Merkel Cell Polyomavirus Expression in Merkel Cell Carcinomas and Its Absence in Combined Tumors and Pulmonary Neuroendocrine Carcinomas

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Abstract: Merkel cell carcinoma (MCC) is the eponym for primary cutaneous neuroendocrine carcinoma. Recently, a new polyoma virus has been identified that is clonally integrated in the genome of the majority of MCCs, with truncating mutations in the viral large T antigen gene. We examined the presence of Merkel cell polyomavirus (MCV) in a set of 17 frozen tumor samples by quantitative polymerase chain reaction; 15 of them (88%) were positive. Sections from corresponding archival material were analyzed by immunohistochemistry (IHC) with the novel monoclonal antibody CM2B4, generated against a predicted antigenic epitope on the MCV T antigen, and tested for the expression of cytokeratin 20 (CK20). Sufficient archival material for IHC was available in only 15 of the 17 cases whose frozen tissue samples had been studied by polymerase chain reaction. Of the 15 tumors analyzed immunohistochemically, 10 (67%) showed positive labeling with CM2B4, 14 (93%) expressed CK20. A tissue microarray of 36 MCCs, 7 combined squamous and neuroendocrine carcinomas of the skin, and 26 pulmonary neuroendocrine carcinomas were also examined by IHC. Of the 36 MCCs assembled on a microarray, 32 (89%) tumors expressed CK20, and 27 (75%) were immunoreactive with CM2B4. The skin tumors with a combined squamous and neuroendocrine phenotype and all pulmonary neuroendocrine carcinomas failed to react with CM2B4. Our study shows that CM2B4 is a useful reagent for the diagnosis of MCC. It labels the majority of MCCs, but fails to react with pulmonary neuroendocrine carcinomas. We also found that neuroendocrine carcinomas of the skin arising in association with a squamous cell carcinoma seem to be independent of MCV.

Key Words: Merkel cell carcinoma, Merkel cell polyomavirus, immunohistochemistry

Primary cutaneous neuroendocrine carcinoma, commonly referred to as Merkel cell carcinoma (MCC), is characterized by distinct histologic, immunohistochemical, and ultrastructural features.[7,22,25,31] Histologically, the tumors are composed of "blue" cells with salt and pepper chromatin pattern and scant cytoplasm; they usually contain many mitotic figures and apoptotic bodies.[3,25] Historically, the tumors were recognized as carcinoma early on, but initially termed "trabecular carcinoma,"[33,35] then renamed "Merkel cell" carcinoma after subsequent ultrastructural studies revealed the presence of electron dense granules, a feature of neuroendocrine differentiation, similar to Merkel cells.[14,28,31] Neuroendocrine differentiation can now readily be documented by immunohistochemical stains for neuroendocrine markers, such as chromogranin or synaptophysin.[5,8,22,25] Epithelial differentiation is confirmed by immunoreactivity for cytokeratins.[5,8,10,19] Classically, a paranuclear dot-like pattern with antibodies to cytokeratin (CK) 20 and/or CAM 5.2 is seen,[5,8,10,19] but occasional unusual patterns of immunoreactivity may be observed.[19]

MCC is an uncommon tumor, but the number of diagnosed cases is on the rise. According to data from the US Surveillance, Epidemiology, and End Results Program of the National Cancer Institute, its reported annual age-adjusted incidence rate in 2001 was 0.44/100,000 persons per year, which represents a 3-fold increase from 0.15 per 100,000 in 1986.[1,17] MCC is often described as one of the more aggressive malignant skin tumors with historically reported high recurrence rates.[16] More recent data, however, suggest that MCC is not uniformly lethal.[7,2,3] Patients with small tumors and disease limited to the skin may not succumb to metastatic disease after complete surgical removal with negative margins.[2,3]

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Among risk factors for MCC, ultraviolet-exposure and immunosuppression play a major role.[7,16] Tumor incidence correlates with regional solar ultraviolet-B indices,[23] and the tumor preferentially affects chronically sun-damaged skin of elderly fair-skinned individuals.[2,7,16] Immunosuppression-associated MCC accounts for nearly 8% of patients with MCC.[16]

The risk for MCC is increased 13-fold among HIV-patients,[12] and 10-fold after solid organ transplantation.[9,16,26] MCC is also found to be more common among patients with lymphoma and those receiving immuno-suppressive therapy.[16,32]

Little is known about the molecular pathways that lead to the formation of MCC.[21,24] A number of cytogenetic aberrations have been noted, among them amplification of the L-Myc and loss of the *pRB1* gene region,[24] but their significance in the biology of MCC remains to be determined. A promising new line of research has been opened by the recent discovery of a novel polyoma virus that is clonally integrated at various sites in the genome of the majority of MCCs.[13] This Merkel cell polyomavirus (MCV) is a 5.4 kbp DNA virus that expresses tumor (T) antigen in MCC tissue.[13,29] It is of interest that tumor-derived viruses carry truncating mutations of the large T gene sequences that are not found in wild-type virus and lead to loss of helicase activity thereby preventing MCV from actively replicating its own genome.[29] Using a polymerase chain reaction (PCR)-based test, MCV has been found to be present in up to 85% of tumors.[6,13,15,20]

With the availability of a monoclonal antibody (mAb) that can recognize a MCV-associated T antigen in formalin fixed and paraffin-embedded tumor tissue,[30] we sought to determine the sensitivity of MCV detection by immunohistochemistry (IHC) and to compare MCV detection by IHC with PCR-analysis of frozen tissue samples from the same tumors. We also examined the expression of MCV by IHC in histologic simulants of MCC (pulmonary neuroendocrine carcinomas) and in a peculiar subset of cutaneous tumors, currently considered a variant of MCC, which shows a combined phenotype of squamous cell and neuroendocrine carcinoma.[4,18,34,36]

MATERIALS AND METHODS

Patients

The study was approved by the Institutional Review Board. All patients were seen at or sought consultations from physicians at Memorial Sloan-Kettering Cancer Center (MSKCC). Consent was obtained for use of tumor tissue for research. Clinical information and follow-up was abstracted from the medical records or by contacting the patient's clinician directly.

Tumor Tissue

Slides and tissues of primary and metastatic tumors were retrieved from the archives and the tissue bank of the institution's department of pathology. The material included 17 snap-frozen tumor samples of MCC, which had been stored at $-70°C$ (MSKCC tumor bank) and formalin-fixed and paraffin-embedded (FFPE) archival tissue, which was used for immunohistochemical studies.

For a tumor to be accepted as MCC and selected for this study, the following criteria had to be present. The tumor had to be predominantly composed of nuclei with pale salt and pepper chromatin pattern, scant cytoplasm, and mitotic figures. Immunohistochemically, the tumor had to be positive for CK20, or, for the rare tumors, which lacked labeling for CK20, positive for the CAM 5.2 in a paranuclear dot-like pattern and/or positive for chromogranin and/or negative for thyroid transcription factor-1 (TTF-1). Furthermore, there had to be clinical evidence in support of a primary skin tumor or, for metastatic lesions, evidence in support of a derivation from a primary skin tumor or at least clear documentation that there was no extracutaneous primary in the rare cases of metastatic MCC with unknown primary. Cases of patients who carried a prior or concurrent diagnosis of an extracutaneous neuroendocrine carcinoma were excluded from this analysis.

In this study, immunohistochemical stains were performed on 3 sets of FFPE Merkel cell tumors:

1. Fifteen tumor samples corresponding to the same tumors, which were analyzed by quantitative PCR (qPCR; a total of 17 frozen tumor samples were tested by qPCR, but suitable archival material for a parallel analysis by IHC was available in 15 of 17 cases).
2. Thirty-six tumor samples spotted on a tissue microarray. Each case was represented in triplicate 1 mm punch biopsy samples from the donor tumor block.
3. A set of 7 combined primary skin tumors, which showed mixed features of squamous cell and CK-20-positive neuroendocrine carcinoma.

To test for the potential presence of MCV in neuroendocrine tumors noncutaneous origin, IHC with mAb CM2B4 was also performed on a series of 26 lung tumors, consisting of 16 primary small cell carcinomas and 10 large cell neuroendocrine carcinomas.

PCR Studies

DNA was extracted from 17 frozen samples of Merkel cell tumors. A corresponding histologic section was reviewed to assure that the tissue sample contained more than 90% tumor tissue. The qPCR testing for MCV was performed using T primer sets amplifying the MCV T antigen promoter region (98 to 184 nucleotide forward: 5′-cccaagggcgggaaactg-3′, reverse: 5′-gcagaaggagtttgca-gaaacag-3′) with an internal probe (5′-ccactccttagtgagg-tagctcatttgc-3′) labeled with FAM and Black Hole Quencher (Biosearch Technologies) and the VP2 region (4563 to 4472 nucleotide, forward: 5′-agtaccagaggaa-gaagccaatc-3′, reverse: 5′-ggccttttatcaggagaggctatattaatt-3′) with an internal probe (5′-gcagagttcctc-3′) labeled with FAM and Minor Groove Binder quencher (Applied Biosystems). Copy numbers were established from standard curves of threshold cycle (Ct) values from serial dilutions of known concentrations of MCV DNA.

RNaseP (Applied Biosystems) or β-actin primer-probe mixtures (forward: 5′-cactggctcgtgtgacaagg-3′, reverse: 5′-cagacctactgtgcgcctacttaa-3′, probe: 5′-tggtgtaaagcggccttg-gagtgtgt-3′; Biosearch Technologies) were used to determine cell genome copy number. qPCR reactions were performed using PRISM 7900HT Fast Real-Time PCR System (Applied Biosystems) and/or Smart Cycler 5RX4Z01 (Cepheid) with TaqMan reagents [uracil N-glycosylase (+) TaqMan Universal PCR Master Mix]. Amplification reactions of all target genes were performed in reaction volumes of 20 μL with following condition: 50°C for 2 minutes, denaturing at 95°C for 10 minutes, then denaturing at 95°C for 15 seconds followed by annealing and extension at 60°C for 1 minute, 40 cycles. Cellular viral DNA copy number equal to or below 1.0×10^{-2} per cell was considered to display low positivity. Samples were randomized and tested in a blinded fashion.

Immunohistochemistry

IHC was performed on standard FFPE archival tissue. Five-micron-thick tissue sections were applied to glass slides (Superfrost Plus, Menzel, Braunschweig, Germany) for IHC and heated overnight at 60°C to ensure proper adherence. Slides were deparaffinized in xylene and rehydrated in series of graded alcohols. As primary reagents, mAbs CM2B4, and Ks 20.8 were used.

The mAb CM2B4 (IgG2b isotype) was generated by standard hybridoma technologies and immunizing mice with keyhole limpet hemocyanin (KLH)-derivatized SRSRKPSSNASRGA peptide from the MCV T antigen exon 2 with a C-terminal cysteine (Epitope Recognition Immunoreagent Core facility, University of Alabama).[30] Immunohistochemical staining with CM2B4 was optimized by testing various dilutions and antigen retrieval techniques. CM2B4 worked best at a dilution of 1:100 (vol/vol) and a heat-based antigen retrieval technique (95°C, 30 min) using a citrate-based commercial buffer solution (DAKO-TRS; DAKO, Carpentaria, CA). The mAb Ks20.8 to CK20 (DAKO) was used at 1:100 (vol/vol), and antigen retrieval was made by heating the slides in ethylenediaminetetraacetic acid buffer solution (pH8, 1 mM) for 30 minutes (95°C). Incubation of primary antibodies was carried out overnight at 6°C. The primary antibody CM2B4 was detected with the Power-vision secondary system (Leitz Microsystems, Wetzlar, Germany), whereas a biotinylated horse-anti-mouse antibody (Vector Labs, Burlingame, CA) in combination with an avidin-biotin system (ABC-Elite, Vector Labs) was used for Ks 20.8. Diaminobenzidine served as a chromogen and counterstaining was made with Gill's (II) hematoxylin. Finally, slides were dehydrated and coverslipped with a permanent mounting media.

Immunostaining of the tissues was estimated based on the amount of immunopositive tumor cells and graded as follows: negative—no immunostaining; focal— < 5% tumor cells; +5% to 25% tumor cells; ++ > 25% to 50% tumor cells; + + > 50% to 75% tumor cells; and + + + + > 75% tumor cells.

RESULTS

MCV Detection in Frozen Tumor Tissue by PCR

Frozen tumor tissue samples of MCC from 17 different patients were examined by qPCR for the presence of MCV DNA. The samples included 9 primary cutaneous tumors (5 from sun-exposed sites, 4 from sun-protected sites), 6 lymph node metastases, and 2 visceral metastases (Table 1). MCV was identified in 15 of 17 tumors (88%), with a positive PCR result seen in 8 of 9 primary and 7 of 8 metastatic tumors.

The one MCV-negative primary tumor was from the right leg of an 85-year-old woman. Its histology was re-reviewed: the primary tumor was a combined superficial squamous cell and neuroendocrine carcinoma that had metastasized to the regional lymph node. The metastasis had a pure neuroendocrine phenotype.

The one MCV-negative metastatic tumor was from a neck lymph node of a 76-year-old man who presented with metastatic MCC in the absence of a known primary tumor. The lymph node metastasis was re-reviewed. The histologic and immunohistochemical features were typical of MCC with positive paranuclear dot-like staining for CK20.

TABLE 1. Comparison of MCV Detection by PCR of Frozen Tumor Samples With Corresponding Sections From FFPE Tissue Blocks

Age	Sex	Site	Primary or Metastasis	PCR-MCV	IHC-MCV	IHC-CK20
80	F	Skin	Primary	Pos	4+	4+
56	M	Skin	Primary	Pos	NA	NA
87	M	Skin	Primary	Pos	4+	4+
58	M	Skin	Primary	Pos	3+	3+
86	M	Skin	Primary	Pos	4+	4+
88	M	Skin	Primary	L Pos	Neg	3+
62	M	Skin	Primary	L Pos	Neg	2+
68	F	Skin	Primary	Pos	3+	3+
85	F	Skin	Primary	Neg	Neg	3+
59	M	Lymph node	Metastasis	Pos	4+	3+
72	F	Lymph node	Metastasis	Pos	3+	3+
52	M	Lymph node	Metastasis	L Pos	4+	1+
60	M	Lymph node	Metastasis	Pos	4+	4+
77	M	Lymph node	Metastasis	Pos	Neg	4+
52	F	Lymph node	Metastasis	Neg	Neg	Neg
76	M	Pancreas	Metastasis	Pos	4+	3+
68	M	Small Bowel	Metastasis	Pos	NA	NA

CK20 indicates cytokeratin 20; F, female; FFPE, formalin-fixed paraffin-embedded; IHC, immuno-histochemistry; L Pos, Low positivity ($< 10^{-2}$ copies/cell); M, male; MCV, Merkel cell virus; NA, not available for analysis (insufficient tissue); Neg, negative; PCR, polymerase chain reaction; Pos, positive.
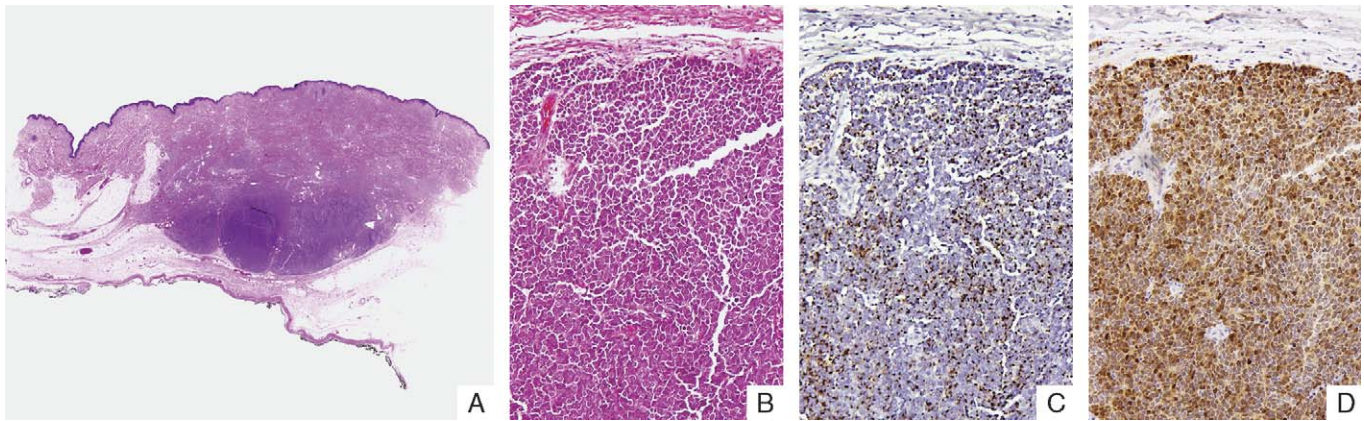
FIGURE 1. A, Merkel cell carcinoma presenting as tumor nodule in the dermis and subcutis. B, Dense population of blue cells (hematoxylin and eosin-stained section). C, Positive staining for cytokeratin 20 in a predominantly paranuclear dot-like pattern. D, Nuclear labeling with monoclonal antibody CM2B4 (this tumor was polymerase chain reaction-positive for Merkel cell polyomavirus). [full color online]

MCV Expression by Immunohistochemistry of Archival Tissue Material Corresponding to the Frozen Tumor Samples Analyzed by PCR

Conventional tissue sections of FFPE portions from the same tumors that had been analyzed by PCR were examined by IHC, using mAb CMB24. An additional section from each tumor was also stained for CK20. Sufficient tissue for this analysis was available in 15 of the 17 cases. Thirteen of them were positive for MCV by PCR.

Ten tumors were immunoreactive for CM2B4 (67% of all tumors with tissue available for IHC, 77% of all PCR-positive tumors). All tumors, which were immunoreactive for CM2B4, were also positive for MCV by PCR. Seven of the CM2B4-positive tumors showed strong nuclear labeling in the vast majority (> 75%) of the tumor cell population (Fig. 1, Table 1). Three tumors showed only partial labeling for CM2B4 (3 + staining, that is, positive labeling of $\geq$ 50% to 75% of tumor cells). All but one tumor was positive for CK20.

The one tumor, which was immunonegative for both CM2B4 and CK20, but contained MCV by PCR, was a lymph node metastasis. The clinical and histologic findings were re-reviewed. The tumor was judged to be metastatic MCC, because there was a known primary cutaneous tumor, which, like the metastasis, showed classic cytologic features of MCC. Furthermore, the tumor was immunohistochemically positive for CAM5.2

in a dot-like paranuclear staining pattern and also positive for chromogranin.

Immunohistochemical Analysis of Merkel Cell Carcinomas by Tissue Microarray

A tissue array with 36 cases of MCC was analyzed by IHC for CK20 and CM2B4. Thirty-two of the 36 tumors (89%) were positive for CK20 (Table 2). Positive staining was seen in 13 of 14 (93%) primary and 19 of 22 (86%) metastatic tumors. The staining was predominantly paranuclear dot-like, but membraneous staining was also focally seen.

Twenty-seven of the 36 tumors (75%) were immunoreactive for CM2B4. In the majority of tumors, the staining of tumor cell nuclei for CM2B4 was strong and homogeneous, labeling more than 75%, and up to 100% of the tumor cell population (Fig. 2). Of the 14 primary tumors, 2 were immunonegative for CM2B4. One of them was a primary cutaneous neuroendocrine carcinoma, which had an associated in situ and invasive squamous cell carcinoma component (combined squamous cell and neuroendocrine carcinoma). Of the 22 metastatic tumors, 15 (68%) were immunopositive for CM2B4. One of the negative metastatic tumors was derived from a primary combined squamous cell and neuroendocrine carcinoma (the metastatic component had a pure neuroendocrine appearance).

TABLE 2. Immunohistochemical Detection of MCV With CM2B4 on a Microarray of FFPE Tissues and Comparison With CK20

Primary MCC	CM2B4 (no. of pos cases/total no. of cases) 12/14 (86%)	CK20 (no. of pos cases/total no. of cases) 13/14 (93%)
Metastatic MCC	CM2B4 (no. of pos cases/total no.of cases) 15/22 (68%)	CK20 (no. of pos cases/total no. of cases) 19/22 (86%)
All MCCs	CM2B4 (no. of pos cases/total no. of cases) 27/36 (75%)	CK20 (no. of pos cases/total no. of cases) 32/36 (89%)

CK indicates cytokeratin; FFPE, formalin-fixed and paraffin-embedded; MCC, Merkel cell carcinoma; pos, positive; no., number.
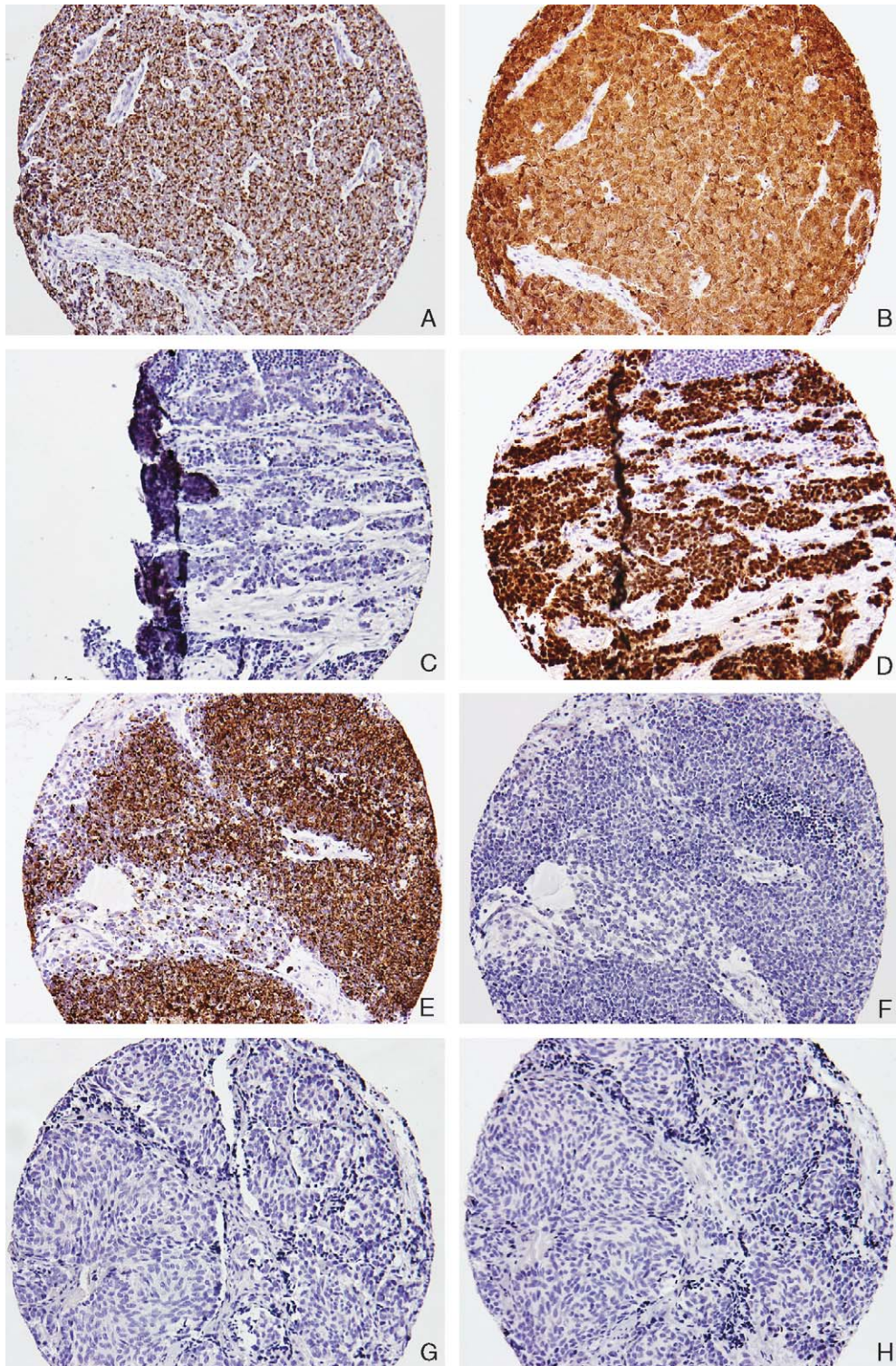
FIGURE 2. Tissue microarray—patterns of colabeling for CK20 and monoclonal antibody CM2B4. A and B, A tumor is positive for both CK20 (A) and CM2B4 (B) [most commonly seen]. C and D, A tumor negative for CK20 (C), but immunreactive for CM2B4 (D). E and F, A tumor expresses CK20 (E), but fails to react with CM2B4 (F). G and H, A tumor is negative for both CK20 (G) and CM2B4 (H). CK20 indicates cytokeratin 20.

Any combination of staining results for CK20 and CM2B4 was seen (Fig. 2). Most (25 of 36) tumors expressed CK20 and stained with CM2B4. All but 2 tumors, which were positive for CM2B4, coexpressed CK20. Six CK20-positive tumors failed to react with CM2B4. Two tumors were negative for both CK20 and CM2B4 (Fig. 2).

The results of the immunohistochemical staining were compared with the patients' clinical information regarding potentially compromised immune status, and sun-exposure to the site of the primary tumor. Seven of the 12 CM2B4-immunopositive primary tumors were from sites of chronic sun-damage (face and extremities with histologic evidence of marked solar elastosis in the adjacent non-neoplastic dermis). Five were from sun-protected sites (buttocks, with no significant solar elastosis). Of the group of patients with immunopositive tumors, one person was known to be clinically immuno-suppressed (had chronic lymphocytic leukemia). There was also one patient with known immunosuppression (status postorgan transplant) in the group of patients with CM2B4-negative tumors.

Immunohistochemial Analysis of Combined Primary Cutaneous Squamous and Neuroendocrine Carcinomas

Seven combined primary skin tumors, which contained an intimately admixed component of both squamous cell and neuroendocrine carcinoma (Fig. 3) were analyzed by IHC for CK20 and CM2B4. Although the neuroendocrine component of all tumors was positive for CK20 (Table 3), none of the cases was immuno-reactive with CM2B4 in either the squamous or neuro-endocrine component (Fig. 3).

Immunohistochemical Analysis of 26 Primary Pulmonary (Small and Large cell) Neuroendocrine Carcinomas

Twenty-six primary pulmonary tumors (16 small cell and 10 large cell neuroendorcrine carcinomas) were analyzed for immunoreactivity with CM2B4. All had been previously characterized by a panel of immunomar-kers for clinical purposes and found to be positive for at least one neuroendocrine marker (chromogranin, synap-tophysin, CD56). IHC for TTF-1 was performed on 21 of the 26 tumors. Sixteen of them (76%) were immuno-reactive for TTF-1. All 26 pulmonary neuroendocrine carcinomas were retested for CK20 expression and confirmed to be negative. None of them showed any nuclear staining with CM2B4.

DISCUSSION

MCC is a rare skin tumor whose reported incidence has tripled in the past 2 decades.[1,17] It affects predominantly the White population, men more often than women.[2,7,16] MCC tends to affect the elderly, with a median age at presentation of 69 years.[2] It is associated with chronic sun-damage[2,7,16] and/or immunosup-
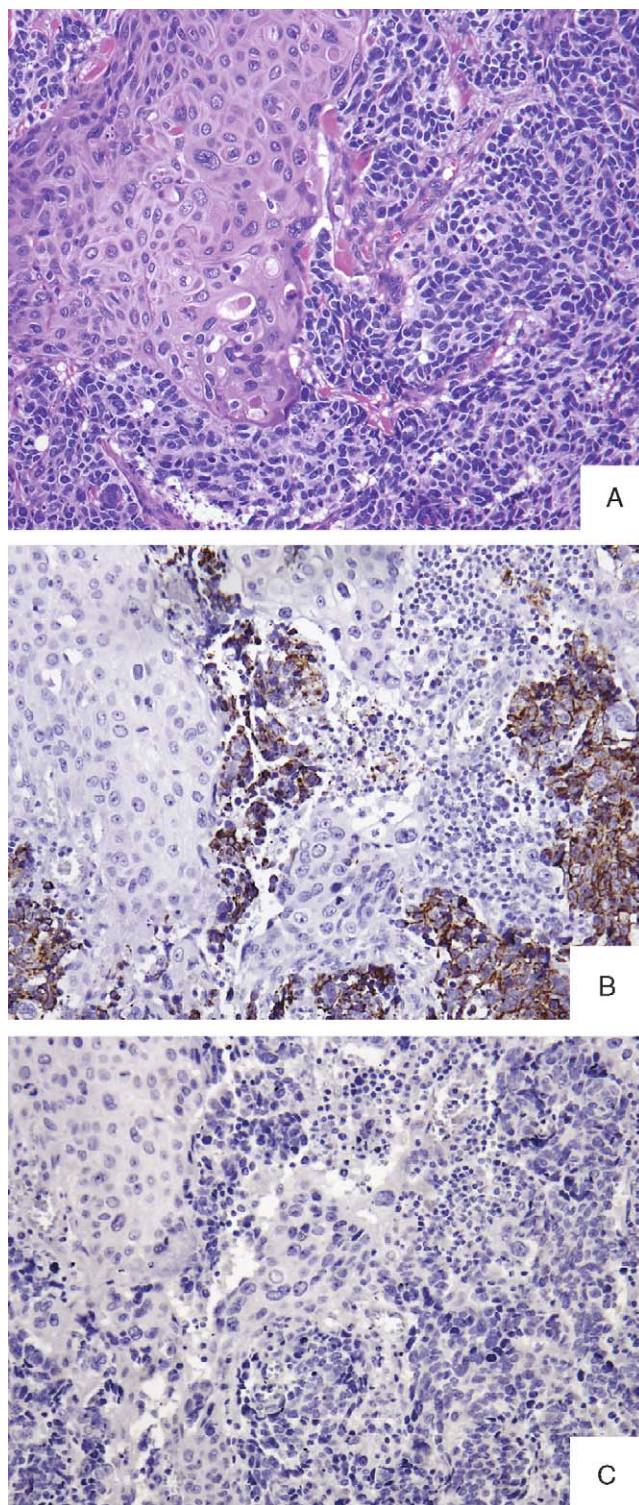

FIGURE 3. Combined cutaneous squamous and neuroendo-crine carcinoma. A, The tumor shows a biphenotypic appearance (hematoxylin and eosin-stained section). It contains a pink squamous and a small cell neuroendocrine component. B, The neuroendocrine component is immuno-reactive for cytokeratin 20. C, The neuroendocrine component fails to react with CM2B4. full color online

TABLE 3. Combined Squamous and Neuroendocrine Carcinomas of the Skin

Age (y)	Sex	Site	Associated Disease	IHC-CM2B4	IHC-CK20
65	M	Skin of scalp	None	Negative	Positive
79	M	Skin of face	None	Negative	Positive
85	F	Skin of face	None	Negative	Positive
78	M	Skin of calf	None	Negative	Positive
74	F	Skin of wrist	None	Negative	Positive
67	M	Skin of face	CLL	Negative	Positive
75	F	Skin of face	CLL	Negative	Positive

CK indicates cytokeratin; CLL, chronic lymphocytic leukemia; F, Female; IHC, immunohistochemistry; M, Male.

pression.[16] The latter includes HIV-associated or lymphoma-associated immunosuppression and patients after solid organ transplantation or undergoing chemotherapy.[9,16,26,32]

Recently, Feng et al[13] identified MCV as a novel polyomavirus clonally integrated into the genome of MCCs. Eight of 10 tumor samples were found to contain the virus. Subsequent studies confirmed this association. The frequency of detection of MCV by PCR in tumor ranged from as low as 24% in a small sample of Australian patients[15] to 70% to 85% in North American and European patients.[6,13,15,20] In our current series, 88% of all MCC were positive for MCV by PCR. Differences in MCV detection rates may be related to different patient populations, technical reasons, or diagnostic criteria of suspected lesions.

The sensitivity of MCV detection by IHC using mAb CM2B4, which was generated against a predicted antigenic epitope on the Merkel cell polyomavirus encoded T antigen, was less than by qPCR (67% vs. 88%, respectively). However, the antibody stained the majority of PCR-positive tumors (77%). The specificity of the immunohistochemical labeling was high. All tumors, which were immunoreactive for CM2B4 were positive for MCV by PCR.

Positive staining was typically homogenous strong nuclear labeling of the majority of tumor cells. However, in a subset of cases, only a portion of the tumor cells was positive. Adjacent normal skin tissue was consistently negative for CM2B4.

There was no apparent association of MCV detection with anatomic site/mode of sun exposure and major clinical immune suppression of the patients. This is not a surprise. Less than 10% of MCC occur in patients with major immunosuppression, such as HIV, associated lymphoma or status postorgan transplantation.[16] As the majority of MCCs had previously been found to be positive for MCV,[6,13,20] it was already evident that MCV-associated MCC could not be restricted to severely immunosuppressed patients. Similarly, given the high frequency of MCV in MCC and the known anatomic distribution of the tumor,[2] it was also expected that MCV could be found in tumors of both sun-exposed and sun-protected sites.

With regard to its potential diagnostic use, our findings suggest that mAb CM2B4 will likely emerge as a valuable adjunct reagent for the differential diagnosis of MCC from histologic mimics, in particular for the distinction of MCC from a cutaneous metastasis of a pulmonary neuroendocrine carcinoma, when current panels of immunomarkers do not allow a definitive diagnosis.[8,10,11,19,22,25,27] None of the 26 neuroendocrine carcinomas of the lung, which we examined in this series, labeled with CM2B4. Further investigations, however, are needed to examine the sensitivity and specificity of CM2B4 on a larger set of tumors, especially neuroendocrine carcinomas from sites other than skin and lung.

Although our results support prior observations that the majority of primary cutaneous neuroendocrine carcinomas are associated with MCV, not all of them are. Lack of virus detection may in part be due to technical reasons, for example, tissue preservation of the specimen, or virus mutation that does not permit PCR-amplification with the primers used, and/or may also lead to lack of immunoreactivity for CM2B4. However, it is also possible that there is a subset of primary cutaneous neuroendocrine carcinomas, which may not be associated with MCV. Consequently, primary cutaneous neuroendocrine carcinomas may be divided into 2 broad categories—MCV-positive type, representing the majority of case, and MCV-negative type.

It is of interest in this regard that none of the 7 combined cutaneous squamous and neuroendocrine carcinomas were immunoreactive with CM2B4. Furthermore, the one PCR-negative primary tumor turned out to be a combined squamous and neuroendocrine carcinoma. Such combined tumors are rare and account for only a minor portion of MCCs. Population-based data are lacking. In the patient review of 29 cases of MCC, Walsh[36] found 3 tumors, in which MCC was associated with a superficial squamous cell carcinoma. In the institutional data set of Merkel cell tumors from MSKCC, approximately 5% of patients with a diagnosis of MCC have a neuroendocrine tumor that is associated with a superficial invasive squamous cell carcinoma (KJ Busam, unpublished observations). Although it is difficult to draw definitive conclusions from such as small sample size, it is tempting to speculate that neuroendocrine carcinomas arising in association with a squamous cell carcinoma may develop via a MCV-independent pathway and are different from "classic" MCCs. One may also question whether or not such combined MCV-negative tumors should be designated as "MCC."

Historically, pathologists have classified the combined tumors as variants of MCC, because phenotypically, they are primary cutaneous carcinomas with a predominant neuroendocrine phenotype (ie, after the differentiation pathway of Merkel cells). Except for the associated presence of a (usually minor) superficial squamous cell carcinoma component, the histologic and immunohistochemical features of the dominant tumor component are indistinguishable from de novo "pure" MCC.[4,18,34,36] Furthermore, the combined tumors tend to

behave clinically similar to pure MCCs,[36] and their metastases tend to have a pure neuroendocrine appearance. It is of interest that among the patients with combined tumors, the proportion of those with chronic lymphocytic leukemia was higher than expected,[16] but this may be related to referral bias to a large cancer center.

In conclusion, our study documents that MCCs frequently express MCV at the molecular and the protein level, with a good correlation of PCR-based and immunohistochemical detection methods. Immunostaining with mAb CM2B4 detects MCV-associated protein the majority of Merkel cell tumors, but not in pulmonary neuroendcorine tumors. Although a broader spectrum of tumors from many different anatomic sites need to be tested, our findings suggest that CM2B4 may be a useful reagent for the diagnosis of MCC. Interestingly, not all primary or metastatic cutaneous neuroendocrine carcinomas appear to be MCV-positive suggesting some biologic heterogeneity in this tumor family.

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