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Detection of mitotic figures and G2+ tumor nuclei with histone markers correlates with worse overall survival in patients with Merkel cell carcinoma

SA Henderson¹, MT Tetzlaff¹, P Pattanaprichakul^{1,2}, P Fox¹, CA Torres-Cabala¹, RL Bassett¹, VG Prieto¹, HW Richards³, and JL Curry¹

¹The University of Texas MD Anderson Cancer Center, Houston, TX, USA ²Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand ³Lawrence Berkeley National Laboratory, Berkeley, CA, USA

Keywords

Merkel cell carcinoma; histones; biomarker; H3K79me3T80ph; PHH3

Introduction

Merkel cell carcinoma (MCC) is an aggressive cutaneous neuroendocrine carcinoma that most commonly arises on the head and neck of elderly Caucasian men.¹ Clinically, the tumors present as firm, rapidly growing, painless violaceous nodules with a smooth surface. Recurrence and metastasis occur frequently.^{2, 3} Up to 25% of tumors may recur, and approximately 80% of patients may develop metastases through the course of their disease.⁴ Ultraviolet radiation, immunosuppression, and Merkel cell polyomavirus (MCV) appear to have etiologic roles in MCC.⁵ The clinicopathologic parameters of MCC that portend poor prognosis include male sex, age greater than 55 years, location on the **head and neck**, tumor size greater than 2.0 cm, infiltrative growth pattern, vascular invasion, and high mitotic count.^{4, 6, 7} However, more recently, primary tumor size failed to correlate with lymph node metastasis.⁸⁻¹⁰ Tumor biomarker expression of p63 and survivin have been shown to correlate with poor prognosis, whereas P-cadherin expression has been associated with prolonged recurrence-free survival.^{11, 12} Mitotic biomarker analyses in MCC have not been thoroughly evaluated.

Mitotic figure count is an important microscopic parameter in tumor classification and prognosis in several human malignancies, including MCC. Chromatin condensation during the G2/M phase of the cell cycle is an essential component of mitosis. An important regulator of chromatin condensation is posttranslational modification of histones. PHH3 (Ser 10) and H3KT (**H3K79me3T80ph**) are histone modifications in mitosis that share similar, strict temporal regulation: both appear in G2 thru M phases of the cell cycle. Neither is

present in other phases of the cell cycle and neither is present in apoptotic cells. PHH3 has been shown to facilitate immunodetection of mitotic figures (evaluation of mitotic figure count) in several tumors (e.g., melanoma, astrocytomas, prostate cancer), and mitotic count combined with the number of G2+ tumor nuclei detected with H3KT identified a subset of primary invasive melanomas with risk of metastasis.^{13, 14, 15, 16} However, the prognostic utility of mitotic histone markers in MCC has not been well characterized. We compared the immunodetection of mitotic figures and G2+ tumor nuclei with anti-PHH3 and anti-H3KT along with the proliferative marker Ki-67 in MCC and correlated these findings with clinical outcome.

Material and methods

Patient cohort

We selected 21 cases of primary cutaneous MCC from our pathology archives (January 1, 2000 to December 31, 2010) with known clinical outcomes and performed immunohistochemical (IHC) studies with PHH3 (Ser 10), H3K79me3T80ph (H3KT), and Ki-67. The study was approved by the Institutional Review Board. Routine hematoxylin and eosin (H&E)-stained sections were reviewed by a dermatopathologist, and mitotic count and other pertinent histopathologic parameters (e.g., tumor size, tumor thickness, mitotic count, pattern of growth, lymphocytic infiltrate, vascular invasion, and invasion beyond subcutaneous tissue) per College of American Pathologists guidelines were collected for each case.¹⁷ Mitotic figure count was determined by the “hot spot” approach in a 1-mm² area of tumor (4.5 high-power fields in an BH40 Olympus microscope) stained with H&E. H3KT, PHH3, and Ki-67+ cells (either positive mitotic chromatin and/or positive G2 tumor nuclei) were counted in a similar manner and recorded as positive cells/mm². Immunodetection of G2+ tumor nuclei was defined as speckled nuclear labeling in an intact nucleus, as previously described.¹⁴

Immunohistochemistry

IHC studies of H3KT, PHH3, and Ki-67 antigen were conducted as previously described.¹⁸ Briefly, 5-μm-thick paraffin-embedded tissues were placed on slides, the slides were deparaffinized, and heat-induced antigen retrieval was performed in 10 mM citrate buffer (pH 6.0). Staining for H3KT was performed by using polyclonal rabbit Ig against H3K79me3T80ph (EMD Milipore, Billerica, MA, USA) at a 1:400 dilution. PHH3 was stained with polyclonal rabbit Ig against Ser 10 PHH3 (EMD Milipore) at a 1:1600 dilution. Ki-67 (clone 7B11) was stained with ready-to-use monoclonal antibody (Life Technologies, Grand Island, NY, USA). The avidin-biotin-peroxidase complex (Thermo Scientific, Rockford, IL, USA) was used to detect immunoreactivity. Slides were counterstained with hematoxylin.

Statistical analyses

Pearson correlation was used to assess mitotic counts on H&E with H3KT and PHH3 (with or without inclusion of G2+ tumor nuclei) and Ki-67+ cells. Comparisons of mitotic counts (by H&E and IHC studies) and G2+ tumor nuclei (detected with H3KT and PHH3) were made with paired Student t-test. Univariate Cox proportional hazards regression models

were performed to assess the relationship between these markers and survival. P-values of < 0.05 were considered statistically significant.

Results

Clinicopathologic parameters

Histopathologic tissue sections from each case consisted of infiltrative proliferations of small round cells arranged in sheets and cords effacing the dermis and/or subcutaneous tissue. Tumor cells demonstrated scant cytoplasm, vesicular nuclei, inconspicuous nucleoli, and a stippled chromatin pattern. Mitotic figures were readily identified on H&E examination. Immunohistochemical studies with antibodies for cytokeratin 20 showed a dot-like perinuclear pattern of positivity in the tumor cells that co-expressed markers of neuroendocrine differentiation (e.g., chromogranin or synaptophysin) and were negative for Thyroid Transcription factor-1 (TTF-1), confirming the diagnosis of MCC. Tissues from 21 patients were amenable to H3KT and PHH3 staining (Table 1) and included primary cutaneous MCC from 17 men and 4 women with a mean age of 70.1 years (range: 56–86 years). The mean tumor size was 13 mm (range: 2 to 24 mm), mean tumor thickness was 10 mm (range: 2 to 23 mm), and mean manual mitotic count per mm² on H&E was 12 (range: 2–34). Vascular invasion was detected in 13 of 21 samples (61.9%) on H&E. Examination of sections with D2-40 identified additional 6 samples with LVI and a total of 19 of 21 samples (91.0%) were positive for LVI. Multivariate analysis of the clinicopathologic parameters in this cohort of patients was not a significant predictor of OS.

Immunodetection of H3KT and PHH3 in MCC

The sensitivity of detecting mitotic figures with H3KT and PHH3 approached 100%. At least one tumor mitotic figure was detected with both H3KT and PHH3 in all 21 cases of MCC analyzed (Fig. 1). In addition, H3KT and PHH3 also labeled tumor nuclei in the G2 cell cycle phase (Fig. 2). The specificity for H3KT and PHH3 was equally high for mitotic figures and G2+ tumor nuclei since labeling of apoptotic tumor nuclei was not evident.

However, the mean mitotic figure count of 58/mm² with H3KT demonstrated a significantly higher sensitivity of this marker ($p < 0.001$) than with PHH3 or manual H&E mitotic count (Table 2). Furthermore, H3KT detected significantly more G2+ tumor nuclei (mean: 10/mm²) than did PHH3 (mean: 7/mm²) ($p = 0.0052$) (Fig. 3).

Association between manual mitotic count with H3KT and PHH3 mitotic count and G2+tumor nuclei

There was a strong correlation between PHH3 and H3KT mitotic count (Pearson: $r = 0.911$) and a moderate correlation with PHH3 G2 and H3KT G2 (Pearson: $r = 0.583$) and PHH3 mitotic count with PHH3 G2 and H3KT mitotic count and PHH3 G2 (Pearson: $r = 0.491$ and 0.421 , respectively). There was no significant correlation with the H&E mitotic figure count or Ki-67+ cells with PHH3 or H3KT immunoreactivity.

There was moderate to mild correlation with manual H&E mitotic count and Ki-67+ tumor cells with tumor size (Spearman r value of 0.560 and 0.369, respectively) and mild

correlation with manual H&E mitotic count and tumor thickness (Spearman r value of 0.335).

Association between mitotic chromatin count and G2+ tumor nuclei and overall survival

We observed for H3KT, the combined mitotic figure count and G2+ tumor nuclei was a significant predictor of worse overall survival (OS) in patients with MCC ($p < 0.035$; HR=1.0172; 95% confidence interval [CI], 1.0012-1.0336) corresponding to a 1.72% increase in the risk of death for each unit increase in H3KT (Table 3). The sum mitotic figure count + G2+ tumor nuclei for H3KT and PHH3 (H3KT + PHH3) was also a predictor of worse OS; however, there was only a 0.97% increased risk of death for each unit increase of these markers combined to that of H3KT alone. The expression of Ki-67 ($p=0.0597$) or H3KT mitotic figure count ($p=0.0518$) had near significant association with worse OS, and mild evidence was seen PHH3 mitotic figure count.

Discussion

The clinicopathologic prognostic parameters in MCC associated with poor survival include male sex, tumor size >2.0 cm, tumors on the **head and neck**, age greater than 55 years, infiltrative growth pattern, presence of vascular invasion, increased mitotic count and Ki-67 expression as well as immunoreactivity for p63 and survivin.^{4, 6, 7, 12, 19, 20} Recently, however, reports about primary tumor size and associated risk of lymph node metastasis have been conflicting. In a study by Tarantola et al.,¹⁰ 240 patients with MCC were examined, and primary tumor size was not a predictor of lymph node metastasis. These findings emphasized the unpredictable natural history of MCC since any size of primary tumor has potential for metastasis.

There have been reports of the utility of a few prognostic biomarkers in MCC. Nuclear expression of p63 and survivin correlated with poor prognosis.^{11, 12} Subsequent studies have further validated the association with p63 expression and worse clinical outcome particularly in cohort of patients from North America and Europe.^{12, 21-24} The association with p63 and clinical outcome however was not apparent in patients from Australia and Asia.^{25, 26} Dabner et. al.²⁵ found a low rate of p63 positivity in their series of MCC and reported that the association with clinical outcome and p63 expression may reflect the higher rate of p63 positivity in MCC from North American and European patients.^{11, 21-24} Other biomarker studies have found matrix metalloproteinase (MMP) 7, MMP 10/2, tissue inhibitor of metalloproteinase 3 (TIMP3), vascular endothelial growth factor, p38, stromal NF-kappa beta, and synaptophysin were all associated with metastasis.²⁷ In contrast, P-cadherin expression (positive labeling in 5-100% of cells) was associated with prolonged recurrence-free survival.²⁸

Evaluation of the proliferative index in MCC measured by Ki-67 has been shown to correlate with disease progression. Expression of Ki-67 measured as the percentage of tumor cell labeling was significantly associated with worse prognosis in a study by Koljonen et al.²⁹ and Llombart et al.³⁰ when cut-off points were $>35\%$ and 50% , respectively. Many experts agree on the prognostic impact of high mitotic figure count in MCC.^{1, 31} However, in one series, the percentage of Ki-67 labeling did not appear to correlate with clinical

outcome.¹² A possible explanation of the differing reports of Ki-67 as a prognostic indicator is that anti-Ki-67 labels G1, G2, S, and M phases of the cell cycle and does not specifically identify cells in the mitotic phase of the cell cycle. Therefore, there appears to be a biologically meaningful difference between the percentage of proliferating cells measured by Ki-67 and the number of mitotically active cells and/or G2+ tumor nuclei detected with H3KT and PHH3 in MCC.

Studies in which the mitotic figure count was examined in MCC have found that the presence of mitotic figures 10 per high-power field or approximately mitotic figure count 5/mm² correlated with increased tumor size and poor prognosis.^{1, 30} To the best of our knowledge, our study was the first to examine the prognostic utility of histone markers associated with mitosis and clinical outcome in patients with MCC. We show that H3KT, which specifically identifies mitosis and G2+ tumor nuclei, better correlates with disease progression. We show that PHH3 and H3KT provided a significantly higher mitotic figure count compared with H&E-stained sections. Both PHH3 and H3KT also identified G2+ tumor nuclei, and the mitotic figure count measured with H3KT combined with the number of G2+ H3KT tumor nuclei was a significant predictor of worse OS. There was a trend to worse OS with increased Ki-67 count and PHH3 positive cells. The failure rate in the detection of mitotic figures was noted with PHH3, similar to what was previously described by Tetzlaff et.al.,¹³ as well as with H3KT. This implies that there is variability in the sensitivity with histone-associated mitotic markers and in our experience, H3KT had significantly lower failure rate in the detection of mitotic figures compared to PHH3.

This retrospective study was limited by its small sample size (21 patients with primary cutaneous MCC). Also, a selection bias toward more aggressive disease may have been introduced in our patient sample since our institution is a referral center for high-risk patients. To further address the prognostic utility of histone-associated mitotic markers that define a subset of patients with higher risk of more aggressive disease, we intend to examine a larger cohort of primary cutaneous MCC.

Our previous studies have demonstrated that H3KT is a specific marker for mitotic figures and G2+ tumor nuclei in melanoma. Recently, it has been shown anti-H3K79me3T80ph detects mitotic figures associated dual modification of histone **H3K9me3S10ph** (H3KS).³² Histone mitotic markers associated with dual modification of residues (e.g. H3KS or H3KT) compared to mono modified histone residues (e.g. PHH3 [Ser10]) appears to identify a subset tumors with more aggressive clinical behavior. Further studies are necessary to examine the clinical significance of histone-associated mitotic marker H3KS in human cancers.

The use of digital image analysis and computer-assisted counting to examine proliferative rate in human cancer may have some clinical utility to provide a means for a standardized, rapid, automated, reproducible assessment of mitotic figure count.^{33, 34, 35} Digital image analysis for Ki-67 was shown to be a strong prognostic indicator in breast cancer.³⁴ Studies, albeit limited thus far, that compare mitotic figure count with computer-assisted methods for PHH3 correlated with Ki-67 when expressed as percentage of positive cells, yet did not provide significant differences in mitotic index compared to visual mitotic figure count on

H&E sections.^{33, 35} As we move towards an era of computer-assisted mitotic figure count with proliferative biomarkers with known clinical significance automated digital image analysis will serve as the cornerstone of pathology reports and will further refine our capacity to more precisely stratify risk in a more reproducible and codified manner. Also, since PHH3 and H3KT are nuclear stains, they can be combined with keratin markers to further solidify their clinical utility.

Conclusion

H3KT appears to be superior to PHH3 in detecting mitotic figures and G2+ tumor nuclei in MCC. Measurement of both mitotic figures and G2+ tumor nuclei appears to have biologic significance in patients with MCC.

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Figure 1.

Representative case cutaneous Merkel cell carcinoma (A and B) with mitotic figures (arrows) (C). Diagram of cell cycle phases in relation to labeling with proliferative markers Ki-67, H3KT and PHH3 (D). Original magnification: (A) $\times 20$; (B) $\times 100$; (C) $\times 400$.

Figure 2.

Immunodetection of proliferating Merkel cells with Ki-67 (A-C), H3KT (D-H), and PHH3 (I-M) from a representative case. Ki-67 (B and C) labeled mitotic figures (arrows) as well as G1, S, G2 nuclei (*) (indistinguishable by immunohistochemistry). Histone proliferative markers H3KT (E) and PHH3 (J) labeled mitotic figures (arrows) and G2+ tumor nuclei (*). Higher magnification of mitotic figures and G2+ tumor nuclei with preserved nuclear contour in a speckled nuclear pattern detected by H3KT (F and G) and PHH3 (K and L). Both H3KT (H) and PHH3 (M) demonstrated failed detection of mitotic figures (arrows). Original magnifications: (A), (D), (I) $\times 40$; (E), (J) $\times 100$; (B) $\times 200$; (C), (F), (G), (H), (K), (L), (M) $\times 400$.

Figure 3.

Mitotic figure count and number of G2+ tumor cells detected on H&E and with histone proliferative markers H3KT and PHH3. H3KT detected higher mean number of mitotic figures when compared to H&E alone and with PHH3 ($p < 0.001$). Greater number of G2+ tumor nuclei were detected with H3KT compared to PHH3 ($p < 0.0052$). H3KT had lower number of failed detection of mitotic figures compared to PHH3 ($p < 0.0001$).

Table 1
Clinicopathologic characteristics of 21 cases of cutaneous Merkel cell carcinoma

Characteristics	Value
Sex	
Male: n (%)	17 (81)
Female: n (%)	4 (19)
Age in years	
Mean (range)	70.1 (56-86)
Histopathologic parameters	
Mean tumor thickness (range) (mm)	10.0 (2.0-23.0)
Mean tumor size (range) (mm)	13.0 (2.0-24.0)
Mean mitotic count (range) (mm ²)	12 (2-34)
Vascular invasion	
H&E: n (%)	13 (62)
D2-40: n (%)	19 (91)
Pattern of growth	
Infiltrative and mixed: n (%)	5 (24)
Nodular: n (%)	16 (76)
Lymphocytic infiltrate	
Absent: n (%)	1 (5)
Non-brisk: n (%)	19 (90)
Brisk: n (%)	1 (5)
Invasion into subcutis: n (%)	18 (86)

Table 2
Immunodetection of mitotic figures and G2+ tumor nuclei with histone proliferative marker H3KT and PHH3

Mitotic count	H&E	H3KT	PHH3	p-value
Mean mitotic figure count (range)/mm ²	12 (2-34)	58 (22-208)	39 (13-143)	< 0.001 (*)
Mean G2+ tumor nuclei (range)/mm ²	N/A	10 (2-25)	7 (1-20)	0.0052
Failed IHC labeling in 1 mitosis; n (%)	N/A	6 (28.6)	18 (85.7)	0.5263
Failed IHC labeling of mitosis: Mean mitotic figure count (range)/mm ²	N/A	0.4 (0-3)	1.95 (0-6)	<0.0001

*
 For each pairwise comparison.

Table 3
Univariate analysis of overall survival with histone proliferative marker H3KT and PHH3 in patients with cutaneous Merkel cell carcinoma

Variable (mm ²)	HR	95% CI		p-value
H&E mitotic figure count	1.0065	0.9415	1.0760	0.8495
Ki-67	1.0015	0.9999	1.0030	0.0597
PHH3 mitotic figure count	1.0181	0.9928	1.0441	0.1618
H3KT mitotic figure count	1.0170	0.9999	1.0344	0.0518
PHH3 G2	1.0883	0.9907	1.1954	0.0775
H3KT G2	1.1030	0.9919	1.2265	0.0704
PHH3 (mitotic figure count + G2)	1.0195	0.9971	1.0423	0.0881
H3KT (mitotic figure count + G2)	1.0172	1.0012	1.0336	0.0355
PHH3 + H3KT (mitotic figure count + G2)	1.0097	1.0003	1.0192	0.0435

MR=mitotic rate; OS=overall survival; HR=hazard ratio; CI=confidence interval