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Title: Immunohistochemical Expression of PAX8, PAX2, and Cytokeratin in Melanomas

Running Title: PAX8, PAX2 and cytokeratin in melanomas

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ABSTRACT

Background: Deviations from the classic melanocytic immunophenotype in melanoma can present a diagnostic challenge. PAX8 and PAX2 are common markers for renal or Müllerian differentiation. While most PAX8+ or PAX2+ carcinomas are seldom confused with melanoma, some cases may show a more ambiguous immunophenotype, especially when MiTF family altered renal cell carcinoma (MiTF-RCC) is in the differential diagnosis. Neither PAX8 nor PAX2 expression has been reported in melanoma to date. We aimed to better characterize PAX8, PAX2, and cytokeratin immunoreactivity in a large series of melanomas.

Methods: Tissue microarrays consisting of 263 melanomas were immunostained for PAX8, PAX2, and cytokeratin and graded by an h-score.

Results: PAX8 expression was seen in 7.9% of melanomas and was significantly associated with spindle cytomorphology. PAX2 was positive in one (0.4%) melanoma. Cytokeratin positivity was seen in three (1.2%) cases and was associated with metastases.

Conclusions: PAX8 is expressed in a subset of melanomas and may be strong/extensive. As PAX8 positivity does not exclude a diagnosis of melanoma, it should be used in conjunction with other immunohistochemical markers, such as cytokeratin and PAX2, when melanoma, MiTF-RCC, and other PAX8+ tumors are in the differential diagnosis.

Key Words: PAX8, PAX2, cytokeratin, melanoma, MiTF, renal cell carcinoma

INTRODUCTION

The classic immunophenotype for melanoma includes positivity for S100, SOX10, Melan-A, HMB45, and MiTF.[1,2] Although melanoma is typically cytokeratin (CK)-negative, aberrant expression of CK has been reported in 2-40% of melanomas, typically in a minority of cells.[3,4] Aberrant expression of other non-melanocytic markers such as CEA, EMA, TTF-1, p63, synaptophysin, desmin, neurofilament, and p63 has also been reported.[4–8] Rare melanomas may demonstrate complete loss of melanocytic markers.[9–12] Such deviations from the classic melanoma immunophenotype could be a diagnostic pitfall. Metastatic melanoma of unknown primary, presumably from a regressed primary cutaneous melanoma,[13] presents yet another diagnostic challenge, as the lack of a preceding diagnosis of primary melanoma does not necessarily exclude the possibility of a metastatic melanoma.

Little is known about the expression of PAX8 and PAX2 in melanoma. PAX8 is a transcription factor expressed in the nuclei of a variety of normal epithelia including renal tubules, thyroid follicles, and Müllerian tract (epithelia of ovary, Fallopian tube, endometrium, and endocervix).[14] Hence it is commonly used to diagnose carcinomas of renal, thyroid, and Müllerian origins.[15] PAX2 is a homolog of PAX8 that is similarly expressed in renal tubular and Müllerian epithelia, but not in thyroid follicular cells.[15] Only two prior studies have examined PAX8 expression in a small number of melanomas in large cohorts of benign and malignant tumors, and found no expression in melanomas.[14,16] Similarly, PAX2 expression has been investigated in only one study in which both melanomas examined were negative.[17]

While most PAX8+ or PAX2+ carcinomas are seldom confused with melanoma due to their relatively consistent expression of cytokeratins and lack of expression of melanocytic markers, a more challenging tumor is MiTF family altered renal cell carcinoma (MiTF-RCC). The microphthalmia-associated transcription factors encoded by genes *MITF*, *TFE3*, *TFEB*, and

TFEC induce tumorigenesis following translocation or amplification events.[18–20] Specifically, *MITF* amplification is associated with melanoma, and *TFE3* and *TFEB* rearrangements are associated with renal cell carcinoma (RCC),[18,19,21,22] perivascular epithelioid cell tumor (PEComa),[23,24] and alveolar soft part sarcoma.[18] Microscopically, RCCs harboring *TFE3* (Xp11) translocation are characterized by papillary or pseudopapillary growth of epithelioid cells with clear or eosinophilic cytoplasm and high-grade nuclei, commonly associated with psammoma bodies.[25] Those harboring translocation of *TFEB* (t(6;11)) are more often biphasic with larger epithelioid nests and smaller cells surrounding basement membrane material,[26,27] whereas RCCs with *TFEB* amplification are high-grade and enriched in papillary and oncocytic features.[19] MiTF-RCCs may mimic melanoma due to frequent patchy expression of melanocytic markers[28–30] and underexpression of epithelial markers.[23,31] Inconsistent expression of PAX8 in MiTF-RCC further adds to this diagnostic challenge.[25,26,32,33] Finally, a subset of renal epithelioid neoplasms may contain melanin pigment, although it remains unclear whether these represent PEComas with *TFE3* translocations or true Xp11 RCCs.[31,34] Of note, metastatic MiTF-RCC to the skin has been rarely reported[35] and may potentially simulate a primary cutaneous melanoma. Although SOX10 expression would generally favor melanoma over MiTF-RCC, to our knowledge SOX10 has only been studied in 13 MiTF-RCCs.[33] Although all 13 cases were negative, this small number of cases precludes a definitive conclusion on the utility of SOX10 in this differential diagnosis.

Herein we explored the frequency and the extent of PAX8, PAX2, and CK expression in a large series of melanomas, in order to assess the utility of these immunostains in distinguishing PAX8+/PAX2+ carcinomas from melanoma. We anticipated our findings to be particularly useful when evaluating a kidney tumor that expresses melanocytic markers in someone with a

history of melanoma, or when evaluating a skin tumor found to express PAX8/PAX2 in someone with a history of MiTF-RCC.

MATERIALS AND METHODS

The study was conducted according to a protocol previously approved by the Institutional Review Board at our institution. Three tissue microarrays (TMAs) previously constructed from formalin-fixed paraffin-embedded melanomas were used for this study. A total of 263 melanomas (176 primary cutaneous and 87 metastatic, unpaired) were included, each represented by triplicate 0.6 mm cores. Sections of 4-μm thickness made from each TMA were deparaffinized, and heat-induced epitope retrieval was performed on the Ventana Benchmark Ultra immunostainer using cell conditioning 1 (CC1) buffer from Ventana Medical Systems (Tucson, AZ, USA). After blocking endogenous peroxidase activity, the slides were incubated with the following antibodies: PAX8 (polyclonal, predilute, Cell Marque) for 32 minutes at 37°C; PAX2 (clone EP235, predilute, Cell Marque) for 60 minutes at 37°C; and CK cocktail (AE1/AE3, predilute, Cell Marque; Cam5.2, 1:40, BD Biosciences) for 32 minutes at 37°C. Immunoreactivity was detected using the Ultraview universal DAB detection kit (Ventana Medical Systems).

Each case was assessed by a board-certified dermatopathologist (M.P.C.) and a pathology trainee (J.M.P.), and was assigned an h-score combining the intensity (0-3) and extent (%) of staining, ranging from 0 to 300. An h-score of ≥20 was considered positive, as this cutoff indicated at least focal but convincing staining that cannot be easily disregarded as negative. An h-score of ≥60 was considered high-positive, as this cutoff indicated readily appreciable staining at low magnification. Presence or absence of spindle cytomorphology was also recorded for each case.

Fisher's exact test was used to compare the number of positive cases between groups (primary vs. metastatic; spindle cytomorphology present vs. absent). Statistical significance was defined as a p-value of <0.05.

Patients' medical records, wherever available, were reviewed for any history of primary renal, Müllerian, or thyroid malignancies.

RESULTS

PAX8

A total of 253 cases were available for PAX8 scoring after elimination of cases with inadequate sections. Table 1 summarizes the results of PAX8 immunostaining. Positive PAX8 expression (h-score ≥ 20) was present in 7.9% of all melanomas, of which almost half were high-positive (h-score ≥ 60). The highest h-score was 210 (Fig 1A). Melanomas with a predominantly spindle cytomorphology were more likely to express PAX8 (Fig 1B) compared to those with a predominantly epithelioid cytomorphology (Fig 1C) ($p=0.02$). A total of 11 spindle melanomas in this series were originally classified as pure or mixed desmoplastic melanomas, 8 of which had immunohistochemical workup at the time of diagnosis and were reported as S100+ while lacking Melan-A, MiTF, and/or HMB45 expression. Two of these desmoplastic melanomas expressed PAX8 with h-scores of 150 and 210, respectively. An association was also found between primary melanoma and positive PAX8 expression ($p=0.01$), which was attributable to the enrichment of spindle melanomas in this group compared to the metastatic group ($p=0.01$). Precisely, 83.0% of the spindle melanomas were primary tumors.

PAX2

A total of 243 cases were available for PAX2 scoring after elimination of cases with inadequate sections. Table 1 summarizes the results of PAX2 immunostaining. PAX2 was positive in only one (0.4%) melanoma with limited staining (h-score =20). It was a metastatic melanoma with epithelioid cytomorphology (Fig 2A), without co-expression of PAX8 or CK cocktail. No statistical significance was detected between groups.

CK cocktail (AE1/AE3/Cam5.2)

A total of 248 cases were available for CK cocktail scoring after elimination of cases with inadequate sections. Table 1 summarizes the results of CK cocktail immunostaining. CK cocktail was positive in three (1.2%) cases, only one of which was high-positive (h-score =60). All three cases were epithelioid metastatic melanomas without spindle cytomorphology (Fig 2B); original diagnosis of metastatic melanoma in these cases was confirmed by positive staining for melanocytic marker(s) at the time of diagnosis. The association between CK cocktail positivity and metastasis (compared to primary melanoma) reached statistical significance (p=0.04). None of the CK cocktail-positive cases co-expressed PAX8 or PAX2.

None of the patients with PAX8, PAX2, or CK cocktail-positive tumors had a history of renal, Müllerian, or thyroid carcinoma.

DISCUSSION

In this study we examined PAX8, PAX2, and CK cocktail expression in primary and metastatic melanomas with correlation to cytomorphology. A few previous studies have broadly surveyed PAX8 (using both polyclonal and monoclonal antibodies) and PAX2 positivity in various normal tissues and tumors, in which melanomas were included in small numbers (2 to 15 cases).[14,16,17] These studies found no evidence of PAX8 or PAX2 expression in melanomas. Contrary to these previous reports, we identified PAX8 staining in a small subset (7.9%) of melanoma cases. This higher rate of PAX8 positivity is likely related to the larger cohort size in our study. Based on h-score which provides information on the degree of staining, about half of the PAX8+ cases showed limited (weak/focal) staining, while the other half showed more significant staining. With the highest h-score being 210 (out of 300), it is important to recognize that rare melanomas may display strong PAX8 staining in a substantial number of cells, and that this finding alone does not necessarily exclude a diagnosis of melanoma. Interestingly, PAX8 expression was significantly associated with spindle cytomorphology. We hypothesize that spindle melanomas tend to be more poorly differentiated, and hence more likely to aberrantly express non-melanocytic markers. We also attribute the apparent association between PAX8 expression and primary melanomas to the fact that our primary melanoma cohort was relatively enriched in spindle melanomas. As it is known that spindle melanomas are also more likely to lose expression for melanocytic marker(s),[36] PAX8 expression in these tumors may result in a greater diagnostic pitfall, although morphologically a PAX8+ epithelioid melanoma may be more prone to misdiagnosis as a carcinoma.

Compared to PAX8, expression of PAX2 was much less frequently observed. Only one (0.4%) melanoma showed weak PAX2 staining that barely met our threshold for positivity (h-score of 20). Considering that PAX2 may be expressed in up to two-thirds of MiTF-RCCs,[31,37–39] we

conclude that any significant PAX2 staining would strongly favor MiTF-RCC over melanoma in the context of this differential diagnosis.

Aberrant expression of CK in melanoma has been well documented.[3–6,43] We performed CK in this study mainly to observe for any coexpression of CK with PAX8 or PAX2, which to our knowledge has not been reported in the literature. Such coexpression, if found, would be expected to pose a greater diagnostic pitfall. Previous studies have shown that MiTF-RCC tends to underexpress CK,[31,40,41] although more recent studies have reported higher rates of CK expression,[33,42] with Cam5.2 being slightly more sensitive than AE1/AE3.[33,40] Using a CK cocktail consisting of both AE1/AE3 and Cam5.2, we found CK expression in 1.2% of all melanomas. Similar to previously described,[4] CK staining was typically focal and weak when present in melanoma. We also found that CK expression was significantly associated with metastatic melanoma (relative to primary melanoma), a finding that was also concordant with prior reports and is presumably a result of antigenic shift associated with melanoma metastasis.[5,6,43] Notably, none of the CK+ melanomas co-expressed PAX8 or PAX2 in our cohort. This finding implies that co-expression of CK and PAX8 or PAX2 would strongly speak against a diagnosis of melanoma.

Our findings support the use of PAX8, PAX2, and CK immunohistochemical stains in the diagnostic workup of challenging tumors when melanoma is in the differential diagnosis of MiTF-RCC and other PAX8+ tumors. While expression of PAX8, especially when strong and diffuse, would favor a diagnosis of MiTF-RCC or other PAX8+ tumors, this finding alone does not completely exclude a diagnosis of melanoma. This is particularly important to keep in mind when dealing with kidney tumors that express melanocytic markers, or when examining a PAX8+/PAX2+/CK- skin tumor in someone with a history of MiTF-RCC. We therefore

recommend that CK and PAX2 be used in conjunction with PAX8 to increase diagnostic accuracy. This relatively basic immunohistochemical panel may be particularly useful in a practice setting where fluorescence in situ hybridization and PCR testing for *TFE3* and *TFEB* alterations are not readily available. It should be noted that TFE3 immunohistochemistry has limited sensitivity and specificity for detecting *TFE3* rearrangement and may suffer from considerable tissue fixation and antibody optimization issues.[42,44] Furthermore, TFE3 expression has been previously reported in a significant subset of melanomas.[45] As such, TFE3 immunohistochemistry has limited utility in distinguishing MiTF-RCC from melanoma, and should not be used alone in the diagnostic workup.[46]

This study has a few limitations. First, the use of TMAs as opposed to whole tissue sections potentially introduced sampling error, which is in part overcome by triplicate sampling of each tumor. Second, heavy melanin pigmentation in some of the melanomas, especially those with weak immunostaining, rendered accurate h-scoring difficult. Careful examination of all cases by an experienced dermatopathologist aimed to mitigate over- or under-interpretation. Third, it is possible that polyclonal and monoclonal antibodies may produce varying results. We did not validate our findings across different clones as only one type of each antibody was readily available in our laboratory. Finally, although the immunophenotype of MiTF-RCC has been well documented,[25-33] we were unable to directly compare the degree of immunostaining between melanoma and MiTF-RCC, in the form of h-score, without assessing the latter in this study.

In conclusion, while tumors expressing PAX8, PAX2, and/or CK are generally less likely to be melanoma, caution should be exercised when evaluating a skin tumor in patients with history of MiTF-RCC, or a renal tumor expressing melanocytic marker(s), as a small subset of primary and metastatic melanomas may demonstrate PAX8, PAX2, or CK staining. These markers should

therefore be used in an immunohistochemical panel rather than in isolation. Ultimately, cytogenetic or molecular studies may be required to distinguish melanoma and MiTF-RCC when immunohistochemistry fails to definitively resolve this differential diagnosis.

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TABLE

Table 1. PAX8, PAX2 and cytokeratin (AE1/AE3/Cam5.2) immunostaining results in melanomas

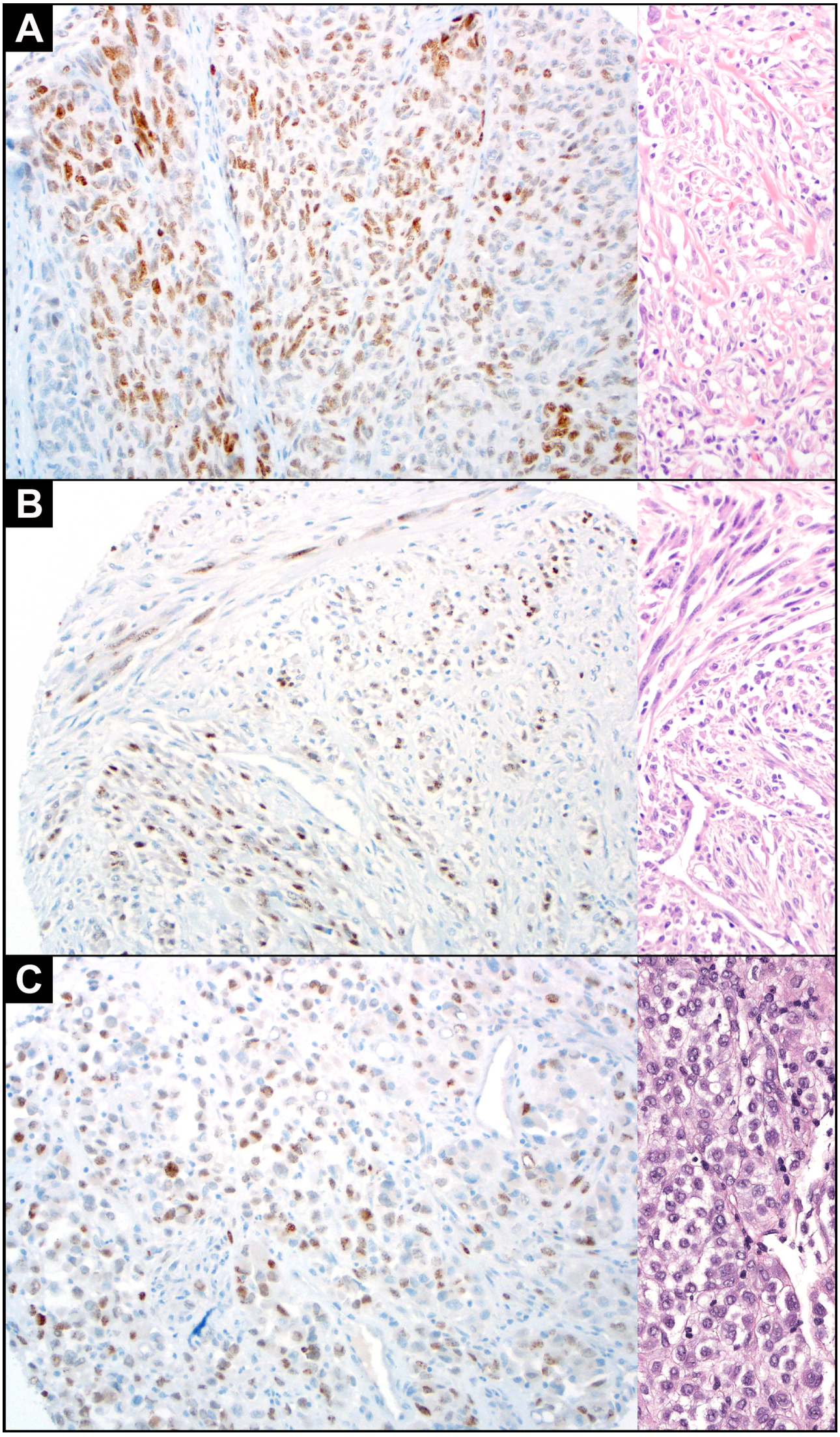
PAX8	Overall (n=253)	Primary (n=166)	Metastatic (n=87)	p-value	Epithelioid (n=206)	Spindle (n=47)	p-value
Positive	20	18	2	**0.01**	12	8	**0.02**
High-positive	9	7	2	0.72	5	4	0.06
PAX2	Overall (n=243)	Primary (n=169)	Metastatic (n=74)	p-value	Epithelioid (n=200)	Spindle (n=43)	p-value
Positive	1	0	1	1.00	1	0	1.00
High-positive	0	0	0	-	0	0	-
Cytokeratin	Overall (n=248)	Primary (n=166)	Metastatic (n=82)	p-value	Epithelioid (n=204)	Spindle (n=44)	p-value
Positive	3	0	3	**0.04**	3	0	1.00
High-positive	1	0	1	0.33	1	0	1.00

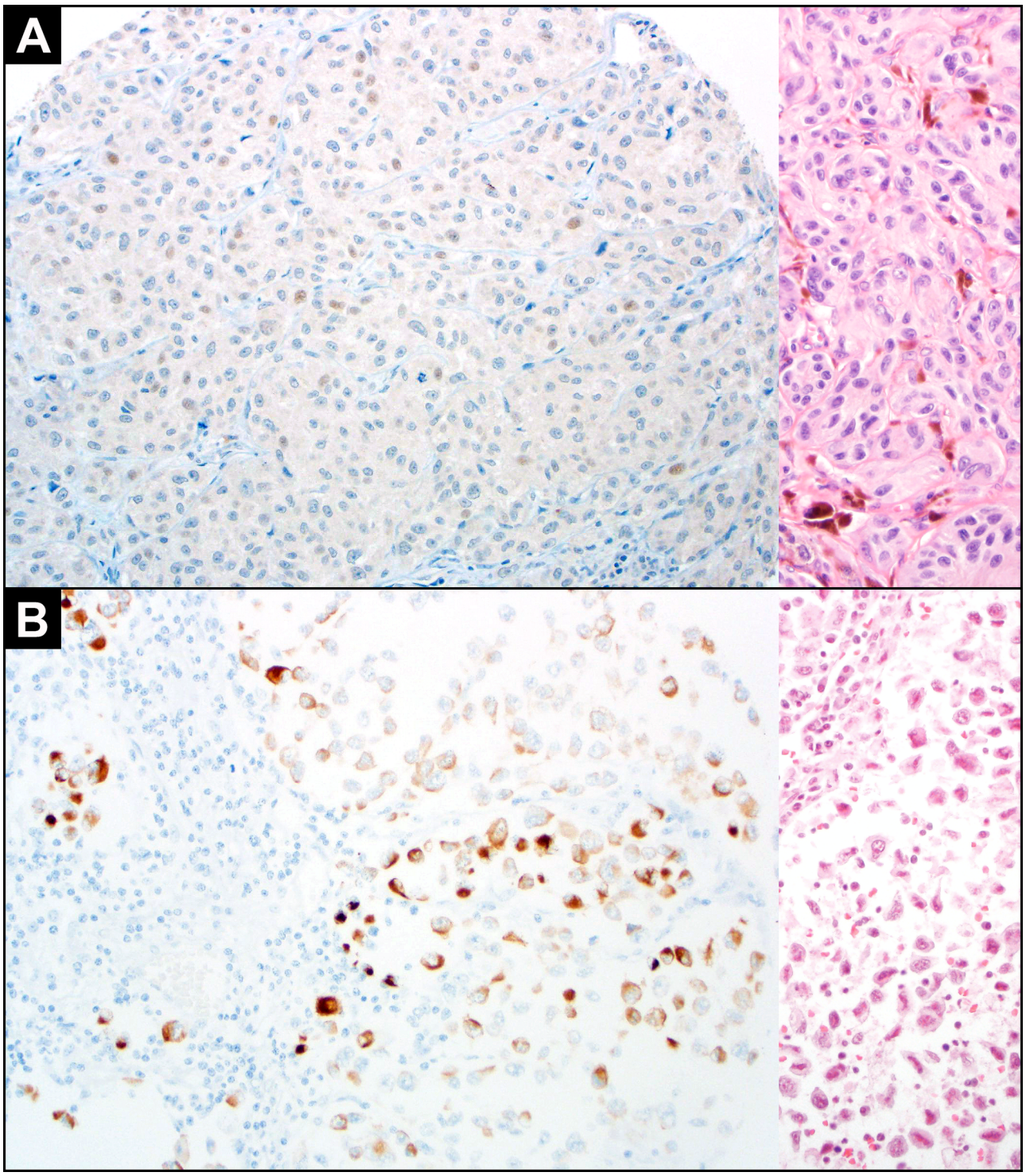
"Positive" refers to an h-score of ≥20. "High-positive" refers to an h-score of ≥60. Boldface indicates statistical significance.

FIGURE LEGENDS

Fig 1. Selected melanoma cases with aberrant PAX8 expression. A) This primary melanoma demonstrated strong nuclear PAX8 expression in a majority of tumor cells, corresponding to the highest PAX8 h-score (210) in this series. B) Another primary melanoma with predominantly spindle cytomorphology demonstrated PAX8 nuclear staining in a subset of the tumor cells. C) This epithelioid melanoma displayed PAX8 positivity in many tumor cells. (Left panel: PAX8 immunohistochemistry; right panel: hematoxylin-eosin; Original magnification: ×200)

Fig 2. Melanoma cases with aberrant PAX2 or cytokeratin expression. A) One melanoma in this series exhibited weak nuclear PAX2 expression in a minority of the tumor cells. B) A metastatic melanoma with epithelioid cytomorphology showed cytoplasmic staining for cytokeratin cocktail. (A, left panel: PAX2 immunohistochemistry, right panel: hematoxylin-eosin; B, left panel: AE1/AE3/Cam5.2 immunohistochemistry, right panel: hematoxylin-eosin; Original magnification: ×200)





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