial growth phase have favorable prognosis; while more invasive cases with involvement of deeper layer should be evaluated for lymph node metastasis and are associated with poor long term survival [3]. Therefore, the selection of proper therapeutic approach heavily depends on the depth of tumor invasion or Breslow thickness [4]. Currently, histopathologic examination using hematoxylin and eosin (H&E) staining is the standard method for the assessment of melanoma tumor thickness, however, presence of isolated or small clusters of melanocytes in dermis, adnexal structures and regression areas make it difficult to evaluate the exact depth of melanoma [5].

For decades, immunohistochemical staining of melanoma associated markers including S-100, Melan-A and HMB-45 has been incorporated as a complementary technique to improve the accuracy of diagnosis [6–8]. The application of these three immunostaining markers can display the melanoma tumoral cells with substantial sensitivity and specificity that is proposed for detection of nodal metastasis by the American Joint Committee on Cancer staging system [9].

Since the precise measurement of thickness of the cutaneous melanoma invasion is a matter of concern, the application of immunohistochemical staining has been suggested recently [10]. Limited studies have been focused on the efficacy of S-100 immunostaining in the depth of melanoma invasion assessment. Despite high sensitivity, low specificity of S-100 in differentiating melanoma associated tumoral cells would necessitate to simultaneously use more specific immunomarkers such as Melan-A and HMB-45 [11, 12].

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To the best of our knowledge, there is lack of evidence in determining the value of the three immunostains in the measurement of depth of tumor invasion and comparison with the result of H&E staining has not been well documented. This study was performed to compare the efficacy of S-100, HMB45 and Melan-A immunohistochemical staining with H&E staining in the evaluation of cutaneous melanoma invasion thickness.

Methods

During 18 months, the histopathology reports of cutaneous melanoma tumors referred to Dermatopathology department of Razi hospital were assessed and invasive melanoma tumors in vertical growth phase were enrolled. Exclusion criteria were melanoma in situ, cases in the radial growth phase (e.g. lentigo maligna, superficial spreading and superficial pagetoid type), desmoplastic and metastatic lesions, improper depth of biopsy as well as poor paraffin- embedded tissue quality. The study protocol conformed to the guidelines of the 1975 Declaration of Helsinki and was approved by the Research Institutional Review Board.

From all included biopsy specimens, the sections with greatest tumor thickness in H&E in the preliminary assessment were selected for comparison with IHC sectioned samples, in order to reduce the measurement biases of three-dimensional structures. Four adjacent tissue sections with 3-μm thickness were obtained from the paraffin-embedded tissue blocks and stained with H&E, and IHC staining was performed on the next serial sections with S-100 (primary polyclonal rabbit antibody, code N1573, Dako, Denmark), Melan- A (primary monoclonal anti-human antibody cytomation isotype: IgG_1 kappa, code N1622, Dako, Denmark) and HMB-45 (primary monoclonal mouse antibody isotype: IgG_1 kappa code N1545, Dako, Denmark) immunostains. Moreover, most of our samples were selected from small lesions in one block. However, in case of larger lesions, only block with greater tumor thickness was included and all sections have been done on the same block. The whole process of H&E and immunomarker staining was performed by a skilled laboratory technician using the standard method of staining. Finally, only cases with acceptable quality of H&E and the three immunohistochemical staining were included in the study.

A skilled dermatopathologist evaluated the Breslow depth of tumor and the Clark level of invasion in H&E and immunomarker slides, independently for each melanoma cases which means four measurements on the same tumor sample. Using an ocular micrometer, distance from top point of granular layer (or the base of ulcer) to deepest point of tumor invasion was measured in hundredths of a millimeter to determine the Breslow tumor thickness. Level of tumor invasion to different anatomic layers of skin was defined as the Clark level [13].

In the cases which were showing significant differences between H&E and immunohistochemical techniques, both H&E- and immunohistochemical stained sections were reassessed regarding the staining quality and dermatopathologist evaluation.

Statistical analysis was performed with SPSS version 16.0 (SPSS Inc., Chicago, IL). In the descriptive analysis, mean and standard deviation were used for quantitative variables, and frequencies and percentages for categorical variables. Paired t-test was used to assess the difference in the Breslow depth of tumor invasion, while the significance of difference in the level of Clark index was evaluated by chi-square test. Finally, kappa value was calculated to determine the rate of agreement on tumor depth of invasion. Differences were considered statistically significant when $p < 0.05$.

Results

In a period of 18 months, 74 cases of invasive malignant melanomas were identified. Thirty- eight cases were excluded due to poor quality of paraffin embedding or depth of biopsy as well as improper H&E and/or immunohistochemical staining. Finally, thirty six paraffin embedded tissue blocks of melanoma (20 men, 16 women; mean age 70.9 years, range 46–86 years) have been enrolled. Basic clinicopathologic characteristics of patients were shown in Table 1.

Table 1 Clinicopathologic characteristics of the evaluated patients

Variables	Values $N = 36$			
Sex				
Male	20			
Female	16			
Mean age	70.9			
(range)	46–86			
Type of melanoma				
Nodular melanoma	9			
Lentigo maligna melanoma	8			
Acral lentigines melanoma	19			
Clark level				
1	–			
2	10			
3	9			
4	10			
5	7			
Breslow thickness	H&E	S100	HMB45	Melan-A
< 1 mm	13	9	10	11
1–2 mm	10	12	11	9
2–4 mm	9	10	10	11
> 4 mm	4	5	5	5

Table 2 The mean and range of tumor thickness in H&E and three immunohistochemical staining

	Mean	95% confidence interval		Minimum	Maximum
		Lower bound	Upper bound		
H&E	2.16	1.48	2.83	0.2	9
S-100	2.38	1.68	3.08	0.3	10.1
HMB-45	2.22	1.55	2.89	0.2	9.1
Melan-A	2.29	1.61	2.96	0.3	9.2

The mean thickness of tumor invasion (the index of Breslow) was 2.16 mm in H&E sections evaluation ranged from 0.2 to 9 mm, while it reported 2.38 mm in S-100 sections ranged from 0.3 to 10.1 mm, 2.22 mm in HMB45 sections ranged from 0.2 to 9.1 mm and 2.29 mm in Melan-A section ranged from 0.3 to 9.2 mm. Using paired- t test analysis, the mean difference of the Breslow thickness between H&E and S-100 stained slides was 0.22 mm (95% Confidence Interval: 0.10–0.35 mm) that was statistically significant ($p = 0.001$). Similarly, there was a significant difference between the Breslow thickness reported from H&E and Melan-A stained sections with the mean difference of 0.13 mm (95% CI: 0.02–0.24 mm; $p = 0.018$). However, no difference was found in the tumor thickness of the H&E and HMB45 staining evaluation ($p = 0.278$). (Table 2).

Greater tumor thickness was observed in 25 lesions (69.4%) with S-100 rather than H&E staining, while H&E sections showed deeper invasion in 5 lesions (13.9%). Furthermore, comparison of Melan-A and H&E resulted in the greater depth of tumor penetration in 20 lesions (55.5%) with Melan-A in contrast to 8 lesion (22.2%) with H&E staining. Likewise, deeper invasion was revealed by HMB-45 in 17 lesions (47.2%); whereas H&E thickness value was higher in 11 lesions (30.5%). With the combination of the three immunohistochemical staining, the size of tumor invasion was greater in 27 lesions (75%) rather than H&E staining (Figs. 1 and 2).

According to the American Joint Committee on Cancer staging system [4], the depth of tumor invasion (T index) divided into <1 mm, 1.01–2 mm, 2.01–4 mm and > 4 mm and the value of agreement of H&E staining with different markers of immunohistochemical staining were assessed. The agreement rate

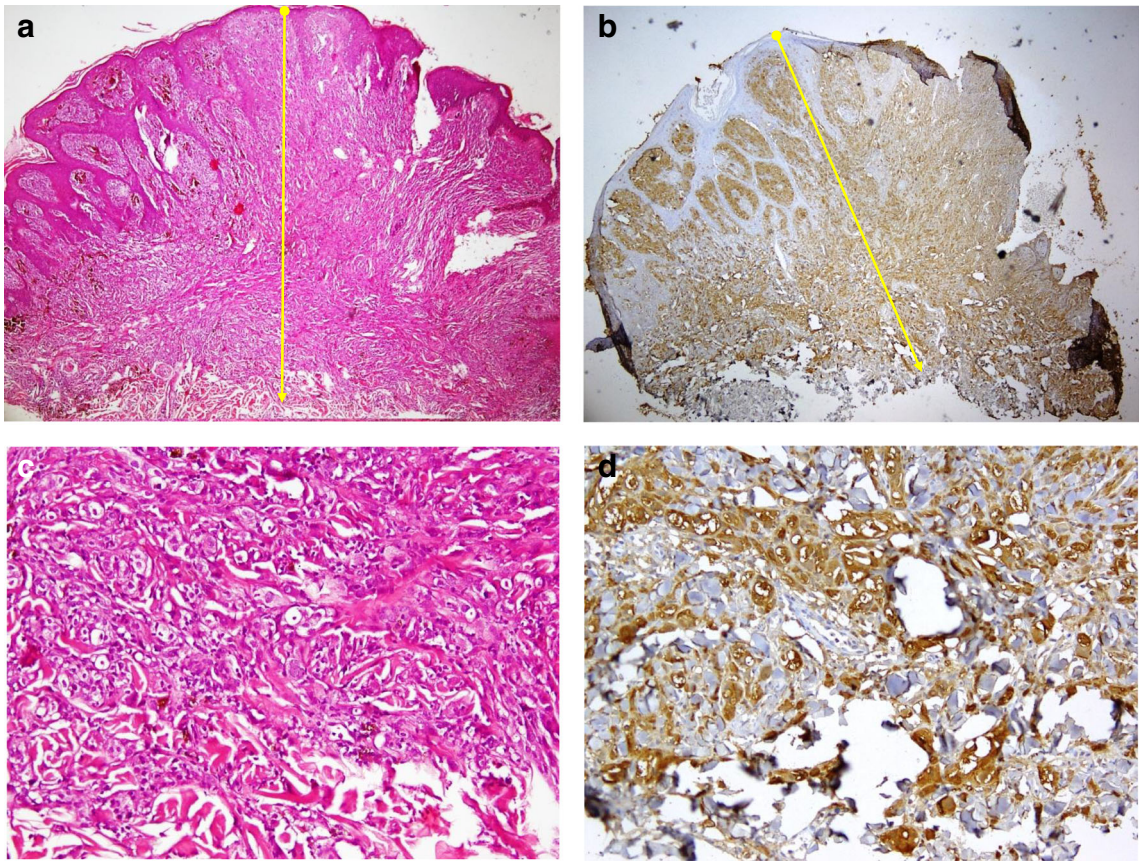

Fig. 1 **a** Nodular malignant melanoma with vertically invasive nests (Breslow: 2.4 mm and Clark level: IV) (H&E × 4), **b** thickness with S-100 measured 2.5 mm (×4), **c** depth of the tumor with difficulty recognized melanocytes (×20), and **d** tumor cells are easily detected with HMB-45 and thickness measured 2.5 mm (×4)

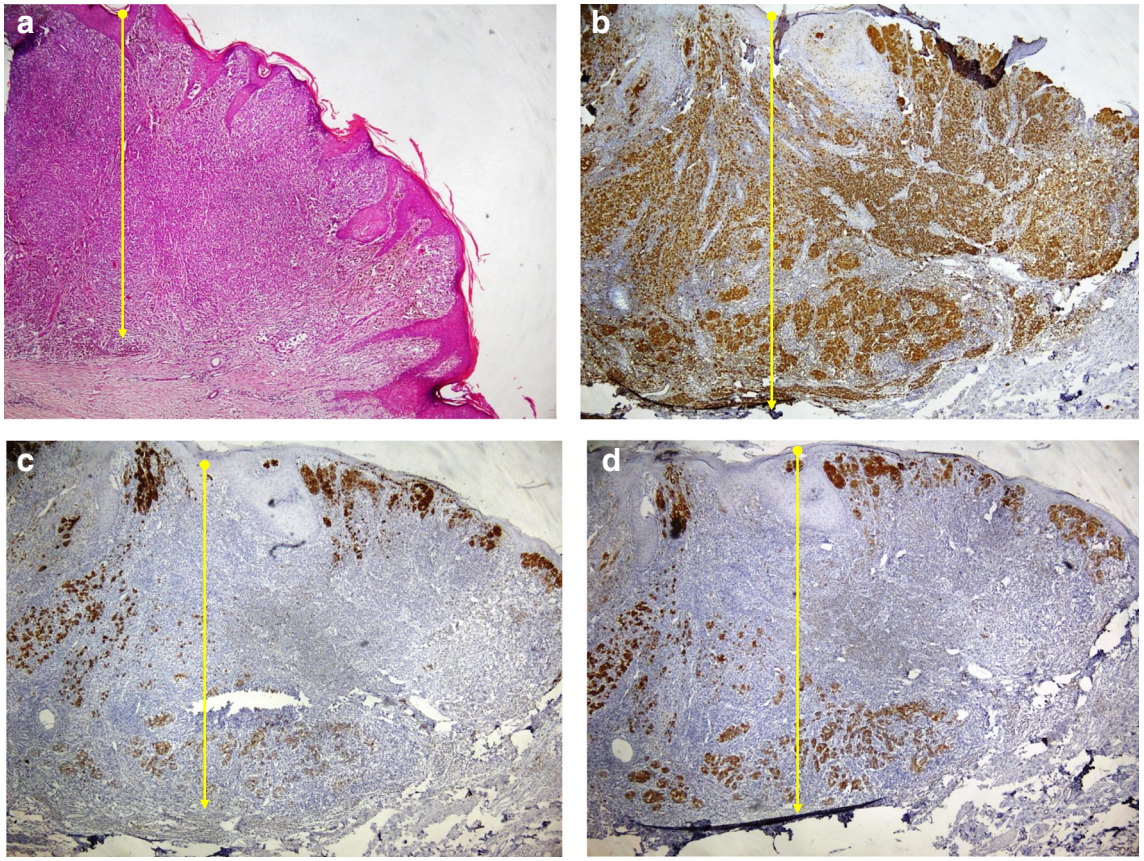

Fig. 2 **a** Nodular malignant melanoma with vertically invasive nests (Breslow: 2.5 mm and Clark level: IV) (H&E × 4), **b** thickness with S-100 measured 2.6 mm (×4), **c** with HMB-45 measured 2.6 mm (×4), and **d** with Melan-A measured 2.6 mm (×4)

between H&E and S-100 staining was substantial with the kappa value of 0.659 that was akin to the result of H&E and HMB-45 (Kappa:0.772) as well as H&E and Melan-A (Kappa:0.695). In Tables 3, 4 and 5, additional details of these agreements have been set out. It should be noticed that T score is raised by S-100, Melan-A and HMB-45 immunostaining evaluation in 8 (22.2%), 7 (19.4%) and 6 cases (16.6%), respectively.

The evaluation of the Clark level of tumor invasion showed similar results of the H&E staining and the three immunohistochemical staining that consisted of 10 cases of level II, 9 cases of level III, 10 cases of level IV as well as 7 cases of level V of the Clark index.

Discussion

This study demonstrated that immunohistochemical evaluation of Breslow thickness of melanoma can improve the accuracy of depth of invasion measurement. In more than half of the cases, deeper location of melanoma cells were identified in the immunohistochemical staining by S-100 or Melan-A rather than H&E staining that reveals some trouble with H&E evaluation. This should be noticed particularly in planning for required surgical free-margin excision of cutaneous melanomas as well as need for sentinel node biopsy and distant metastasis examination [14].

Table 3 The value of agreement of H&E staining with S-100

		S-100 immunostaining			
		< 1 mm	1.01–2 mm	2.01–4 mm	> 4 mm
H&E staining	< 1 mm	9 (25%)	4 (11.1%)	–	–
	1.01–2 mm	–	7 (19.5%)	3 (8.3%)	–
	2.01–4 mm	–	1 (2.8%)	7 (19.4%)	1 (2.8%)
	> 4 mm	–	–	–	4 (11.1%)

Table 4 The value of agreement of H&E staining with HMB-45

		HMB-45 immunostaining			
		< 1 mm	1.01–2 mm	2.01–4 mm	> 4 mm
H&E staining	< 1 mm	10 (27.8%)	3 (8.3%)	–	–
	1.01–2 mm	–	8 (22.2%)	2 (5.6%)	–
	2.01–4 mm	–	–	8 (22.2%)	1 (2.8%)
	> 4 mm	–	–	–	4 (11.1%)

A study by Dyson et al. has shown that obtaining additional slides reveals a greater tumor thickness than is reported from the original slide in 43% of samples. Additionally, the new tumor thickness could change the surgical management of the patients. They concluded that extensive block sampling could help in more accurate histologic information of melanoma, but this must be considered with the extra charge of materials, labor, time, and the risk of not retaining tissue for future use [15]. For this reason, Immunohistochemical analysis are using increasingly for the diagnosis of melanocytic lesions especially lesions with atypical clinical or pathological features [16]. Several immunohistochemical markers may be used, among them S-100, HMB-45 and Melan-A are the immunostains most widely used [17]. As these markers have different sensitivity and specificity in diagnosis of melanocytic lesions, using three methods of immunostaining would increase the accuracy of evaluation of melanoma thickness.

In a study by Penneys et al. microinvasion of tumoral cells documented in about 15% of lentigo maligna specimens (14 over 91 cases) by S-100 immunohistochemical staining. With reevaluation of the H&E staining sections, melanoma cells were not found in 6 lesions, although the reminders had focal atypical cell that was difficult to differentiate, especially in the setting of fibrotic reaction of papillary dermis and inflammatory infiltrates [18]. Moreover, Flügge and Rassner reported deeper penetration of tumor in 48.5% of melanoma cases by S-100 staining in addition to dermal invasion of four cases with previous diagnosis of "melanoma in situ" [19].

In our study the mean differences of the Breslow thickness between H&E and S-100 stained slides and H&E and Melan-A were statistically significant, but no difference was found in the tumor thickness of the H&E and HMB45 staining evaluation. It appears that S-100 has the highest potency of the Breslow measurement on account of thickness deviation to H&E staining in up to 70% of our cases. On the contrary, HMB-45 seems not to provide additional benefit as a reason of high kappa value of agreement to H&E staining. Interestingly, the combination of the three immunohistochemical staining showed deeper invasion of tumoral cell in 75% of our cases that consequently resulted in one-step raise in T score of 22% of lesions.

It is suggested that similarities in the appearance of melanoma cells with dermal structural tissue can lead to ignore the true location of tumor cells that results in inaccuracy of the Breslow tumor thickness [20]. It seems that immunostaining of melanoma biomarkers can differentiate the melanoma cells from surrounded tissues. Although, most of the available studies have been focused on S-100 staining, low specificity of this immunohistochemical marker can make some potential misinterpretation. Regarding to the staining of some normal dermal cells such as eccrine cells, myoepithelial cells, schwann cell, and histiocytes with S-100 immunomarkers, the exclusive use of this marker is not recommended [21]. Therefore, simultaneous immunostaining of more specific markers such as Melan-A and HMB-45 is valuable.

In a study by Drabeni et al. in 59.6% of cases, they found higher tumor thickness measurements in Melan-A than in H&E, and 33% of those with diagnosis of in situ melanoma in H&E were reclassified as microinvasive melanoma, with thickness ranging from 0.15 to 0.35 mm. In 23 lesions, the values were identical with both techniques, however in 17 biopsies, thickness measured with H&E staining were slightly

Table 5 The value of agreement of H&E staining with Melan-A

		Melan-A immunostaining			
		< 1 mm	1.01–2 mm	2.01–4 mm	> 4 mm
H&E staining	< 1 mm	10 (27.8%)	3 (8.3%)	–	–
	1.01–2 mm	1 (2.8%)	6 (16.7%)	3 (8.3%)	–
	2.01–4 mm	–	–	8 (22.2%)	1 (2.8%)
	> 4 mm	–	–	–	4 (11.1%)

higher (from 0.01 to 0.18 mm) [10]. In another study by Megahed et al. in 104 cases of in situ melanomas, immunohistochemical staining with Melan-A permitted detection of dermal invasion in 30 cases (29%). In their study, none of the 74 patients with confirmed diagnosis of melanoma in situ progressed to metastatic disease, however, 2 of 27 cases with diagnosis of invasive melanoma by using Melan-A developed distant metastases. So, they suggested that immunohistochemical evaluation should be a diagnostic confirmation tool for in situ melanoma [22].

As like S-100, expression of Melan-A is not specific for melanocytes lineage and staining has also been observed in tumors like atypical fibroxanthoma, pleomorphic sarcoma and angiomyolipoma, as well as, non-melanocytic cells damaged by inflammatory processes [23, 24]. In some studies, it was recommended that the results of study by Melan-A should be considered in the context of other melanocytic markers such as HMB-45, S-100 and MITF [25].

The standards of staging and treatment protocol of melanoma, as well as, prognostic models are based on the Breslow thickness determined by H&E techniques, so using immunohistochemical analysis for measurement of tumor thickness can result in some misinterpretations, like different results of H&E and immunohistochemical techniques in determination of mitotic counts [26]. Accordingly, additional studies are needed to define the value and effect of immunohistochemical techniques in the staging, treatment and prognosis of primary melanoma.

In Conclusion, it appears that H&E staining cannot prove the actual size of melanoma invasion in some cases and immunohistochemical examination can be a complementary method for the Breslow melanoma staging procedure. Of the melanoma associated immunomarkers, the combination of S-100 and Melan-A staining may suffice to measure depth of tumor invasion.

Compliance with Ethical Standards

Conflict of Interest There is no conflict of interest to declare.

References

1. Breslow A (1980) Prognosis in cutaneous melanoma: tumor thickness as a guide to treatment. Pathol Annu 15:1–22
2. Shaikh WR, Dusza SW, Weinstock MA et al (2015) Melanoma thickness and survival trends in the United States, 1989–2009. J Natl Cancer Inst 12:108(1)
3. Scolyer RA, Shaw HM, Thompson JF, Li LXL, Colman MH, Lo SK, McCarthy SW, Palmer AA, Nicoll KD, Dutta B, Slobedman E, Watson GF, Stretch JR (2003) Interobserver reproducibility of histopathologic prognostic variables in primary cutaneous melanomas. Am J Surg Pathol 27:1571–1576
4. Haydu LE, Stollman JT, Scolyer RA, Spillane AJ, Quinn MJ, Saw RPM, Shannon KF, Stretch JR, Bonenkamp JJ, Thompson JF (2016) Minimum safe pathologic excision margins for primary cutaneous melanomas (1–2 mm in thickness): analysis of 2131 patients treated at a single center. Ann Surg Oncol 23:1071–1081
5. Hurt MA, Santa Cruz DJ (1994) Malignant melanoma microstaging. History, premises, methods, problems, and recommendations—a call for standardization. Pathol Annu 29: 51–74
6. Ohsie SJ, Sarantopoulos GP, Cochran AJ, Binder SW (2008) Immunohistochemical characteristics of melanoma. J Cutan Pathol 35:433–444
7. Plaza JA, Suster D, Perez-Montiel D (2007) Expression of immunohistochemical markers in primary and metastatic malignant melanoma: a comparative study in 70 patients using a tissue microarray technique. Appl Immunohistochem Mol Morphol 15:421–425
8. Paradela S, Fonseca E, Pita S, Kantrow SM, Goncharuk VN, Diwan H, Prieto VG (2009) Spitzoid melanoma in children: clinicopathological study and application of immunohistochemistry as an adjunct diagnostic tool. J Cutan Pathol 36:740–752
9. Buzaid AC, Gershenwald JE, Atkins MB, Ross ME (2016) Tumor node metastasis (TNM) staging system and other prognostic factors in cutaneous melanoma. UpToDate. UpToDate, Waltham
10. Drabeni M, Lopez-Vilaró L, Barranco C, Trevisan G, Gallardo F, Pujol RM (2013) Differences in tumor thickness between hematoxylin and eosin and Melan-A immunohistochemically stained primary cutaneous melanomas. Am J Dermatopathol 35:56–63
11. Xia J, Wang Y, Li F, Wang J, Mu Y, Mei X, Li X, Zhu W, Jin X, Yu K (2016) Expression of microphthalmia transcription factor, S100 protein, and HMB-45 in malignant melanoma and pigmented nevi. Biomed Rep 5:327–331
12. Orchard GE (1998) Melan A (MART-1): a new monoclonal antibody for malignant melanoma diagnosis. Br J Biomed Sci 55:8–9
13. Morton DL, Davtyan DG, Wanek LA, Foshag LJ, Cochran AJ (1993) Multivariate analysis of the relationship between survival and the microstage of primary melanoma by Clark level and Breslow thickness. Cancer. 71:3737–3743
14. Urist MM, Balch CM, Soong SJ et al (1984) Head and neck melanoma in 534 clinical Stage I patients. A prognostic factors analysis and results of surgical treatment. Ann Surg 200:769–775
15. Dyson SW, Bass J, Pomeranz J, Jaworsky C, Sigel J, Somach S (2005) Impact of thorough block sampling in the histologic evaluation of melanomas. Arch Dermatol 141:734–736
16. de Vries TJ, Smeets M, de Graaf R, Hou-Jensen K, Bröcker EB, Renard N, Eggermont AMM, van Muijen GNP, Ruiter DJ (2001) Expression of gp100, MART-1, tyrosinase, and S100 in paraffin-embedded primary melanomas and locoregional lymph node, and visceral metastases: implications for diagnosis and immunotherapy. A study conducted by the EORTC melanoma cooperative group. J Pathol 193:13–20
17. Kim RH, Meehan SA (2017) Immunostain use in the diagnosis of melanomas referred to a tertiary medical center: a 15-year retrospective review (2001–2015). J Cutan Pathol 44:221–227
18. Penneys NS (1987) Microinvasive lentigo maligna melanoma. J Am Acad Dermatol 17:675–680
19. Flügge G, Rassner G (1989) Demonstration of S 100 protein in malignant melanoma of the skin. Pattern of distribution and significance for determination of tumor thickness. Hautarzt. 40:290–295
20. Prade M, Sancho-Garnier H, Cesarini JP, Cochran A (1980) Difficulties encountered in the application of Clark classification and the Breslow thickness measurement in cutaneous malignant melanoma. Int J Cancer 26:159–163
21. Prieto VG, Shea CR (2008) Use of immunohistochemistry in melanocytic lesions. J Cutan Pathol 35:1–10
22. Megahed M, Schön M, Selimovic D, Schön MP (2002) Reliability of diagnosis of melanoma in situ. Lancet. 359:1921–1922
23. Thum C, Hollowood K, Birch J, Goodlad JR, Brenn T (2011) Aberrant Melan-a expression in atypical fibroxanthoma and

undifferentiated pleomorphic sarcoma of the skin. J Cutan Pathol 38:954–960

24. Bax MJ, Johnson TM, Harms PW, Schwartz JL, Zhao L, Fullen DR, Chan MP (2016) Detection of occult invasion in melanoma in situ. JAMA Dermatol 152:1201–1208
25. Kim J, Taube J, McCalmont TH, Glusac EJ (2011) Quantitative comparison of MiTF, Melan-a, HMB-45 and Mel-5 in solar lentigines and melanoma in situ. J Cutan Pathol 38:775–779
26. Helm TN, Helm KF (2014) Breslow thickness determined with the use of Immunohistochemical techniques could provide misleading information when used with prognostic models based on data obtained by conventional means. Am J Dermatopathol 36:757