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The diagnostic utility of immunohistochemistry in distinguishing primary skin adnexal carcinomas from metastatic adenocarcinoma to skin: an immunohistochemical reappraisal using cytokeratin 15, nestin, p63, D2-40, and calretinin

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Often the distinction of primary adnexal carcinoma from metastatic adenocarcinoma to skin from breast, lung, and other sites can be a diagnostic dilemma. Current markers purportedly of utility as diagnostic adjuncts include p63 and D2-40; however, their expression has been demonstrated in 11–22% and 5% of metastatic cutaneous metastases, respectively. Both cytokeratin (CK) 15 and nestin have been reported as follicular stem cell markers. We performed CK15 and nestin, as well as previously reported stains (such as p63, D2-40, and calretinin) on 113 cases (59 primary adnexal carcinomas and 54 cutaneous metastases). Expressions of p63, CK15, nestin, D2-40, and calretinin were observed in 91, 40, 37, 44, and 14% of primary adnexal carcinoma, respectively, and in 8, 2, 8, 4, and 10% of cutaneous metastases, respectively. p63 appeared to be the most sensitive marker (with a sensitivity of 91%) in detecting primary adnexal carcinomas. CK15 appeared to be the most specific marker with a specificity of 98%. Using χ^2 analysis, statistically significant *P*-values (<0.05) were observed for p63, CK15, nestin, and D2-40 in the distinction of primary adnexal carcinoma *versus* cutaneous metastases. In logistic regression and stepwise selection for predicting a primary adnexal carcinoma, statistical significance was observed for p63, CK15, and D2-40 (*P*-values: <0.001, 0.0275, and 0.0298, respectively) but not for nestin (*P*-value $=0.4573$). Our study indicates that diagnostic sensitivity and specificity are significantly improved using a selected panel of immunohistochemical markers, including p63, CK15, and D2-40. Positive staining with all three markers argues in favor of a primary cutaneous adnexal neoplasm.
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Cutaneous metastases from internal malignancies are often a marker for a widely disseminated neoplasm and may be a consequence of failure of ongoing therapy, the manifestation of an unsuspected occult neoplasm, or the result of a recurrence of a neoplasm that was believed to have been eradicated previously. Given this, understandably

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enough, the appearance of cutaneous metastases is a sign of poor prognosis, with survival from detection and/or diagnosis typically not exceeding 12 months.[1] The frequency of cutaneous metastases ranges from 0.7 to 10% in patients with visceral tumors. The most common sites for cutaneous metastases are the head and neck region and the trunk.[2] The primary carcinoma is typically of the breast, lung, gastrointestinal, and renal origin.[2]

Often the distinction of primary adnexal carcinoma from metastatic adenocarcinoma to skin from breast, lung, and other sites can be a diagnostic dilemma.[3–10] Current markers purportedly of utility as diagnostic adjuncts include p63 and D2-40, both reportedly preferentially expressed in the primary sweat gland carcinomas.[3–6,9,10] However, the utility of these is somewhat conflicting, given that frequent expression of p63 has been shown in 11–22% of metastatic cutaneous adenocarcinomas and that positive staining with D2-40, although focal, may be seen in up to 5% of cutaneous metastases.[3,6,9,10] Others markers cited include calretinin, found to be positive in 64% of primary cutaneous neoplasms and negative in 72% of cutaneous metastases.[10] Although Qureshi *et al*[3] suggested that CK5/6 may also be diagnostically useful, other studies have shown that CK5/6 expression can be observed in 9–15% of adenocarcinomas of various primary sites.[11,12] Although not completely specific, the differential expression of CK7 and CK20 may also be helpful particularly in delineating the site of origin of the primary carcinoma.[11]

During embryogenesis, hair follicles develop two or three bulges on their undersurface in the early bulbous stage.[13] The uppermost bulge, if present, either involutes or develops into an apocrine gland.[13] The remaining lower two bulges develop into sites for pilar muscle attachment and sebaceous gland, respectively.[13] Cytokeratin (CK) 15 and nestin have been reported to label hair follicle progenitor cells.[14–17] In a recent study (unpublished data, MM, MPH), we observed CK15 and nestin expression in 11 of 23 (48%) and 7 of 23 (30%) cases of adnexal carcinoma, respectively, including microcystic adnexal carcinoma, porocarcinoma, and eccrine carcinoma. In light of these findings and as follicular germinative cells are believed to give rise to folliculo-sebaceous-apocrine units,[13] we hypothesize that metastatic carcinoma from other sites should not express the skin/follicular stem cell markers CK15 and/or nestin.

In this study, we performed CK15 and nestin, as well as previously reported stains (such as p63, D2-40, and calretinin) in a series of cases to determine the following:

- Sensitivity and specificity of the stem cell markers CK15 and nestin in distinguishing primary adnexal carcinoma from cutaneous metastatic adenocarcinoma.
- Predictive value of each of the five stains studied (CK15, nestin, p63, D2-40, and calretinin) or in combination in distinguishing primary adnexal carcinoma from cutaneous metastatic adenocarcinoma using logistic regression analysis.

Materials and methods

The study was approved by the Institutional Review Boards of the Massachusetts General Hospital (2009-P-001210) and the Boston University Medical Center (H-28387). Archival materials obtained from 1989 to 2008 with a diagnosis of primary adnexal carcinomas and metastatic adenocarcinomas to the skin were retrieved from the database of both institutions. A total of 113 cases, 59 primary adnexal carcinomas and 54 cutaneous metastases, with available archival materials were retrieved from the pathology files of the Massachusetts General Hospital and Skin Pathology Laboratory of Boston University School of Medicine (Boston, MA, USA) (Table 1). The histological sections of all cases were re-reviewed and the diagnoses confirmed by two dermatopathologists (MM and MPH). Clinical information was extracted from medical records. All patient data were de-identified.

Immunohistochemical Analysis

Sections of 5-μm thickness were obtained for immunohistochemical studies, which were conducted on formalin-fixed, paraffin-embedded tissues using standard peroxidase immunohistochemistry techniques, heat-induced epitope retrieval buffer, and primary antibodies against CK15 (LHK15; 1:250, NeoMarkers, Fremont, CA, USA), nestin (MAB5326, 1:200, Chemicon, Temecula, CA, USA), p63 (7JUL, 1:25, NovoCastra, Newcastle upon Tyne, UK), D2-40 (1:40, Signet Pathology Systems, Dedham, MA, USA), and calretinin (1:100, Invitrogen, Camarillo, CA, USA). Appropriate positive and negative controls were included. The immunostains were reviewed by two dermatopathologists (MM and MPH), and disagreements were reviewed together to achieve a consensus score. Positive staining of CK15, nestin, p63, D2-40, and calretinin were scored as 3+ (51–100% of the tumor cells), 2+ (26–50%), 1+ (6–25%), and 0/negative ($\leq$5%).

Statistical Analysis

The statistical association of CK15, nestin, p63, D2-40, and calretinin immunohistochemical expressions in primary cutaneous carcinomas *versus* metastatic adenocarcinoma to the skin was analyzed by χ^2 analysis, with respect to each immunostain assessed in this study. A two-tailed P-value <0.05 was considered to be statistically significant. Logistic regression and stepwise selection for predicting a primary adnexal carcinoma were also

conducted. All variables were dichotomized for values >0 and 0.

Results

Immunohistochemical Evaluation

The immunohistochemical results for p63, CK15, nestin, D2-40, and calretinin are summarized in Tables 1 and 2, and in Figure 1. Positive staining of CK15, nestin, D2-40, and calretinin were noted by ascertaining expression in the cytoplasm and any nuclear staining was considered as background artifact. Nuclear staining was considered positive for p63. In each case, CK15 expression in the basal layer of the epidermis and secretory cells of eccrine glands and, expression of nestin within endothelial cells served as the internal control. D2-40 expression was noted in the basal keratinocytes, sebaceous glands, and some dermal lymphatics. Nuclear p63 expression was observed within basal keratinocytes and sebocytes. The innermost aspect of the outer root sheath of the hair follicle was labeled with calretinin.

Primary Adnexal Carcinoma

p63 appeared to be the most sensitive marker in detecting primary adnexal carcinomas (Tables 1 and 2,

Table 1 Immunohistochemical results of p63, cytokeratin 15, nestin, D2-40, and calretinin in primary adnexal carcinoma and cutaneous metastases

	N	*p63*	*Cytokeratin 15*	*Nestin*	*D2-40*	*Calretinin*
Primary adnexal carcinoma						
Eccrine carcinoma	11	8/10 (80%)	5/11 (45%)	4/11 (36%)	5/11 (45%)	1/11 (9%)
Apocrine carcinoma	2	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
Hidradenocarcinoma	9	9/9 (100%)	3/9 (33%)	5/9 (56%)	5/9 (56%)	1/9 (11%)
Porocarcinoma	17	16/16 (100%)	7/17 (41%)	6/17 (35%)	3/17 (18%)	0/17 (0%)
Microcystic adnexal carcinoma	15	12/12 (100%)	5/9 (56%)	6/15 (40%)	11/15 (73%)	4/15 (27%)
Trichilemmal carcinoma	3	3/3 (100%)	0/2 (0%)	0/3 (0%)	2/3 (67%)	2/3 (67%)
Mucinous carcinoma	1	0/1 (0%)	0/1 (0%)	0/1 (0%)	0/1 (0%)	0/1 (0%)
Adenoid cystic carcinoma	1	1/1 (100%)	1/1 (100%)	1/1 (100%)	0/1 (0%)	0/1 (0%)
Total	59	49/54 (91%)	21/52 (40%)	22/59 (37%)	26/59 (44%)	8/59 (14%)
Primary site of cutaneous metastases						
Breast	16	0/15 (0%)	1/16 (6%)	2/15 (13%)	0/16 (0%)	3/14 (21%)
Lung	8	2/8 (25%)	0/8 (0%)	1/8 (13%)	0/8 (0%)	0/8 (0%)
Ovary	2	0/2 (0%)	0/2 (0%)	1/2 (50%)	2/2 (100%)	1/2 (50%)
Endometrium	2	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
Stomach	2	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
Pancreas	4	2/4 (50%)	0/4 (0%)	0/4 (0%)	0/4 (0%)	0/4 (0%)
Thyroid	3	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	1/3 (33%)
Kidney	3	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)
Paget's disease	5	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)
Extramammary Paget's disease	9	0/9 (0%)	0/9 (0%)	0/9 (0%)	0/9 (0%)	0/7 (0%)
Total	54	4/53 (8%)	1/54 (2%)	4/53 (8%)	2/54 (4%)	5/50 (10%)
P-value		<0.0001*	<0.0001*	0.0002*	<0.0001*	0.5678
Sensitivity		91%	40%	37%	44%	14%
Specificity		92%	98%	92%	96%	90%

*Statistically significant.

Table 2 Scoring summary of p63, cytokeratin 15, nestin, D2-40, and calretinin immunohistochemical stains

Markers	*Primary adnexal carcinoma*					*Metastatic adenocarcinoma to skin*				
	Grade of reactivity					*Grade of reactivity*				
	+ Cases (%)	*0 (%)*	*1+ (%)*	*2+ (%)*	*3+ (%)*	*+ Cases (%)*	*0 (%)*	*1+ (%)*	*2+ (%)*	*3+ (%)*
p63	44/54 (81%)	5 (9%)	5 (9%)	10 (19%)	34 (63%)	1/53 (2%)	49 (92%)	3 (6%)	1 (2%)	0 (0%)
CK15	21/52 (40%)	31 (60%)	4 (8%)	8 (15%)	9 (17%)	1/54 (2%)	53 (98%)	0 (0%)	0 (0%)	1 (2%)
Nestin	22/59 (37%)	37 (63%)	3 (5%)	10 (17%)	9 (15%)	4/53 (8%)	49 (92%)	2 (4%)	2 (4%)	0 (0%)
D2-40	26/59 (44%)	33 (56%)	2 (3%)	15 (25%)	9 (15%)	2/54 (4%)	52 (96%)	0 (0%)	2 (4%)	0 (0%)
Calretinin	8/59 (14%)	51 (86%)	7 (12%)	1 (2%)	0 (0%)	5/50 (10%)	45 (90%)	4 (8%)	1 (2%)	0 (0%)

0/negative ($\leq$5% of the tumor cells), 1+ (6–25%), 2+ (26–50%), and 3+ (51–100%).

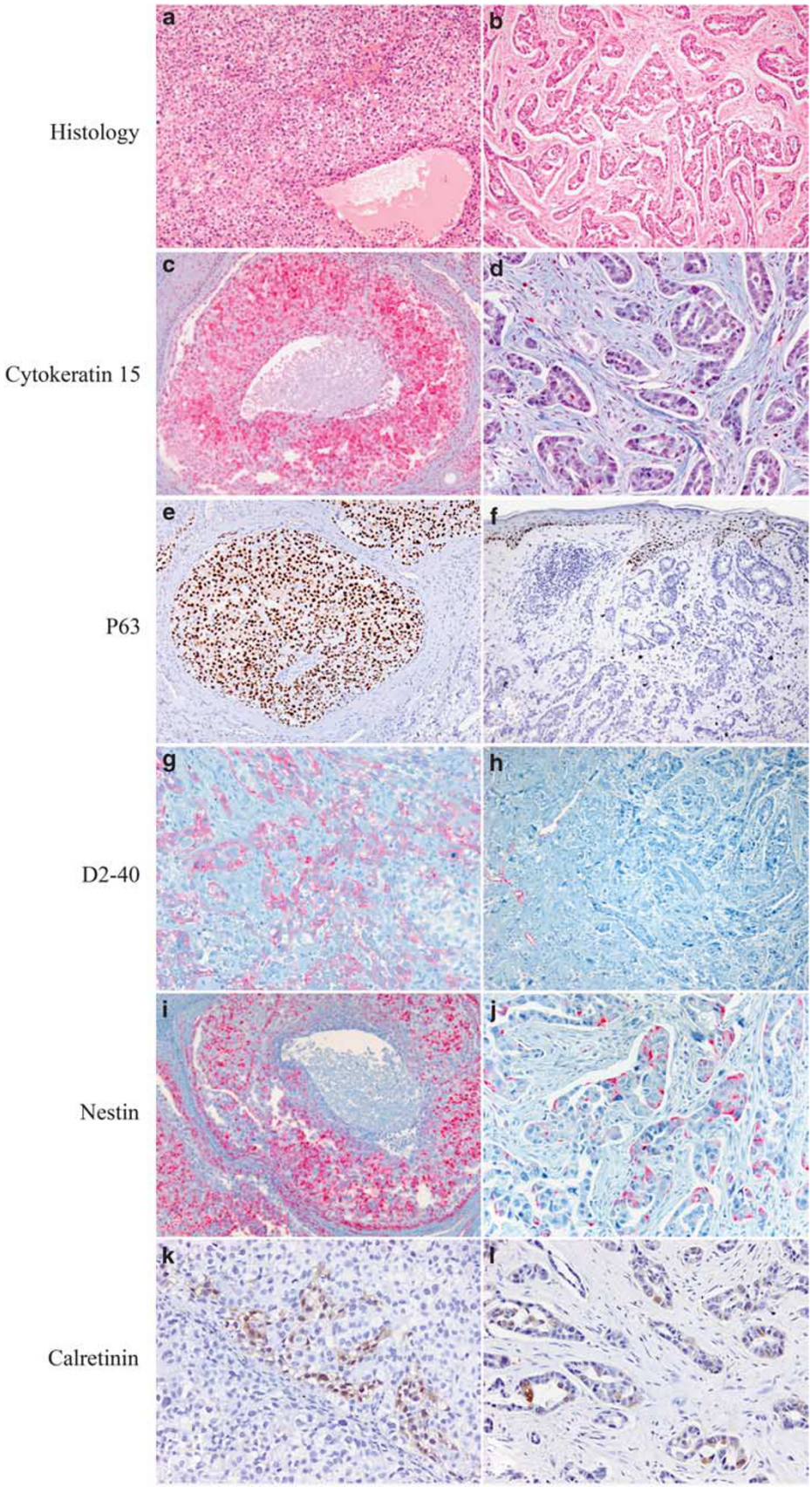
Histology
Cytokeratin 15
P63
D2-40
Nestin
Calretinin
a
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Figure 1). With the exception of apocrine and mucinous carcinoma, p63 expression was seen in the majority of the cases. The expression of CK15 and nestin was similar with the sensitivity of 40 and 37%, respectively, with the exception of apocrine, mucinous, and trichilemmal carcinoma. D2-40 expression was seen in 44% of cases, including eccrine carcinoma, hidradenocarcinoma, microcystic adnexal carcinoma, and trichilemmal carcinoma. Calretinin expression was seen in 14% of cases, most frequently in trichilemmal carcinoma (2/3, 67%).

Cutaneous Metastases

The expression of the five studied markers was most often seen in metastatic breast carcinoma, and also in metastatic lung, ovarian, pancreatic, and thyroid carcinomas (Tables 1 and 2, Figure 1). In the 16 cases of metastatic breast carcinoma, CK15, nestin, and calretinin expressions were seen in 6, 13, and 21%, respectively. In the 8 cases of metastatic lung carcinoma, p63 and nestin expressions were seen in 25 and 13% of cases, respectively. Nestin and D2-40 expressions were seen in one and both cases of metastatic ovarian carcinomas, respectively. p63 and calretinin expressions were seen in 2 of 4 and 1 of 3 cases of metastatic pancreatic and thyroid carcinomas, respectively.

Statistical Analysis

The sensitivity and specificity of each immunostain were calculated and are listed in Table 1. Statistically significant *P*-values were observed for p63, CK15, nestin, and D2-40 in the distinction of primary adnexal carcinoma *versus* cutaneous metastases. In logistic regression for predicting a primary adnexal carcinoma, statistical significance was observed for p63, CK15, and D2-40 (*P*-values: <0.001, 0.0275, and 0.0298, respectively) and not for nestin (*P*-value $= 0.4573$). In further application of stepwise selection, the odds ratio estimates were 64.410, 21.658, and 11.461 for p63, CK15, and D2-40, respectively.

Discussion

As previously reported, we observed that p63 is the most sensitive marker for primary adnexal carcinoma.[3–6,10] In studies published by Ivan *et al*,[4,5] tumors with $<25\%$ p63 nuclear staining were considered negative. Thus, with this higher cutoff value, the authors reported a 100% specificity with the majority of the cutaneous metastases being negative for p63, although focal staining was reported in 5 of 14 (36%) cases. Using this same cutoff value, although we observed a comparably high specificity (98%) with p63 in our cases, we found that the sensitivity was only 78%. Using the lower cutoff values used by Qureshi *et al*,[3] Kanitakis and Chouvet,[6] and Sariya *et al*[10] increased the sensitivity (91% in our study) in detecting primary adnexal carcinoma. Overall, 8% of our cutaneous metastases stained with p63, compared with 13, 11, and 22% (7/32) reported by Qureshi *et al*,[3] Kanitakis and Chouvet,[6] and Sariya *et al*,[10] respectively. Thus, if p63 was to be used as a single diagnostic marker, a number of cutaneous metastases would be classified as primary adnexal carcinoma.

p63, a nuclear transcription factor that triggers keratinocyte differentiation, is downregulated in terminally differentiated cells.[18–20] In previous studies, p63 expression was noted in epidermal and adnexal basal/myoepithelial cells.[21,22] Similar to the series by Ivan *et al*,[4] all cases of trichilemmal carcinomas, microcystic adnexal carcinoma, and eccrine carcinoma expressed p63 in our series. Ivan *et al*[5] and Sariya *et al*[10] reported that both primary cutaneous mucinous carcinoma and apocrine carcinoma are p63 negative. Similarly, our two apocrine carcinomas and one mucinous carcinoma were negative for p63, as well as for CK15, nestin, D2-40, and calretinin.

Of the five markers included in the current study, the stem cell marker CK15 appeared to be the most specific with a specificity of 98%. Thus, the expression of CK15 in a malignant cutaneous tumor would support the diagnosis of a primary adnexal carcinoma opposed to a metastasis. However, we found the sensitivity of CK15 to be only 40%. CK15 has been cited as a relatively specific marker of hair follicle stem (germinative) cells in the bulge region.[14] CK15 expression was reported in follicular neoplasm,[23] microcystic adnexal carcinoma,[24] apocrine mixed tumors of the skin, and other benign neoplasms with apocrine differentiation.[25,26] Our own experience indicates that CK15 expression may be seen in desmoplastic trichoepithelioma, basal cell carcinoma (only the nodular and keratotic subtypes), and microcystic adnexal carcinoma with understandably enough, negative expression in squamous cell carcinoma.[16,24] More recently, we have also found strong CK15 expression in benign adnexal neoplasms, such as chondroid syringoma, hidradenoma papilliferum, apocrine hidrocystoma, and cylindroma/spiradenoma (unpublished data, MM, MPH).

D2-40 monoclonal antibody specifically detects a fixation-resistant epitope on podoplanin.[27] Although podoplanin is one of the most highly

Figure 1 In an eccrine carcinoma (**a**), the tumor cells were strongly positive for cytokeratin 15 (**c**), p63 (**e**), D2-40 (**g**), and nestin (**i**), and focally for calretinin (**k**). In contrast, in a case of metastatic breast carcinoma (**b**), the tumor cells were negative for cytokeratin 15 (**d**), p63 (**f**), and D2-40 (**h**), whereas focally positive for nestin (**j**) and calretinin (**l**). As internal control, the basal keratinocytes were positive for p63 (panel f) and the small vessels adjacent to the tumor were D2-40 positive (panel h).

expressed lymphatic endothelium-specific genes, the clinical utility of D2-40 immunostain is not confined to the detection of lymphatic endothelial cells.[28,29] To date, there has been only one series studying the expression of D2-40 in primary skin adnexal carcinomas in comparison with cutaneous metastases.[9] Our results somewhat conflict those obtained by Liang *et al*[9] in that we observed a similar high specificity (96 *versus* 97.2% by Liang *et al*[9]), yet a significant difference was noted in the sensitivity (44 *versus* 94.5% by Liang *et al*[9]). Furthermore, in contrast to the 100% positivity reported in the series by Liang *et al*,[9] we observed only 18, 45, 56, and 73% D2-40 positivity for porocarcinoma, eccrine carcinoma, hidradenocarcinoma, and microcystic adnexal carcinoma, respectively. A simplistic explanation is that the results are reflective of differences relating to the clone of antibody or dilutions used. Although we used the same clone of antibody, our dilution was optimized to 1:40 compared with 1:25 in the study by Liang *et al*.[9] Furthermore, the authors also included 10 cases of squamous cell carcinoma, which is technically not a primary adnexal carcinoma, although we also observed strong D2-40 positivity in the same (unpublished data, MM, MPH). Last but not least, the observed differences are a consequence of different cutoffs; staining of even 1% was considered positive in the series by Liang *et al*,[9] whereas we used a cutoff value of 5%.

The expression of the intermediate protein nestin, a marker for neural progenitor cells, initially noted in the bulge area of nestin-GF transgenic mice, has recently been noted in the epidermis and the upper two-third of the hair follicles in normal human scalp.[15–17] Although the expression of nestin in cutaneous metastases, has to date, not been previously evaluated, our recent experience with expression of the same in benign adnexal neoplasms indicates that it parallels that of CK15. In the current study, we found nestin to have a good specificity (92%), yet low sensitivity (37%). Thus, in our experience, nestin does not appear to be of utility as an immunohistochemical adjunct in a screening panel.

Calretinin is a calcium-binding protein of 29 kDa that belongs to the EF-hand family, and calcium-dependent regulation is believed to be important for hair follicle development.[30] Calretinin expression is seen in various normal and neoplastic tissues.[31] It has been reported to label the innermost layer or the companion cell layer of the outer root sheath of hair follicle.[32] In the only series investigating the diagnostic utility of calretinin in cutaneous tumors, Sariya *et al*[10] found calretinin expression in 64% (16/25) of primary adnexal carcinoma and 28% (9/32) of cutaneous metastases. In contrast, we observed calretinin positivity in only 14 and 10% of primary adnexal carcinoma and cutaneous metastases, respectively. Although differences observed might relate to the utilization of antibodies directed perhaps against different epitopes, Sariya *et al*[10] add the qualifier that staining with calretinin was observed only a sub-population of tumor cells. Although its specificity is 90% in our study, we observed a low sensitivity of 14%. Thus, in our experience, calretinin, similar to nestin, does not appear to be of utility as an immunohistochemical adjunct in a screening panel.

The morphological distinction between a metastatic tumor and a primary adnexal neoplasm can be difficult if not impossible. Although the presence of an epidermal connection, might argue in favor of a primary adnexal neoplasm, metastases from several primary malignancies such as adenocarcinomas of the prostate, breast, or colon, although infrequently, have been reported to exhibit epidermotropism.[33] Clinical findings such as single *versus* multiple nodules and duration of skin nodule either greater or less than 6 months have been found to be very useful in the distinction of primary *versus* metastatic skin tumor. However, pertinent clinical history is often not available and lesions can masquerade as dermatoses, and the metastases may be 'precocious' or the first indication of a visceral cancer.[34]

In summary, our findings underscore the diagnostic utility of ancillary adjuncts such as immunohistochemistry when confronted with a neoplasm of unknown origin. Data from the current study, although small in size yet the largest to date on primary adnexal neoplasms *versus* cutaneous metastases, indicate that both diagnostic sensitivity and specificity are significantly improved using a selected panel of immunohistochemical markers, including p63, CK15, and D2-40. Positive staining with all three markers argues in favor of a primary cutaneous adnexal neoplasm.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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