# The Application of Immunocytochemistry to Cytologic Direct Smears of Metastatic Merkel Cell Carcinoma

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*Merkel cell carcinoma represents a highly aggressive cutaneous malignancy characterized by regional recurrences, lymph node metastases, distant metastases, and high mortality. As the cytomorphology of Merkel cell carcinoma can be mimicked by other malignancies, especially lymphoma and pulmonary small cell carcinoma, immunocytochemistry is often useful in confirming the diagnosis. Cell blocks, which are traditionally utilized for immunocytochemistry, occasionally exhibit insufficient cellularity. Hence, we prospectively investigated the application of CK20 immunocytochemistry to air-dried, unstained direct smears in the diagnosis of Merkel cell carcinoma fine needle aspirates (FNAs). Eight consecutive FNAs of Merkel cell carcinoma were prospectively examined in this series; seven (88%) cases exhibited immunoreactivity for CK20 in the tumor cells. The one CK20-negative Merkel cell carcinoma was immunoreactive for synaptophysin and CD56. This immunophenotype was identical to that of the original primary tumor. For comparison, air-dried direct smears prepared from three pulmonary small cell carcinoma FNAs were examined by CK20 immunocytochemistry. In all cases, no CK20 immunoreactivity was seen in any of the tumor cells. In conclusion, direct smears represent a feasible and robust source of cellular material for immunocytochemical studies to diagnose Merkel cell carcinoma. This methodology allows the cytologist to confirm on site that material for diagnostic immunocytochemistry is present thereby serving as a safeguard in instances where insufficient cell block cellularity is anticipated or encountered.* Diagn. Cytopathol. 2013;41:729–733. 

*Key Words:* Merkel cell carcinoma; CK20; immunocytochemistry; direct smear; cytology

Merkel cell carcinoma represents a highly aggressive primary cutaneous neuroendocrine malignancy. Although Merkel cell carcinoma is a rare entity, the incidence of this cancer has been increasing steadily.[1,2] These tumors are characterized by a high incidence of local recurrences, lymph node and distant metastases, and high mortality.[1] The exact etiology is unknown; nonetheless, risk factors include increased age, immunosuppression, and skin radiation exposure.[1] Recently, the possibility of a viral etiology has been raised as a new polyomavirus, Merkel cell polyomavirus, was characterized from Merkel cell carcinoma tumor tissues.[3–5]

Fine-needle aspiration (FNA) represents a minimally invasive technique and an accurate, safe, affordable means to achieve a tissue diagnosis of metastatic Merkel cell carcinoma.[6–10] An early definitive diagnosis of metastatic Merkel cell carcinoma allows for accurate staging and prompt, appropriate management including surgical removal and/or chemotherapy. The cytomorphology of Merkel cell carcinoma is typically characterized by small, round to oval cells with scant cytoplasm and nuclei exhibiting finely granular chromatin without prominent nucleoli.[7,9,11] The cells can be present as a discohesive population or as cohesive clusters of cells. In the latter scenario, nuclear molding is common and rosettes can

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occasionally be observed. Regardless, these cytomorphologic patterns are not specific for Merkel cell carcinoma as other malignancies such as lymphoma, small cell carcinoma of the lung, and melanoma can mimic one or both of these patterns.

Immunohistochemistry serves as a useful ancillary technique in differentiating Merkel cell carcinoma from morphologically similar malignancies. Nonetheless, there exist only a limited number of reports in the literature that specifically examine the role of immunocytochemistry in the diagnosis of Merkel cell carcinoma in cytology specimens. For instance, unlike lymphomas, Merkel cell carcinoma is negative for leukocyte common antigen (LCA) and immunostaining with antibodies directed against broad spectrum cytokeratins characteristically highlight the tumor cells in a perinuclear dot-like pattern.[7] Neuroendocrine differentiation can be confirmed by demonstrating immunoreactivity of the tumor cells for one or more of the following markers: neuron-specific enolase (NSE); CD56; chromogranin; and synaptophysin. Nonetheless, this overall immunophenotype does not allow for the distinction between Merkel cell carcinoma and pulmonary small cell carcinoma.

Immunocytochemistry utilizing antibodies directed against cytokeratin 7 (CK7) and cytokeratin 20 (CK20) often allows for the distinction between Merkel cell carcinoma and pulmonary small cell carcinoma. Whereas the majority of small cell carcinomas of the lung display CK7 positivity, only a small subset of Merkel cell carcinomas are CK7 positive.[12] In contrast, the vast majority of Merkel cell carcinomas display immunoreactivity for CK20 whereas pulmonary small cell carcinomas are essentially CK20 negative.[12,13]

Ancillary immunocytochemical studies routinely employ cell blocks prepared from Merkel cell carcinoma FNAs. Occasionally, insufficient cellularity of the cell blocks can represent a problematic issue thereby impeding immunophenotypic characterization of the tumor cells. This can result in repeat procedures to obtain additional cellular material for the cell block, which are not without potential complications, and can result in delays in treatment. We recently reported the diagnostic utility of immunocytochemistry on direct smears in the subclassification of pulmonary non-small cell carcinomas and the diagnosis of metastatic melanomas.[14,15] Hence, we prospectively investigated the application of immunocytochemistry for CK20 to cytologic direct smears prepared from Merkel cell carcinoma FNAs and describe our experience herein.

## Materials and Methods

The study was approved by the Institutional Review Board at University of Michigan. Eight consecutive cases of metastatic Merkel cell carcinoma were prospectively evaluated in which unstained direct smears were prepared, using positively charged slides, for confirmatory immunocytochemistry. Immunocytochemistry was performed on air-dried, unstained direct smears, following formalin fixation, using the Ventana Autostainer (Ventana Medical Systems, Tucson, AZ) as performed previously.[14,15] Immunostaining for CK20 (clone Ks20.8; 1:100 dilution; Dako, Carpenteria, CA) was performed after protease 2 pretreatment (Ventana Medical Systems, Tucson, AZ) for 8 min. Immunocytochemistry for CD56 (clone 123C3; predilute; Cell Marque, Rocklin, CA) and synaptophysin (clone SP11; predilute; Ventana Medical Systems, Tucson, AZ) were performed following 30-min pretreatment in CC1 buffer at 95°C. Finally, immunocytochemistry for CK20 was applied to direct smears in three cases of pulmonary small cell carcinoma.

**Table I.** Immunocytochemistry on Direct Smears of Merkel Cell Carcinoma

| *Case no.* | *FNA site* | *CK20 immunophenotype* |
|---|---|---|
| 1 | Right preauricular nodule | Positive |
| 2 | Anterior neck lymph node | Positive |
| 3 | Left postauricular mass | Negative |
| 4 | Right dorsal foot mass | Positive |
| 5 | Right axillary lymph node | Positive |
| 6 | Left submental lymph node | Positive |
| 7 | Left inguinal nodule | Positive |
| 8 | Right proximal foot nodule | Positive |

Abbreviations: CK20, cytokeratin 20; FNA, fine needle aspiration.

## Results

Eight consecutive cases of Merkel cell carcinoma, in which unstained, air-dried direct smears were obtained on-site, were analyzed by immunocytochemistry for CK20. The sites of the FNA procedures and the CK20 immunophenotypes are summarized in Table I. The aspirates in each case were cellular and composed of a discohesive and/or loosely cohesive population of small, round blue cells exhibiting nuclei with finely granular chromatin and high nuclear-to-cytoplasmic ratios (Figs. 1 and 2). In seven (88%) of the eight cases, immunoreactivity for CK20 was observed (Fig. 1). In one case (Table I; Case 3), none of the tumor cells were CK20 positive (Fig. 2). The tumor cells were diffusely immunopositive for the neuroendocrine markers, CD56 and synaptophysin. This CK20(-)/CD56(+)/synaptophysin(+) immunophenotype was identical to that observed in the tumor cells within the primary site (Fig. 2). For comparison, immunocytochemistry for CK20 was applied to direct smears prepared from three FNA cases of pulmonary small cell carcinoma, a morphologic mimic of Merkel cell carcinoma. In all three cases, the tumor cells did not exhibit immunoreactivity for CK20.

## Discussion

Metastatic Merkel cell carcinoma typically presents cytomorphologically as a discohesive, loosely cohesive, and/

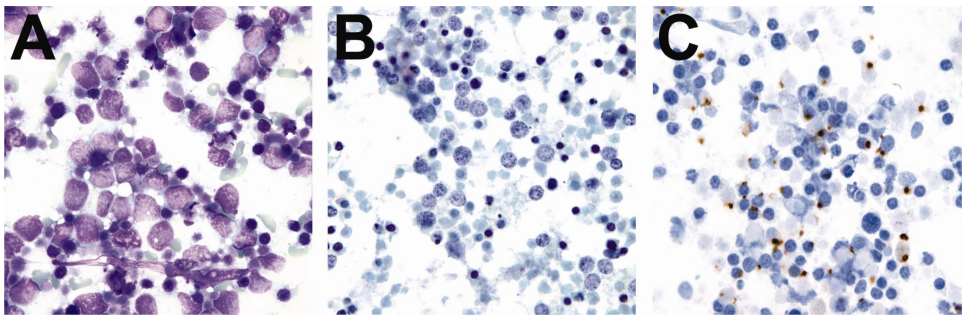


**Fig. 1.** Immunocytochemistry of a CK20-positive Merkel cell carcinoma. (**A**) The Diff-Quik and (**B**) Papanicolaou stained smears demonstrate the presence of small round blue cells exhibiting nuclei with finely granular chromatin and scant cytoplasm. Nuclear molding can be appreciated on the Diff-Quik stained slide. (×1,000) (**C**) The malignant cells exhibit immunoreactivity for CK20 in a perinuclear dot-like pattern (×1,000). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

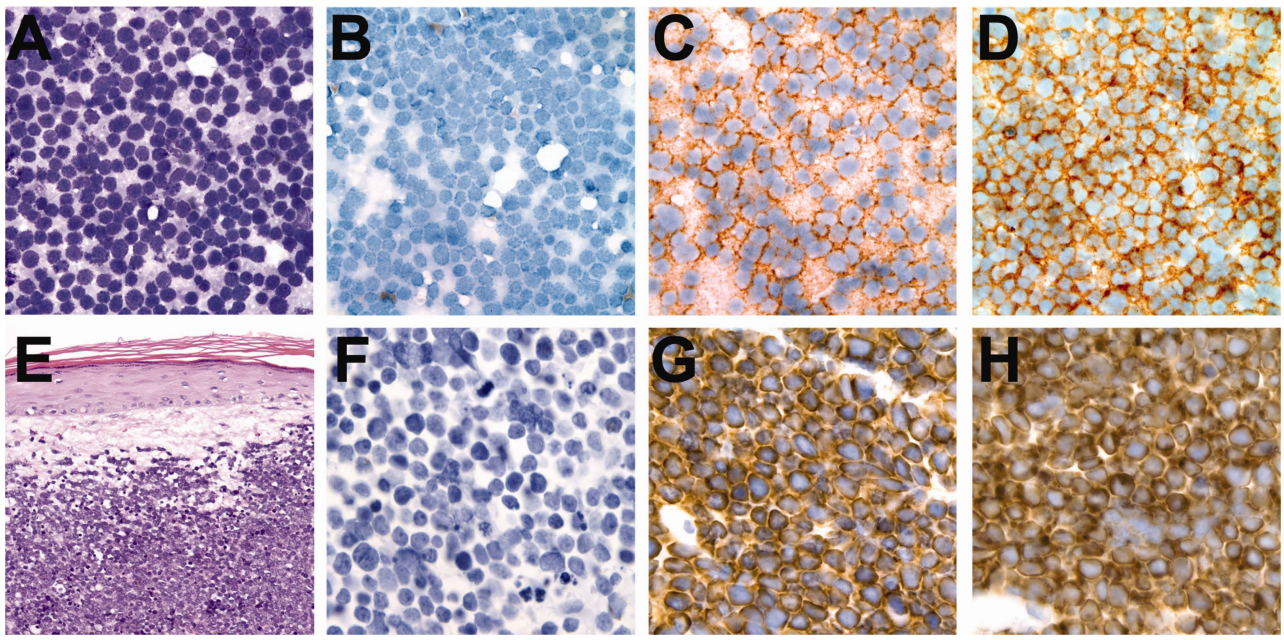


**Fig. 2.** Immunocytochemistry of a CK20-negative Merkel cell carcinoma. (**A**) A predominantly discohesive population of small round blue cells with nuclei exhibiting finely granular chromatin is seen in this Diff-Quik stained smear (×1,000). (**B–D**) Immunostains for CK20, CD56, and synaptophysin are shown, respectively (×1,000). The tumor cells are CK20-negative but are positive for CD56 and synaptophysin. (**E**) The cutaneous primary Merkel cell carcinoma is displayed (H&E, ×400). (**F–H**) Immunostains for CK20, CD56, and synaptophysin, respectively, reveal that the tumor cells display the identical immunophenotype as those demonstrated on the cytologic direct smears (×1,000). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

or cohesive population of small round blue cells in FNA specimens. As these patterns can mimic lymphoma and especially small cell carcinoma of the lung, difficulties can be encountered in the cytodiagnosis of metastatic Merkel cell carcinoma. Accurate diagnosis of this entity allows for efficacious staging of patients afflicted by this disease allowing for prompt formulation of appropriate next steps in management.

An established history of Merkel cell carcinoma is often helpful and immunocytochemistry serves as an essential adjunct in confirming the diagnosis and distinguishing this tumor from pulmonary small cell carcinoma. Cell blocks, traditionally, have represented the primary platform for immunocytochemistry. Nonetheless, insufficiencies in cellular material are occasionally encountered in these preparations thereby posing a problematic issue with regards to further workup of Merkel cell carcinoma FNAs. Furthermore, the cellularity of the cell blocks, as determined by examining H&E sections prepared after processing and embedding, is not immediately known. Cell block cellularity is influenced by several variables including: the overall cellularity and percent tumor cellularity of the targeted lesion; the precision in targeting the lesion in successive FNA passes; effective sampling during dedicated passes for the cell block; and handling of the needle rinse specimen in the cytopreparative labora-

tory. Dedicated passes for the needle rinse are often performed to increase the probability that the cell block exhibits adequate tumor cellularity for ancillary studies. Nonetheless, it is not possible to ensure the effectiveness of this approach at the time of the FNA procedure. On the basis of our experience, we recognized the realistic possibility that cell blocks can occasionally provide inadequate material especially in scenarios where the need for ancillary studies is the greatest. Hence, we sought to investigate the application and diagnostic utility of immunocytochemistry using direct smears prepared from FNAs of metastatic Merkel cell carcinoma.

In this study, we prospectively performed immunocytochemistry for CK20 using unstained, air-dried direct smears in eight consecutive FNAs performed on patients with either clinically suspected or previously biopsy-proven Merkel cell carcinoma. In seven (88%) of these cases, the tumor cells exhibited immunoreactivity for CK20. In one case, the CK20 immunostain was negative in the tumor cells. The previously biopsied cutaneous primary tumor was reviewed in this case and the CK20 immunophenotype was compared. The CK20(-) immunophenotype was also observed in the primary tumor. Workup of the primary tumor biopsy revealed immunoreactivity for the neuroendocrine markers, CD56, and synaptophysin. Thus, in this instance, we also performed immunocytochemistry for these two markers on unstained direct smears. The CD56 and synaptophysin immunostains were positive in the FNA specimen reflecting the identical immunophenotype in the biopsy specimen. As patients with Merkel cell carcinoma typically have had tissue biopsy performed from the primary tumor, this case illustrates the importance of correlating the cytomorphology and immunophenotypes of the primary site and metastatic focus. Although a limited number of cases were examined in this study, owing to the rarity of the disease and the prospective manner in which the cases were examined, the CK20 positivity rate of 88% is in keeping with prior reports that have specifically analyzed this marker in Merkel cell carcinomas.[12,13] Of note, a small number of Merkel cell carcinomas that are immunoreactive for CK7 have been reported in the literature.[12,13,16,17] Immunocytochemistry for CK7, however, would be of limited utility as CK7 positivity would confirm the diagnosis of a carcinoma but not allow for distinction from small cell carcinoma of the lung. In this context, demonstrating nuclear immunoreactivity for TTF-1 would serve an important role in confirming the diagnosis of pulmonary small cell carcinoma. In this study, we focused on CK20 immunocytochemistry due to the high specificity of this marker in the diagnosis of Merkel cell carcinoma. The specificity of CK20 expression in Merkel cell carcinoma is highlighted by our observation of the CK20 negative immunophenotype observed on immunostained direct smears prepared from FNAs of pulmonary small cell carcinoma.

These findings corroborate our previous reports describing the efficacy of performing confirmatory immunocytochemistry on air-dried, unstained direct smears.[14,15] The advantage of this approach is mainly 2-fold. First, additional unstained direct smears on positively charged slides can be prepared from a given FNA pass, in addition to the routine Diff-Quik stained and alcohol-fixed Papanicolaou stained smears, simply by distributing the FNA material over multiple slides. This would allow for the cytologist to confirm, during the on-site procedure, that adequate material has been obtained not only for cytomorphologic evaluation but also for ancillary studies. Specifically, visualization of tumor cells on the Diff-Quik stained smear would signify the presence of tumor cells in the additional unstained direct smears. Second, the unstained smears can be directly and promptly submitted for immunostaining. In each of these eight cases of metastatic Merkel cell carcinoma, a final diagnosis inclusive of the results of confirmatory immunostains was reported on the same day of the procedure. Hence, this approach can lead to improved turnaround time in diagnosis.

In addition to unstained direct smears, unstained cytospin preparations also represent an analogous platform for immunocytochemistry that is especially useful when encountering limited material on FNAs. The use of cytospins also allows for dispersal of cellular material over multiple slides thereby enabling the use of panels of immunostains.[18] The efficacy of cytospin preparations in performing immunocytochemistry is dependent on the cellularity of the needle-rinse. Immunostaining of smears and cytospins can both be occasionally associated with relatively higher background staining. This can pose challenges in the interpretation of some immunostains, especially those geared toward immunophenotypic confirmation of clonality in lymphoproliferative disorders, which represent mimics of Merkel cell carcinoma. The needle-rinse specimen, therefore, remains an integral component of FNA specimen triage as a portion of or the entire needle-rinse can be submitted for flow cytometric analysis in the diagnostic workup of lymphomas.

We acknowledge that continued utilization of needle rinses and dedicated passes for the cell block are essential to preserve the remainder of the cellular material in formalin-fixed, paraffin-embedded form for additional future studies as needed. The use of unstained direct smears for ancillary immunocytochemistry, however, would reduce and potentially eliminate the sole reliance on the cell block for immunocytochemistry. This has important implications in the optimal triage of FNA material especially in an era during which the number of ancillary immunocytochemical and molecular studies to be performed using cytology specimens is likely to increase.

For instance, the use of FNA material to detect BRAF mutations in metastatic melanoma along with EGFR and KRAS mutations in pulmonary non-small cell carcinoma is becoming increasingly appreciated.[14,19,20] In these settings, we have demonstrated that direct smears represent a robust source of cellular material for the performance of these molecular studies. Hence, optimization of FNA specimen triage during on-site assessments represents an important opportunity for cytopathologists to meet this challenge. This would further cement the essential role of FNA cytology in the management of patients with metastatic cancer while preventing additional invasive procedures that could result from scenarios in which the cell blocks exhibit insufficient cellularity.

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