

**USE OF IMMUNOHISTOCHEMISTRY IN MELANOCYTIC LESIONS  
EXAMINATION OF SENTINEL LYMPH NODES IN PATIENTS WITH MELANOMA**

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Melanocytic lesions constitute a very important topic in Pathology, not only for dermatopathologists but also for general pathologists. In this talk we will review how we apply immunohistochemistry as a tool to help in the differential diagnosis of melanocytic lesions. And we would like to stress that, as in other fields of pathology, immunohistochemistry should be considered as an adjunct to the diagnosis. A diagnosis should always be based on morphologic grounds and then immunohistochemistry should help in either confirming or ruling out the diagnosis.

*Introduction:*

In a majority of laboratories, the most utilized chromogen is diaminobenzidine (DAB). Due to its brown color, sometimes it may be difficult to distinguish between DAB and melanin pigment. For the evaluation of such pigmented lesions, there are several options. Other chromogens, such as Fast red or AEC result in red immunoperoxidase reaction product. Also, rather than using hematoxylin as the counterstain, techniques such as Giemsa or Azure B change melanin color from brown to green. Finally, bleaching techniques eliminate melanin from the slides. However, these latter techniques may induce a change in the expression of several antigens. As an example, HMB-45 may become positive in macrophages. Therefore, those laboratories using bleaching techniques should standardize the immunohistochemical processing to ensure consistent results.

- S-100 protein remains the most sensitive (if non-specific) marker of melanocytic differentiation. In general, melanomas initially considered S-100-negative become positive on additional testing after changing the settings (enzymatic pretreatment, detection system). Conversely, there is occasional expression of markers of epithelial differentiation, such as cytokeratin, in melanoma, especially in metastasis, reinforcing the adage that immunohistochemistry should be performed with a battery of reagents. The majority of melanocytic lesions express this marker, thus we predominantly use it as a second line of antibodies in cases in which we suspect melanoma but the cells lack other melanocytic markers (see below).

- Gp100 (with the antibody HMB-45) remains one of the most useful melanocytic markers. While not absolutely specific, the other lesions that also express this marker (angiomyolipomas, sugar cell tumors of the lung, "pecomas") do not usually enter in the differential diagnosis of skin tumors. HMB-45 labels the junctional component of nevi and, with the exception of blue nevi, does not label the deep dermal component ("maturation" of nevi). In contrast, most melanomas in the skin (except for desmoplastic melanoma, see also below) at least focally label with HMB-45 in the dermal component (see below). Another possible utility of HMB45 is that it seems to label more strongly and consistently than anti-S100 protein or anti-MART1 in nasal melanomas.

- MIB1 (anti-Ki67, a proliferation marker expressed in proliferating cells) has also proven to be helpful in the analysis of melanocytic lesions. In a study of 384 melanocytic lesions, they found significant differences in the amount and pattern of cell proliferation among various types of nevus and melanoma. Specifically, common nevi and dysplastic nevi exhibited reactivity in <1% of cells, generally disposed at the dermal-epidermal junction or in the more superficial dermal compartment. In contrast, melanomas did not show this orderly pattern, but instead had a random pattern of immunoreactivity, and a mean growth fraction of 16.4%, particularly at the deep edge of the lesion. These authors reclassified 112 lesions that

had posed diagnostic problems on routine histology; on subsequent clinical review, systemic progression was demonstrated in 70.7% of the cases finally classified as melanoma and in none of the finally classified benign lesions, a highly significant result that indicates the potential clinical utility of this approach. Melan-A (MART1, Melanoma Antigen Recognized by T cells), an antigen first detected in melanoma metastases is widely expressed in both nevi and melanoma, to a higher degree than that seen in gp100. However, and similar to gp100, it is usually lost in desmoplastic melanoma. Due to the widespread expression we use it to try to detect melanocytic differentiation. One possible drawback is that it may label macrophages, particularly pigmented ones.

- Tyrosinase is an enzyme involved in melanogenesis. In our hands, tyrosinase expression is very similar to HMB-45 labeling.

- Mitf (Microphthalmia Transcription Factor) is a nuclear protein involved in the development of melanocytes and the regulation of melanin synthesis. It is expressed in many melanocytic lesions. However, it is also expressed macrophages, lymphocytes, fibroblasts, Schwann cells, and smooth muscle cells.

- A relatively recent development is the combination of one or more than one antibodies in a single cocktail. One such combination is panmel (HMB45, anti-MART1, Melan A, and anti-tyrosinase), as a pan-melanocytic cocktail to try to detect any possible melanocytic differentiation. In our hospital, we have combined HMB45 just with anti-tyrosinase, to try to avoid a false positivity in cases with melanophages. Another type of combination is to include a nuclear and a cytoplasmic marker, such as HMB45/MIB1 or anti-MART1/MIB1; in such way it is easier to determine the fraction of melanocytes that are proliferating (since they will have both cytoplasmic and nuclear positivity)

A recent study has examined in different types of nevi the expression of multiple melanocytic markers (tyrosinase, tyrosinase-related protein-1 [TRP1], dopachrome tautomerase [TRP2], Pmel-17/gp100, P-protein, and microphthalmia-associated transcription factor [MITF]). All melanogenesis-related genes examined in the junctional type were expressed in the basal epidermal layer. In the compound and intradermal nevi, mRNA for tyrosinase, TRP2, and MITF were expressed in all of the cells. However, the expression of TRP1, P-protein, and Pmel-17/gp100 in the compound type and TRP1 in the intradermal type became weaker in accordance with the depth of the dermis layer, as compared to those levels in the basal to upper dermis layer.

#### *Use of immunohistochemistry in particular situations:*

- Diagnosis of melanocytic differentiation: When encountered with a poorly differentiated neoplasm in the skin, the differential diagnosis mainly includes carcinoma (primary or metastatic), melanoma (primary or metastatic), mesenchymal tumor (primary or metastatic), and lymphoid proliferation. A possible approach is to use a panel (anti-keratin, panmel, anti-LCA [leukocyte common antigen]). If negative for all three, we then use anti-S100 protein to try to detect any melanocytic marker at all.

- Determination of depth of invasion: In melanoma cases with epidermal hyperplasia it may be difficult to determine how much of the melanoma is in situ or invasive. Anti-keratin will label keratinocytes thus determining that in that particular area the melanoma is in situ. It also will help determine if the melanoma cells are around hair follicles, a situation that will modify the measurement of the Breslow thickness.

- Detection of maturation: Benign melanocytic lesions will show a differential pattern of expression of Ki67 and gp100. With the exception of blue nevi, all nevi show expression of gp100 limited to the epidermal component as well as to some of the cells located in the upper papillary dermis. Blue nevi show diffuse, strong expression of gp100 throughout the lesion. Finally, primary melanomas show patchy expression of gp100, including at least some cells in the deep dermal component. Ki67 is also similarly expressed. In nevi, proliferating (Ki67 positive) cells are located in the upper dermis but not in the deep dermal component. Blue nevi do not show this pattern of maturation but still show only rare positive cells.

Melanomas have at least some cells in the deep dermal component, without clear-cut distinction between the top and the bottom of the lesion.

- Distinction between compound nevus and nevoid melanoma: It is our opinion that immunohistochemistry is very helpful in this differential diagnosis. In particular, labeling with HMB45 and MIB1 in the cells of the deep dermal component strongly suggests melanoma.

- Distinction between desmoplastic melanoma and desmoplastic nevus: The similarities between desmoplastic nevi and desmoplastic melanomas include the presence of atypical cells, perineural involvement, lymphoid infiltrates and HMB-45 labeling in the superficial portion of the lesions. However, the infrequent location on the head or neck, the absence of mitotic figures, the significantly lower number of Ki-67-reactive cells, and the decrease in HMB-45 expression in the deep area of the lesions, help distinguish desmoplastic nevi from desmoplastic melanoma.

- Distinction between scar and desmoplastic melanoma: Since most desmoplastic melanomas lack expression of gp100, MART1, or tyrosinase, S100 is the most useful marker to try to distinguish desmoplastic melanoma from scar. Scars show scattered dendritic cells expressing S100 while most of the spindle cells in desmoplastic melanoma are positive. In addition, we use the panmel cocktail to try to detect any labeling at all. If there is clear expression of any of the atypical, spindle cells in the lesion, that finding further supports the diagnosis of melanoma.

Immunohistochemistry in all cases from the AFIP showed positive expression of S100 protein and lack of reactivity with HMB-45 and Leu-7. In a follow-up study, the AFIP group retrieved 56 cases of spindle cell melanoma and determined that tumors reacting with HMB-45 had a significantly worse prognosis than the HMB-45-negative spindle cell melanomas. An immunohistochemical study from the UK examined 25 cases of desmoplastic melanoma; S100 and neuron-specific enolase were the most sensitive markers for this tumor, while NKIC3 was strongly positive in only 27% of cases, and HMB-45 in 22%, mainly in small groups of cells near the surface. Longacre and colleagues from Stanford reported that all cases of desmoplastic melanoma in their series were negative, while a minority of non-desmoplastic spindle cell melanomas was HMB-45+. They found a number of cases in which S100 staining was restricted to <5% of the tumor cells. They also found many cases expressing smooth-muscle actin, which they interpreted as a population of myofibroblasts in the stroma. It is to be noted that reaction of spindle cell melanomas with HMB-45 in the most recent series is higher than in several older reports, due to the modern use of sensitive antigen retrieval techniques, and may prove helpful in problematic cases. P75NTR was expressed strongly in 11/11 spindle-cell melanomas and may mediate the greater tendency of spindle-cell melanomas to undergo neurotropic spread.

- Distinction between spindle cell melanoma and blue / desmoplastic nevus: As mentioned above, spindle cell melanomas may lose expression of gp100, MART1, or even S100 protein. Therefore, when examining a spindle cell melanocytic lesion, if there is *strong, diffuse* expression of the three markers, it usually is a nevus.

- Distinction between melanoma and malignant peripheral nerve sheath tumor (MPNST): Most MPNST show just weak, patchy expression of S100 protein. Therefore, strong, diffuse expression of S100 (even better, along with any other melanocytic marker) supports the diagnosis of melanoma.

**Examination of sentinel lymph nodes**: Within the last 15 years, evaluation of sentinel lymph nodes (SLN) has become the most popular method of early staging of several malignancies, including melanoma. In order to detect early metastasis, those lymph nodes are examined by the pathologists in a more intense manner than that used in standard lymphadenectomy specimens. The main goal of

examination of SLNs is to provide accurate staging of early lesions, which more accurately defines the prognosis of these patients and provides more consistent grouping in clinical trials.

Some authors recommend frozen sections to examine SLN, particularly in breast, to try to render an immediate diagnosis of metastatic disease during the surgical procedure. However, frozen sections provide a sub-optimal morphology and may lack the subcapsular region of the lymph node (which is the area most likely involved in melanoma metastases). Furthermore, since processing of the frozen tissue requires embedding in paraffin, facing off the block, and new sectioning, then micrometastases may be lost in the discarded tissue. Therefore, at least for SLN from melanoma patients, most authors consider only routinely processed material (formalin-fixed, paraffin-embedded) and discourage frozen sections.

The classical processing used in lymph nodes, i.e., bivalving of the node and examination of a single, routine hematoxylin and eosin (H&E) slide misses a number of small metastases. In an early study from our institution with 243 patients with SLN initially diagnosed negative when examining one H&E slide per block, 10 (4.3%) presented a recurrence in the same lymphatic basin. Of those 10 patients, when the original SLN was re-examined using new serial sections or immunohistochemistry, eight (80%) were re-classified as positive. In another study, 3 of 7 patients with recurrent disease had metastatic MM in the originally negative SLN after re-examination with serial sections and immunohistochemistry. Based upon these studies, most current protocols for examination of SLN require additional sectioning or immunohistochemistry. Regarding other studies, some clinical trials call for preserving a portion of the node for PCR analysis to try to detect mRNA associated with melanocytic differentiation.

The original protocol proposed by Cochran et al called for bivalving the SLN through the hilum to allow examination of the afferent lymphatic vessels of the lymph node. In order to simplify the processing, at our institution we recommend breadloafing of the SLN to allow examination of a large area of the subcapsular region. Then we study one H&E slide; if this is positive we report it as such or else we submit the block again to the laboratory to obtain a new H&E deeper section slide (~200 microns deeper in the block) and two unstained slides. One of them is reacted with a pan-melanocytic cocktail (HMB45, anti-MART1, and anti-tyrosinase). Using this method the results are comparable with those reported for SLN examined by bivalving (~20% of patients)

Approximately 20% of patients with cutaneous melanoma show deposits of melanoma cells in the SLN. The amount of tumor in the SLN (tumor burden) ranges from solitary, rare cells up to complete replacement of the lymph node. At our institution we measure the largest tumor deposit as a two-dimensional value in mm (long axis x short axis). The majority of these metastatic deposits are located within or close to the subcapsular sinus. Less frequently tumor cells are located within the parenchyma closer to the center of the lymph node and even more exceptional is to see melanoma cells located in the intracapsular lymphatic vessels (see below). Less than 5% of metastases show extracapsular extension.

Regarding the cytologic features, metastatic melanoma cells may display a large variety of morphologies, although most commonly they resemble the cells in the primary lesion. This fact is very important at the time of establishing a differential diagnosis (see next).

Immunohistochemical studies are very helpful when trying to detect small metastatic deposits and also to differentiate metastasis from nodal (capsular) nevus. Of the approximately 20% of patients that have positive SLN, 16% are detected in the initial hematoxylin and eosin slide and the remaining 4% are detected with the serial sections or immunoperoxidase. Some authors propose the use of anti-S100 protein. However, since S100 labels lymph node dendritic cells in addition to melanocytes, in our opinion, it is difficult to distinguish single melanoma cells from a background of dendritic cells. Therefore other markers may be more useful. Among the different options, we recommend a pan-melanocytic cocktail (HMB45, anti-MART1, and anti-tyrosinase). In addition, since MART1 can be expressed by macrophages, we sometimes use HMB45 by itself when trying to differentiate between macrophages and melanoma cells (HMB45 usually does not label macrophages). In cases of spindle cell melanoma in which the tumor cells do not express MART1 or gp100 (with HMB45) we may run anti-S100; furthermore spindle melanocytes that express S100 but not MART1 or gp100 are likely malignant.

The differential diagnosis also includes capsular nevi. Up to 20% of lymphadenectomies from the axilla or groin contain such benign collections of melanocytes. The characteristic capsular location of these nevus deposits is different from the common subcapsular location of metastatic melanoma. Therefore, clusters of melanocytes in the capsule are usually benign (nodal nevus) while subcapsular / intraparenchymal clusters are malignant. However, a potential problem is the presence of vascular metastasis in the intracapsular lymphatic vessels of the node thus mimicking a capsular nevus (in our experience, it is extremely rare to detect nevus aggregates within the vessels of the SLN capsule). Immunohistochemistry against vascular markers (CD31, CD34, or D2-40) may be helpful in detecting the rim of endothelial cells, thus confirming the intravascular location of the melanoma cells.

Capsular nevi may rarely extend into the underlying node parenchyma. In general, those lymph nodes contain similar melanocytes in the capsular region, lack gp100 expression (with HMB45), and show very low Ki67 expression, thus consistent with benign melanocytes. In order to facilitate the identification of proliferating melanocytes, we use a homegrown cocktail that includes anti-MART1 and MIB1 (against Ki67). Since these two markers are expressed in different cellular components (Ki67 in the nucleus and MART1 in the cytoplasm) it is relatively easy to determine how many of the melanocytes (i.e., cells expressing MART1) are proliferating (i.e., expressing Ki67).

We recommend issuing a pathology report that includes the number of positive nodes and the total count, both spelled out and as numbers to avoid possible typographical errors (e.g., “one of three lymph nodes (1/3)”). SLN positivity is associated with decreased survival, along with Breslow thickness, and ulceration. Quantification of melanoma metastasis size in SLN correlates with subsequent involvement of non-sentinel lymph nodes from the same anatomic region and with prognosis. Some authors recommend a modification of Breslow thickness (measurement of the distance between the capsule and the most deeply located deposit). Based upon our results, we measure the tumor burden in the SLN as determined by the size of the largest tumor deposit (in two dimensions in mm), the location (subcapsular versus other), and presence or absence of extracapsular extension. Not all studies have detected an association between tumor location (subcapsular/intraparenchymal) and survival but at any rate, a majority of responders to a recent survey in Europe also report the size of the largest tumor deposit in the SLN.

Our preliminary data on 237 positive SLN out of 1417 patients, suggest a stratification in three groups with progressive worse prognosis: 1) involvement of one or two SLN AND metastasis size  $\leq 2$  mm (in the largest nest), AND no ulceration (in the primary lesion), 2) ulceration in the primary lesion OR any metastatic nest  $>2$  mm, 3) involvement of 3 or more SLN OR ulceration in the primary lesion AND any metastatic nest  $>2$  mm. Additional studies are necessary to determine if such stratification scheme provide clinically significant prognostic information.

Regarding the relationship between tumor size and prognosis, an unexpected finding in our study was the lack of a definite cut-off. Unlike breast carcinomas, in which SLN with tumor deposits smaller than 0.2 mm are not considered positive by some authors, we have seen at least two cases in which only a single melanoma cell was identified in the SLN, and both lesions had recurred as multiple distant metastases within 4 years of diagnosis.

Regarding additional techniques, some studies have indicated that PCR detection of melanocytic mRNA in SLN correlates with decreased survival, a finding not shown by other authors. A possible explanation for these differences may be the presence of nodal nevi in some SLN that would result in positive PCR results. Therefore, unless mRNA specific for melanoma cells becomes available for PCR studies, it seems that histologic examination will remain the gold standard in SLN for melanoma.

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