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Decreased H3K27me3 Expression Is Associated With Merkel Cell Polyomavirus-negative Merkel Cell Carcinoma, Especially Combined With Cutaneous Squamous Cell Carcinoma

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Abstract. *Background/Aim: Merkel cell carcinoma (MCC) is a rare and aggressive neuroendocrine skin cancer, frequently infected with Merkel cell polyomavirus (MCPyV). H3K27me3 acts as a repressive histone modification that epigenetically controls gene transcription. The aim of this study was to examine H3K27me3 expression in MCC. Materials and Methods: H3K27me3 expression levels were immunohistochemically analyzed in 20 MCPyV-positive MCCs, 15 MCPyV-negative MCCs with squamous cell carcinoma (SCC) (combined MCCs), and six MCPyV-negative pure MCCs. Results: Reduced H3K27me3 expression was variously observed in MCCs. H3K27me3 H-score was significantly lower in MCPyV-negative MCCs than in MCPyV-positive MCCs ($p=0.002$). H3K27me3 expression was significantly lower in MCPyV-negative combined MCC component than in MCPyV-positive MCCs ($p<0.001$), MCPyV-negative pure MCCs ($p=0.036$), or pure MCC histology ($p<0.001$). Kaplan–Meier analysis showed no association of H3K27me3 with outcome. Conclusion: Differential reduction in H3K27me3 expression was observed based on MCPyV status and morphological type. These results implicate H3K27me3-mediated epigenetic changes in tumorigenesis of MCC, especially in MCPyV-negative MCC combined with SCC.*

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Key Words: Merkel cell carcinoma, Merkel cell polyomavirus, immunohistochemistry, H3K27me3, epigenetics.

Merkel cell carcinoma (MCC) is an aggressive neuroendocrine skin cancer that mostly occurs in the elderly (1, 2). Merkel cell polyomavirus (MCPyV) is detected in approximately 80% MCCs and associated with carcinogenesis (3). MCPyV infection is associated with morphological differences (4) as well as better survival prognosis (5, 6). Morphologically, MCPyV-infected MCCs are uniform tumor cells with round nuclei and less cytoplasm, whereas MCPyV-negative MCCs are more pleomorphic tumor cells with small and large irregular nuclei and more cytoplasm (4). MCCs combined with squamous cell carcinoma (SCC) are consistently negative for MCPyV (2, 4). Sequence analysis has recently revealed that MCPyV-negative MCC has a high frequency of DNA mutations associated with UV damage, whereas MCPyV-positive MCC generally has few somatic mutations and little evidence of UV damage (2, 7-9).

The initiation and progression of cancer may be the result of genetic mutations as well as of aberrant epigenetic regulation, such as DNA methylation and histones acetylation (10). Systematic analyses of genetic and epigenetic alterations in a variety of pediatric cancers have identified tumor types with few or no mutations, suggesting that epigenetic derangements can drive these cancers (11). Methylation of lysine 27 on histone H3 (H3K27me), a modification associated with gene repression, plays a critical role in regulating the expression of genes that determine the balance between cell differentiation and proliferation.

Alteration in the levels of this histone modification has emerged as a recurrent theme in several cancer types, demonstrating that either excess or lack of H3K27 methylation can have oncogenic effects (12). H3K27me3 is a state of trimethylation of lysine 27 on histone 3. This histone modification of H3K27me3 acts as an epigenetic regulator that is associated with transcriptional repression (13). The Polycomb repressive complex 2 (PRC2) is one of the central epigenetic regulators that catalyzes H3K27me3 (12, 14). Loss of histone modification of H3K27me3 is observed in several cancers, such as malignant peripheral nerve sheet tumors (15) and pediatric high-grade gliomas (16). In addition, reduced H3K27me3 levels are associated with unfavorable prognosis in breast, ovarian, and pancreatic cancers (17, 18). There is only one study on the expression of H3K27me3 in MCC (14), which used 35 MCCs with pure histological features and five MCCs with combined squamous and neuroendocrine carcinomas of the skin. The results showed a strong reduction in H3K27me3 staining in tumors with 30 virus-positive MCCs and pure histological features. The present study used 20 MCPyV-positive MCCs and 21 MCPyV-negative MCCs, including 15 MCCs combined with SCC or SCC *in situ* (combined MCCs) to investigate the association of H3K27me3 expression with MCPyV status, morphology and prognosis in MCCs, and showed results different from the previous report. We will describe the results in detail and discuss the reasons of the difference.

Patients and Methods

Samples. A total of 41 formalin-fixed paraffin-embedded MCC samples were used. These included 20 MCPyV-positive MCCs (15 samples from the United Kingdom and five from Japan) and 21 MCPyV-negative MCCs (16 samples from the United Kingdom and five from Japan). The MCPyV-negative MCC samples contained 15 MCC samples combined with SCC or SCC *in situ*. Sample data are summarized in Table I. Tumors were diagnosed as MCC if the presentation of a primary cutaneous tumor was clinically obvious (no prior history of neuroendocrine carcinoma elsewhere) and/or the tumors were positive for at least one of the following markers: CK20 with a perinuclear dot-like staining pattern; neuroendocrine markers, such as chromogranin A, synaptophysin, or CD56; and also negative for TTF1. The presence of MCPyV was determined by quantitative PCR in addition to immunohistochemistry for MCPyV-large T antigen (CM2B4) (4).

Immunohistochemistry. Formalin-fixed, paraffin-embedded samples were sectioned into 4-μm-thick slices, followed by deparaffinization and rehydration. Antigen retrieval (pH 6) was performed by incubating the sections for 40 min at 100°C in Nichirei Heat Pro II (Tokyo, Japan). After blocking endogenous peroxidase activity for 5 min, sections were incubated with the first antibody at room temperature for 60 min and then with the secondary antibody for 30 min. Thereafter, the sections were incubated with diaminobenzidine for 10 min. All processing steps used a Nichirei Histo Stainer. After washing the sections using phosphate-buffered saline, the sections were counterstained with hematoxylin for 5 sec, rehydrated, and mounted. The primary antibody used in this experiment was tri-methyl-histone H3 (Lys27) rabbit monoclonal antibody (clone C36B11, dilution 1/150; Cell Signaling Technology, Danvers, MA, USA). The presence of positive internal controls (endothelial cells, stromal cells, and/or inflammatory cells) was strictly checked to assess the immunohistochemical H3K27me3 signal. After staining, nuclear immunostaining was evaluated using the modified H-score; the percentage of cells stained was summed and multiplied by values according to the staining intensity level (0, 1, 2, and 3=not stained, weakly, moderately, and strongly stained, respectively). The H-score ranged from 0 to a maximum of 300 (19, 20).

Statistical analysis. Immunohistochemical H-scores were analyzed with respect to MCPyV status using the Mann–Whitney *U*-test. For survival analysis, the Kaplan–Meier analysis was performed to assess survival time distributions according to the expression of analyzed H3K27me3, and differences in the distribution were evaluated using the log-rank test. Data were statistically analyzed using SPSS software (version 21.0J; SPSS Japan Inc, Tokyo, Japan). A p-value of <0.05 was considered statistically significant.

Results

H3K27me3 expression in histological subtypes based on MCPyV status in MCCs. The H-score results of immunohistochemical staining for H3K27me3 are summarized in Table I. The representative histology and immunostaining features of H3K27me3 in MCPyV-positive and -negative MCC samples are shown in Figure 1. The H-score of H3K27me3 were differentially reduced in most MCCs, with an H-score of <100 in 17/21 MCPyV-negative MCCs and 12/20 MCPyV-positive MCCs (Table I). The H-score of H3K27me3 was significantly lower in MCPyV-negative MCCs than that in MCPyV-positive MCCs (p=0.002; Table II). There was no difference in H3K27me3 modification between MCPyV-positive MCCs and pure histologic MCPyV-negative MCCs (p=0.744). The H-score of H3K27me3 was significantly lower in MCPyV-negative combined MCC component than that in MCPyV-positive MCCs (p<0.001), pure histologic MCPyV-negative MCCs (p=0.036), and pure histologic MCCs (regardless of MCPyV status; p<0.001).

Prognostic analysis. The summary of prognostic analysis related to clinicopathological factors is shown in Table III. The survival analysis revealed that the presence of MCPyV was associated with favorable overall survival (OS, p=0.028) but not with disease specific survival (DSS, p=0.228). However, there were no significant differences between other factors and outcome, including H3K27me3 immunostaining and OS (p=0.209) or DSS (p=0.658), combined SCC (no or yes; OS, p=0.08; DSS, p=0.89), MCPyV-negative pure MCCs or MCPyV-negative combined MCCs (OS, p=0.987; DSS, p=0.154), sex (OS, p=0.05; DSS, p=0.244), or race (Japanese or UK Caucasian; OS, p=0.078; DSS, p=0.163).

Table I. *Summary of sample data with MCPyV status and H3K27me3 H-score in MCCs*.

					MCPyV status		H3K27me3 IHC		
					q-PCR	IHC	Pure MCC or combined MCC	Combined SCC (or SCC *in situ*)	
Sample No.	Age (year)	Gender	Outcome	Histologic type	MCPyV-DNA	MCPyV-LT (CM2B4)	H-score	Combined SCC within MCC component H-score	Combined SCC in epidermis (and dermis) H-score
UK-M-3	85	F	DOC (21)	Combined MCC & SCC	–	–	105	SCC differentiation: 40	None
UK-M-5	81	F	DOD (18)	Combined MCC & SCC	–	–	25	SCC differentiation: 0	None
UK-M-6	82	F	DOC (4)	Combined MCC & SCC *in situ*	–	–	5	None	SCC *in situ*: 20
UK-M-14	86	F	DOC (11)	Combined MCC & SCC	–	–	115	SCC differentiation: 15	SCC *in situ*: 0
UK-M-15	83	F	DOC (2)	Combined MCC & SCC	–	–	0	None	SCC *in situ*: 0. Invasive SCC: 0
UK-M-20	82	M	DOD (31)	Combined MCC & SCC	–	–	0	None	SCC *in situ*: 0
UK-M-35	78	F	DOC (10)	Combined MCC & SCC	–	–	0	SCC differentiation: 0	None
UK-M-41	85	M	DOC (40)	Combined MCC & SCC	–	–	45	None	SCC *in situ* like: 30
UK-M-44	86	F	NED (35)	Combined MCC & SCC	–	–	0	None	SCC *in situ*: 0
UK-M-46	75	M	NED (32)	Combined MCC & SCC, superficial squamous cell atypia	–	–	25	SCC differentiation: N.A.*	Superficial squamous cell atypia: 50
UK-M-50	85	F	NED (16)	Combined MCC & SCC	–	–	0	None	SCC *in situ*: 0
MCC81	85	M	DOC (13)	Combined MCC & SCC	–	–	0	SCC differentiation: 0	SCC *in situ*: 0
MCC99	102	F	DOD (19)	Combined MCC & SCC	–	–	4	None	Invasive SCC: 50
MCC100	90	F	N.A. (19)	Combined MCC & SCC	–	–	0	SCC differentiation: 0	SCC *in situ*: 40
MCC107	100	M	N.A. (6)	Combined MCC & SCC	–	+	0.02	None	SCC *in situ*: 2
UK-M-10	94	F	DOD (16)	Pure MCC	–	–	65	None	None
UK-M-13	61	M	DOD (23)	Pure MCC	–	–	70	None	None
UK-M-18	94	F	DOC (6)	Pure MCC	–	–	0	None	None
UK-M-45	84	F	NED (31)	Pure MCC	–	–	85	None	None
UK-M-54	68	M	AWD (14)	Pure MCC	–	–	115	None	None
MCC102	86	F	DOD (9)	Pure MCC	–	–	180	None	None
UK-M-7	68	M	NED (59)	Pure MCC	+	+	75	None	None
UK-M-9	69	M	NED (55)	Pure MCC	+	+	36	None	None
UK-M-11	61	F	NED (18)	Pure MCC	+	–	120	None	None
UK-M-16	63	F	NED (70)	Pure MCC	+	+	47	None	None
UK-M-19	85	F	DOC (31)	Pure MCC	+	+	22	None	None
UK-M-22	74	F	DOD (72)	Pure MCC	+	+	20	None	None
UK-M-36	84	F	DOC (3)	Pure MCC	+	+	60	None	None
UK-M-37	76	M	NED (48)	Pure MCC	+	+	10	None	None
UK-M-40	76	M	NED (43)	Pure MCC	+	+	85	None	None
UK-M-42	76	F	DOD (9)	Pure MCC	+	+	110	None	None
UK-M-43	95	F	N.A.	Pure MCC	+	+	205	None	None
UK-M-48	83	F	DOC (15)	Pure MCC	+	+	120	None	None
UK-M-51	83	F	DOD (8)	Pure MCC	+	–	90	None	None
UK-M-52	86	F	NED (11)	Pure MCC	+	+	99	None	None
UK-M-53	Unknown	F	NED (12)	Pure MCC	+	+	110	None	None
MCC86	74	F	N.A.	Pure MCC	+	+	80	None	None
MCC93	84	F	NED (12)	Pure MCC	+	+	195	None	None
MCC94	86	F	NED (4)	Pure MCC	+	+	125	None	None
MCC95	76	F	NED (4.5)	Pure MCC	+	+	65	None	None
MCC126	83	F	NED (1)	Pure MCC	+	+	190	None	None

AWD: Alive with disease; DOC: dead from other causes; DOD: dead of disease; F: female; IHC: immunohistochemistry; M: male; MCC: Merkel cell carcinoma; MCPyV: Merkel cell polyomavirus; N.A.: not available; q-PCR: quantitative polymerase chain reaction; SCC: squamous cell carcinoma.

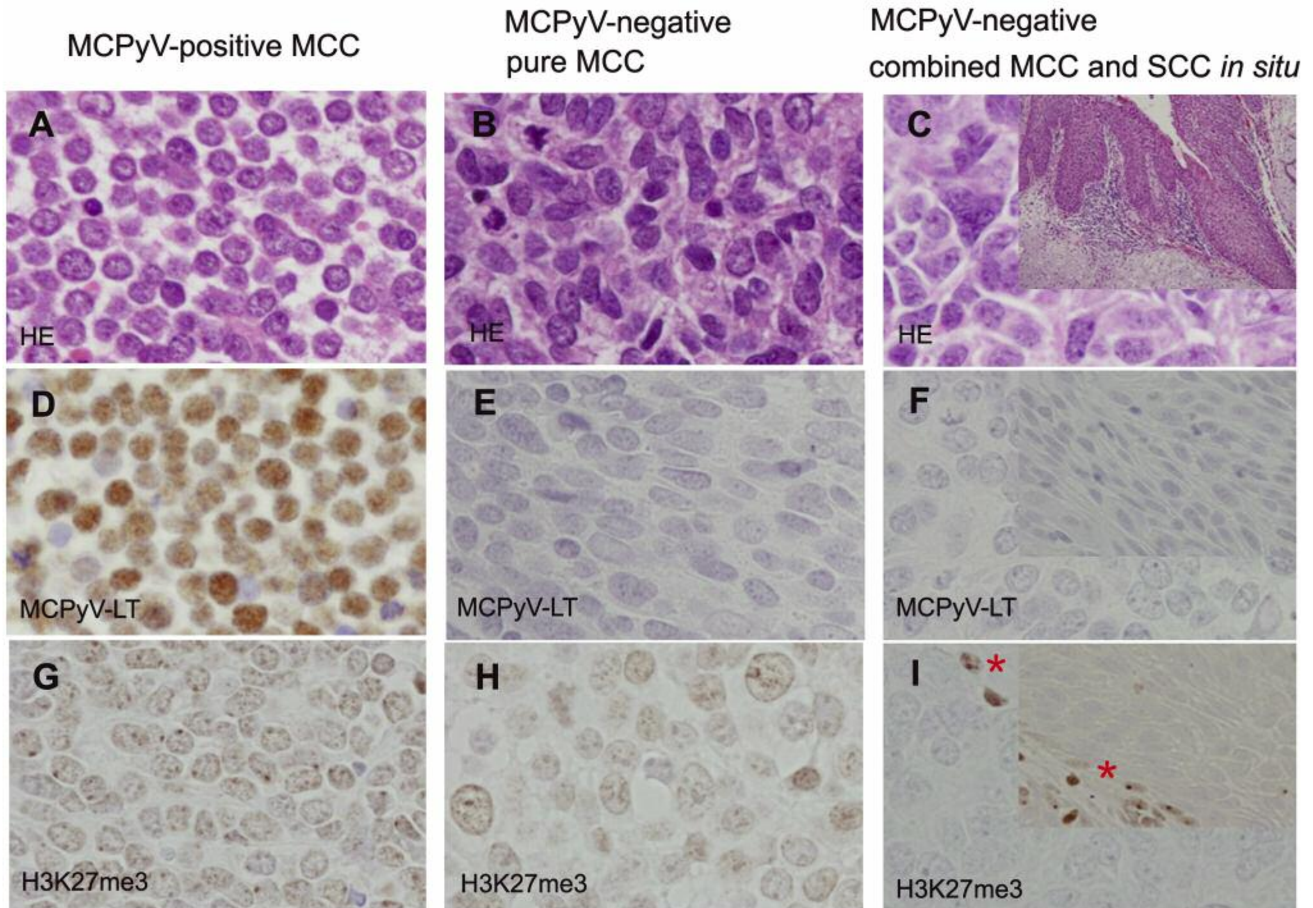

Figure 1. *Representative histology and immunohistochemistry of MCPyV-positive MCC, MCPyV-negative pure MCC, and MCPyV-negative combined MCC and SCC in situ. The morphology and immunostaining of MCPyV-positive MCC (A, D, and G), MCPyV-negative pure MCC (B, E, and H), and MCPyV-negative combined MCC (C, F, and I) and SCC in situ (inset of C, F, and I) are shown. MCPyV-positive MCC cells with rounded nuclei and less cytoplasm (A) are positive for MCPyV-large T antigen (MCPyV-LT) (D), whereas MCPyV-negative pure MCC cells with pleomorphic nuclei and abundant cytoplasm (B) are negative for MCPyV-LT (E). In the MCPyV-negative combined MCC and SCC in situ case, both the components of MCC (C) and SCC in situ (inset of C) are negative for MCPyV-LT (F and its inset). In MCPyV-positive MCCs (G) and MCPyV-negative pure MCCs (H), weak expression of H3K27me3 is observed, but the H-score of H3K27me3 in most cases reduced to <100 (a maximum H-score is 300) because of the weak intensity of immunoreaction compared with the positive internal control stromal cells (*in Figure 1-I). The H-scores were not different in MCPyV-positive MCCs (G) and MCPyV-negative pure MCCs (H) ($p=0.744$), whereas the loss of H3K27me3 expression was frequently observed in MCPyV-negative combined MCC (I) and SCC in situ (inset of I). The H-score of H3K27me3 expression in MCPyV-negative combined MCC component (I) was significantly lower than that in MCPyV-positive MCC (G) and MCPyV-negative pure MCCs (H) ($p\leq0.001$ and $p=0.036$, respectively). *in Figure1I, H3K27me3-positive internal control stromal cells.*

Discussion

MCC is a rare cutaneous neuroendocrine carcinoma frequently infected with MCPyV. Clinicopathologically, MCPyV-negative MCC is different from the MCPyV-positive subset. In the present study, we reconfirmed the previous findings that patients with MCPyV-positive MCC have more favorable outcomes. The tumorigenesis mechanism is different in MCPyV-negative and -positive MCCs; MCPyV-negative MCC is induced by frequent UV-mediated mutations, whereas MCPyV-positive MCC develops by viral infection as well as UV-induced immunosuppression (2). Compared with the frequent mutations in MCPyV-negative MCC, the fewer mutations in MCPyV-positive MCC could be indicative of epigenetic tumorigenesis in virus-positive MCC.

PRC2, a chromatin modifying complex, is responsible for catalyzing H3K27me3. PRC2-mediated H3K27me is essential for the development and differentiation of cells. In all cell types, H3K27me3 modification is associated with repression of regulator genes that encode alternative lineages (21).

Table II. *Relation of H3K27me3 H-score to MCPyV status based histological subtypes in MCCs.*

	H3K27me3 H-score (mean±SD)	*p*-Value[a]
MCPyV-positive MCCs (n=20)	93.2±56.4	0.002
MCPyV-negative MCCs (n=21)	40.0±52.9	
MCPyV-positive MCCs (n=20)	93.2±56.4	0.744
MCPyV-negative pure MCCs (n=6)	85.8±59.6	
MCPyV-positive MCCs (n=20)	93.2±56.4	<0.001*
MCPyV-negative combined MCC component (n=15)	21.6±38.3	
MCPyV-negative pure MCCs (n=6)	85.8±59.6	0.036*
MCPyV-negative combined MCC component (n=15)	21.6±38.3	
Pure MCC histology (MCPyV-positive and MCPyV-negative) (n=26)	91.5±56.0	<0.001*
MCPyV-negative combined MCC component (n=15)	21.6±38.3	

[a]Mann–Whitney *U*-test; *, statistically significant. MCC: Merkel cell carcinoma; MCPyV: Merkel cell polyomavirus.

Cancer genome sequencing studies have revealed that several genes that encode PRC2 components or histone H3 are mutated in several cancer types (21). Loss or reduced expression of H3K27me3 has been reported in several cancers, such as malignant peripheral nerve sheet tumors (15) and pediatric high-grade gliomas (16). In addition, reduced H3K27me3 expression is associated with unfavorable outcome in breast, ovarian, pancreatic, and metastatic colon cancers (17, 18, 22).

Busam *et al*. (14) has reported a strong reduction in H3K27me3 expression in MCCs with pure histologic features (n=35) and MCPyV-positive MCCs (n=30), whereas combined MCC and SCC (n=5) showed minimal or no loss of H3K27me3 expression. In addition, we analyzed the expression of H3K27me3 in MCC according to MCPyV status (20 MCPyV-positive MCCs with pure histology and 21 MCPyV-negative MCCs) and histologic type of pure MCC or combined MCC and SCC (6 MCCs with pure histology and 15 MCCs combined with SCC or SCC *in situ*). Unlike previous findings, the results of the present study showed that the expression of H3K27me3 was significantly lower in MCPyV-negative MCCs than that in MCPyV-positive MCCs. Furthermore, the expression of H3K27me3 was lower in MCPyV-negative combined MCC component (n=15) than that in pure histologic MCCs (n=26, 6 MCPyV-negative and 20 MCPyV-positive MCCs) ($p\leq 0.001$). Interestingly, the expression level of H3K27me3 was not different between MCPyV-positive MCC with pure histology and MCPyV-negative pure MCC ($p=0.744$). However, MCPyV-negative combined MCC component (n=15) showed significantly lower expression of H3K27me3 than that in not only MCPyV-positive MCC with pure histology ($p\leq 0.001$), but also in MCPyV-negative pure MCC ($p=0.036$). Lower H3K27me3 expression in MCPyV-negative MCC may be due to the well-known fact that MCPyV-negative MCC has frequent somatic mutations (2, 7-9). Moreover, the present study showed a reduced mean H-score of H3K27me3 in both MCPyV-positive and -negative MCCs (mean H-score, 93.2 and 40.0, respectively) compared with the maximum H-score of 300. This suggests that H3K27me3-related epigenetic deregulation may play a role in tumorigenesis of both subsets of virus-positive and -negative MCCs, particularly in virus-negative MCC combined with SCC or SCC *in situ* (mean H-score, 21.6). Our findings also support the notion that cutaneous squamous and neuroendocrine (Merkel cell) carcinoma of skin is genetically and immunohistochemically different from pure MCC (23).

Table III. *Correlation between clinicopathological data including H3K27me3 expression and OS or DSS.*

Factor	OS (*p*-Value)	DSS (*p*-Value)
H3K27me3 negative/positive	0.209	0.658
Combined SCC no/yes	0.08	0.89
Pure MCPyV-negative MCCs/ Combined MCPyV-negative MCCs	0.987	0.154
MCPyV negative/positive	0.028	0.228
Gender female/male	0.05	0.244
Race Japanese/UK Caucasian	0.078	0.163

DSS: Disease-specific survival; MCC: Merkel cell carcinoma; MCPyV: Merkel cell polyomavirus; OS: overall survival.

Several factors may contribute to our findings compared with those of the previous studies. The most important factor may be the sample size of MCPyV-negative MCCs; our study used 21 virus-negative MCCs, including 15 MCCs combined with SCC, whereas the previous study used 10 virus-negative MCCs, including five MCCs combined with SCC. The second factor is the difference in immunohistochemistry scoring method. The present study employed modified H-scores based on the percentage of stained tumor cells and the staining intensity level, and the H-score ranged from 0 to a maximum of 300. The previous study scored according to the percentage of immunoreactive tumor cells per total tumor cells. The third

factor is the different methods used to determine the presence of MCPyV. In the present study, the presence of MCPyV was determined basically by quantitative PCR data but not only by immunohistochemistry for MCPyV-LT (CM2B4), whereas the previous study employed only immunohistochemistry for MCPyV-LT (CM2B4). We have previously reported pseudo-negative and -positive immunoreactions for MCPyV-LT (CM2B4) in MCC (24), whereas in the present study, we found two and one pseudo-negative and -positive case, respectively, using only immunohistochemistry (Table I). It is necessary to continue studying H3K27me3 expression in more MCC cases, especially in cases combining MCC and SCC to identify the nature of discrepancy between the findings of the previous and present study. In the previous report (14), the relation of H3K27me expression to outcome was not reported. In the present study, there was no significant association of H3K27me3 expression with prognosis.

Epigenetic studies are important for tumorigenesis and have a possible application in target therapy (25, 26). It is important to develop novel therapies by targeting the components involved in the epigenetic system for patients with advanced MCC because around 50% patients with advanced-stage MCC do not respond to recently-developed immune checkpoint blockade, and a substantial number of patients develop acquired resistance (2). Therefore, additional new therapies combined with immunotherapy are necessary, including treatment targeting the epigenetic changes in tumorigenesis.

Conflicts of Interest

There are no conflicts of interest regarding this study.

Authors' Contributions

MM and KH designed this study and wrote the manuscript. MM and LOW performed the experiments. DN contributed to sample preparation. SK performed histological re-evaluation of samples and confirmed the diagnosis. TI contributed to the statistical analyses of the results. KN and MK contributed to the interpretation of the results. KH and YK supervised the experiments.

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