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Detection of Merkel cell polyomavirus in formalin-fixed, paraffin-embedded tissue of Merkel cell carcinoma and correlation with prognosis

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Abstract

Merkel cell carcinoma (MCC) is a rare, but highly aggressive primary cutaneous malignancy, showing neuroendocrine differentiation. In 2008, a novel member of the polyomavirus family, named Merkel cell polyomavirus (MCPyV) was identified in the genome of MCC tumors raising the possibility of an involvement in its pathogenesis. Due to the rarity of this tumor and current pathology practices, the most readily available tissue is archival formalin-fixed, paraffin-embedded (FFPE) material. In this study, we evaluated the presence of MCPyV in FFPE tissue and correlated its presence with tumor progression. Representative FFPE specimens from 18 tumors belonging to 14 patients with a diagnosis of MCC spanning the period from 2003 to 2008 were retrieved. Following DNA extraction, we performed PCR amplification and sequencing with four different MCPyV-specific primer pairs mapping within the T antigen and VP1 region. Overall, we detected MCPyV amplicons in 8/18 (44.4%) analyzed tumors from 7/14 (50%) cases. Two-year survival rate and median survival for the MCPyV-positive MCCs were 48% and 22.5 months, respectively and for the negative ones 69% and 51.3 months, respectively; however, the difference did not reach statistical significance (p=0.8). There was no significant correlation between the presence of the virus and the stage at presentation; however, tumors in the head and neck area had a lower frequency of viral positivity compared to those arising in the extremities suggesting a MCPyV-independent oncogenetic pathway perhaps, dependent on UV exposure, in a subset of these cases.

Keywords: Merkel cell carcinoma, Merkel cell polyomavirus, prognosis.

Introduction

Merkel cells are specialized epidermal cells containing dense neurosecretory granules located in the basal layer of the epidermis, especially in acral skin. They are closely associated with the expanded terminal bulb of afferent myelinated nerve fibers. Merkel cell carcinoma (MCC) is a primary cutaneous malignant, small blue cell tumor regarded as having differentiation towards Merkel cells due to the expression of neuroendocrine markers and the ultrastructural presence of dense core neuroendocrine granules. Immunohistochemistry (IHC) reveals a characteristic paranuclear dot-like pattern of cytokeratin 20 [1].

MCC is a rare tumor and is considered one of the most aggressive neuroendocrine skin cancers with an incidence of 0.24–0.44 cases per 100 000 person-years [2–4]. About half of the patients with advanced MCC live less than nine months after diagnosis and overall more than 33% of patients diagnosed with MCC will die from their disease, resulting in a mortality rate considerably higher than melanoma [5]. MCC tumors have a high recurrence rate and readily metastasize *via* both lymphogenous and hematogenous spread.

Since MCC is uncommon, little has been known about this neoplasm; however, recent investigations have shed some light on the epidemiology, etiology, and potential basis for therapy. MCC is more common in elderly Caucasian men and is linked to both increased ultraviolet light exposure and immunosuppression. Approximately 90% of patients diagnosed with MCC are over the age of 50 [5]. There is a higher incidence of MCC near the equator with over 80% of primary skin lesions occurring over sun-exposed regions [2, 5]. Also, a clear association exists with MCC and immunosuppressed patients, as they have a 15 times greater risk of developing MCC than the general population [5]. Patients with T-lymphocyte immune suppression, such as from HIV infection and solid organ transplantation have been shown to have a significantly higher incidence of MCC; furthermore, MCC in immunosuppressed patients has been reported to be more aggressive, with a mortality up to 56% [6–8]. A few studies have even reported MCC regression following HIV treatment or cessation of Cyclosporine [9, 10]. The strong association with immunological status has suggested the hypothesis of an infectious agent that plays a role in causing or sustaining MCC.

In 2008, Feng *et al.* discovered and cloned a virus present in the genome of MCC tumor cells and named it Merkel cell polyoma virus (MCPyV) [11].

Polyomaviruses are non-enveloped double stranded DNA viruses, circa 40–45 nm in size, whose genome consists of approximately 4700–5400 base pairs and encodes for three structural proteins and a few early and late proteins (Figure 1).

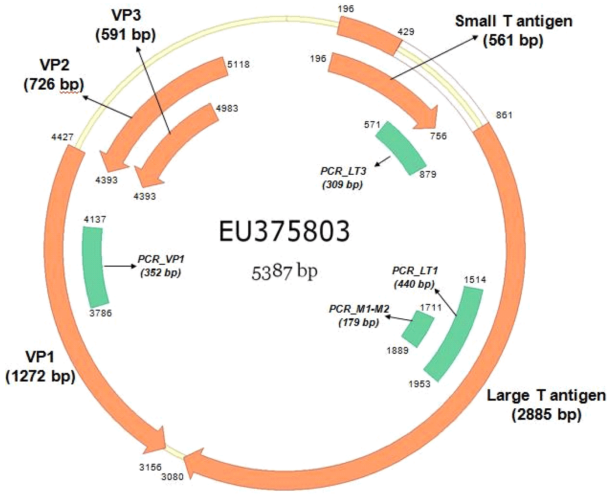

Figure 1 – ***Vector map of the 5387 bp Merkel cell polyoma virus genome. Green-colored fragments (PCR_LT1, PCR_LT3, PCR_M1-M2 and PCR_VP1) denote areas amplified by PCR for viral detection.***

Early expressed genes give rise to small and large T antigens, which promote host cells to enter the S phase of the cell cycle. The late expressed genes generate the viral capsid proteins VP1, VP2 and VP3 and components for lysis [12, 13]. The T antigen locus generates three different gene products *via* alternative splicing, a large T antigen (LT), a small T antigen (sT) and a 57 kT antigen (57 kt) [14, 15]. LT is a protein having the ability to bind DNA polymerase, primase, and topoisomerase I. LT also binds the tumor suppressor genes pRb (due to the presence of an LxCxE motif) and p53, which is likely a factor in its putative oncogenic potential [16]. LT also contains at the carboxyl terminal end an origin-binding domain and a helicase domain involved in viral DNA replication [17]. The sT has the ability to bind protein phosphatase 2A and is thought to participate in the process of viral DNA replication and cellular transformation [16, 18, 19]. In the original study by Feng *et al.*, 80% of the MCC cases contained entire viral genomes or fragments of it; however, the integration site was not consistent suggesting that the viral infection likely precedes clonal expansion of the tumor [11, 20].

Due to the relative rarity of MCC and also to the current practices in pathology, the most readily available tissue material is formalin-fixed and paraffin-embedded (FFPE). In this study, we aimed to investigate the feasibility of detecting MCPyV specific DNA sequences in a series of FFPE tissue samples from primary and metastatic MCC cases. Also, since little is known about the clinical significance of MCPyV integration in the MCC genome, we set out to evaluate the potential correlations between viral presence, clinical parameters and patient outcome.

Materials and Methods

Patients

The study was approved by the Institutional Review Board (IRB). A search of the pathology electronic records from 2003 to 2008 identified 14 patients with a diagnosis of MCC and sufficient tumor tissue for DNA extraction. Pathology reports and all available slides and paraffin blocks were retrieved from the archives of the pathology department. For four patients, more than one tumor specimen was available for a total of 18 tumors. Clinical outcome data were collected for the entire cohort with a median follow-up interval of 20 months.

Viral DNA detection

DNA was extracted from the primary lesion in eight patients while in two patients a metastatic lesion was investigated. For four patients, two separate lesions were studied (the primary focus and a metastasis in three patients and two separate metastatic foci in one patient). For each case, 10 μm thick sections were obtained. By comparison with HE-stained sections, the area involved by tumor was marked on the unstained slides. Tumor was microdissected with a surgical blade from the unstained slides. Depending on the surface area covered by tumor, between three and 10 slides were processed per case, aiming for a total of 20 mg of tissue per case. Following deparaffinization with xylene, DNA was extracted using QIAamp® DNA FFPE Tissue kit (Qiagen, Valencia, CA, USA) following manufacturer's instructions.

Reference sequences for MCV gene were obtained from the *NCBI Database* (NC_010277, GenBank accession no. EU375803). Previously published primers [11] were used to amplify three sequences within the T antigen (LT1, LT3, M1-M2) and one within the VP1 gene of MCV (Table 1).

Table 1 – ***Primer sets specific for MCPyV***

Primer	Forward (5'–3')	Tm [^{0}C]	Reverse (5'–3')	Tm [^{0}C]	Product size [bp]
LT1	TACAAGCACTCCACCAAAGC	58.93	TCCAATTACAGCTGGCCTCT	59.84	440
LT3	TTGTCTCGCCAGCATTGTAG	60.01	ATATAGGGGCCTCGTCAACC	60.70	309
M1-M2	GGCATGCCTGTGAATTAGGA	61.00	TTGCAGTAATTTGTAAGGGGACT	63.03	179
VP1	TGGATCTAGGCCCTGATTTTT	59.92	TTTGCCAGCTTACAGTGTGG	59.90	352

Tm – Melting temperature.

PCR reactions were carried out using the Platinum PCR supermix (Invitrogen, Carlsbad, CA, USA) following manufacturer's recommendations. Reaction volume was 50 μL (45 μL PCR mix, 1 μL DNA, 1 μL forward primer, 1 μL reverse primer, 2 μL water). The following PCR protocol was used: 98^{0}C for 2 minutes, followed by 50 cycles of denaturation at 98^{0}C for 40 seconds, annealing at 58^{0}C for 30 seconds, and elongation at 72^{0}C for 40 seconds, and a final elongation at 72^{0}C for 10 minutes. A nested PCR technique was also employed using a first round of PCR with LT3 primers followed by a second round with M1–M2 primers. The PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Valencia, CA). Direct sequencing was performed in both forward and reverse direction with the original PCR primers using the BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Identification of the PCR fragments was confirmed using the VectorNTI

software packet (Invitrogen, Carlsbad, CA) and Mutation Surveyor (SoftGenetics, LLC, State College, PA, USA).

Statistical analysis

Analysis of disease-specific survival was performed using Kaplan–Meier curves and the *log*-rank statistic test. Two and 5-year survival rates were estimated using follow-up life tables. For all analyses, the SPSS 9.0.0 statistical software package was used.

Results

The study series was composed of 13 males and one female. The average age at diagnosis was 73 years (range 56–93 years). Distribution of stages I, II, III and IV at presentation was 33%, 22%, 22% and 22%, respectively. In 11 (79%) patients, the primary tumors were located in the head and neck area, in two (14%) they were on the extremities and one patient (7%) presented with a positive axillary lymph node with no known cutaneous primary.

Cases with bands on gel electrophoresis corresponding to specific amplified viral genome segments were purified and sequenced. Figures 2 and 3 show agarose gels with PCR amplicons from the M1–M2 and VP1 fragments, respectively (179 bp and 352 bp in size, respectively). Similar PCR bands with a size of 309 bp were found for the LT3 segment. The sequences of these fragments were compared to the reference sequence for MCPyV using Vector NTI software and Mutation Surveyor (SoftGenetics, LLC, State College, PA) and showed a 99–100% similarity to the corresponding segments of VP1 and large T antigen viral genes.

PCR followed by sequencing identified at least one MCPyV amplicon in eight of 18 (44.4%) analyzed tumors from seven of 14 (50%) cases. M1–M2, LT1, LT3 and VP1 amplicons were detected in five (28%), 0 (0%), four (22%) and two (11%) cases, respectively. PCR products from the T antigen portion of MCPyV (M1–M2 and/or LT3) were found in six of 18 (33%) tumors and from the VP1 gene in two of 18 (11%) tumors (Table 2). Rate of detection was inversely correlated with the size of the PCR products being 28% for M1–M2 (179 bp), 22% for LT3 (309 bp), 11% for VT1 (352 bp) and 0% for LT1 (440 bp).

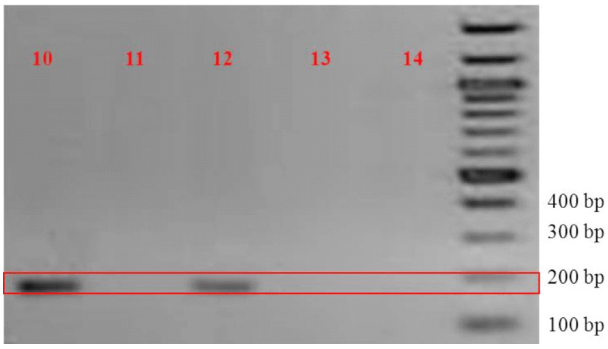

Figure 2 – *Agarose gel electrophoresis for large T antigen LT3 and M1–M2 nested PCR products. Samples had undergone PCR amplification for the LT1 (440 bp) portion of the virus. Afterwards nested PCR was performed on the purified LT1 product to amplify the M1–M2 (179 bp) portion. Samples #10 and #12 showed bands for M1–M2, while #11, #13 and #14 did not.*

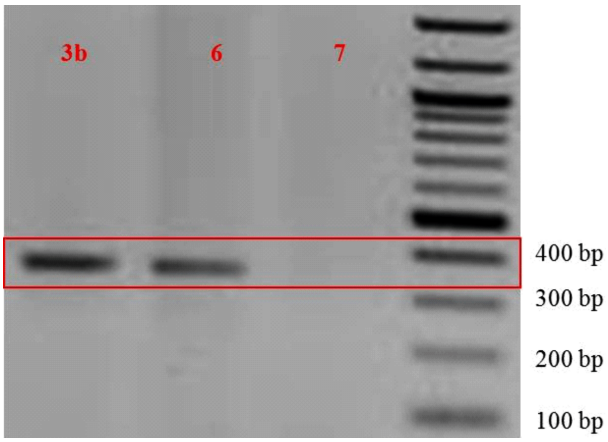

Figure 3 – *Agarose gel electrophoresis for VP1 PCR products. Samples had undergone PCR amplification for a region of the VP1 (352 bp) portion of the virus. Samples #3b and #6 showed bands for VP1, while sample #7 did not.*

Table 2 – *MCPyV detection results. A total of 18 tumors from 14 patents were studied. Cases #1, #2, #3, and #7 had two tumors (designated a and b). MCV was detected in 8/18 tumor samples (44%) and 6/14 patients (33%)*

Case #	Age [years]	Gender	Site	LT1	LT1/M1 (nested PCR)	M1	LT3	VP1	Any viral product	FU [months]
1a.	72	M	H&N – primary	N	N	Y	Y	N	Y	DOD, 19
1b.			Neck LN – metastasis	N	N	N	N	N	N	
2a.	73	M	H&N – primary	N	N	Y	N	N	Y	DOD, 27
2b.			H&N skin – metastasis	N	N	N	N	N	N	
3a.	78	M	Extremity – primary	N	N	Y	Y	N	Y	DOD, 7
3b.			Groin LN – metastasis	N	N	N	N	Y	Y	
4.	76	M	Neck LN – metastasis	N	N	N	N	N	N	DOD, 59
5.	73	M	Neck LN – metastasis	N	N	N	N	N	N	NED, 20
6.	64	M	H&N – primary	N	N	N	N	Y	Y	DOD, 5
7a.	77	M	H&N parotid – metastasis	N	Y	N	Y	N	Y	NED, 39
7b.			H&N skin – metastasis	N	N	N	N	N	N	
8.	63	M	H&N – primary	N	N	N	N	N	N	AWD, 21
9.	72	F	LN axilla – primary	N	N	N	N	N	N	DOD, 0.7
10.	56	M	Extremity – primary	N	Y	Y	Y	N	Y	NA
11.	71	M	H&N – primary	N	N	N	N	N	N	NED, 38
12.	93	M	H&N – primary	N	Y	Y	N	N	Y	NED, 20

Case #	Age [years]	Gender	Site	LT1	LT1/M1 (nested PCR)	M1	LT3	VP1	Any viral product	FU [months]
13.	78	M	H&N – primary	N	N	N	N	N	N	AWD, 8
14.	72	M	H&N – primary	N	N	N	N	N	N	DOD, 1
Total				0	3	5	4	2	8	

M – Male; F – Female; H&N – Head and neck; LN – Lymph node; N – Not detected; Y – Detected; NA – Not available; FU – Follow-up; DOD – Dead of disease; NED – No evidence of disease; AWD – Alive with disease.

Although, one-step PCR did not amplify the LT1 segment in any case, nested PCR with primers for LT1 followed by amplification with primers for the M1–M2 segment (which maps within the LT1 segment) showed a PCR product in three (17%) cases.

For three patients (Cases #1, #2, and #3 in Table 2), MCPyV detection was performed in both the primary and metastatic tumors. In Case #3, the virus was found in both lesions while in the other two, only the primary tumor demonstrated the virus. In Case #7, MCPyV was found in one of the two separate metastatic foci investigated.

Two-year survival rate and median survival for the MCPyV-positive MCCs were 48% and 22.5 months, respectively and for the negative ones were 69% and 51.3 months, respectively; however, the difference did not reach statistical significance (p=0.8). Kaplan–Meier survival curves for MCC patients comparing those with MCPyV-positive *versus* negative tumors are shown in Figure 4.

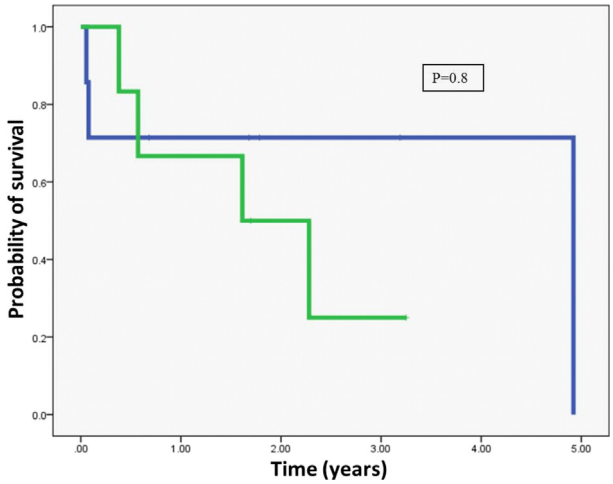

Figure 4 – ***Kaplan–Meier survival curves by MCPyV status. The blue line represents patients with MCPyV-negative tumors, whereas the green line shows patients with MCPyV-positive ones.***

Both MCC tumors from the extremities were positive for MCPyV (100%), while only five of 11 (45.5%) of the head and neck MCCs were positive. The primary MCC in an axillary lymph node was also negative. The difference was not statistically significant (p=0.4 on Fisher's exact test); however, this may have been due to the small number of cases investigated. The frequency of MCPyV-positive tumors by stage was 66.6%, 50%, 50% and 50% for stages I, II, III and IV, respectively.

Discussion

The discovery of MCPyV integrated in the genome of MCC tumors has opened the door for new research venues in the pathogenesis of this disease. Generally, it is ideal to use fresh frozen tissue to investigate tumors due to better tissue preservation; however, the relative rarity of MCC tumors and the current protocols in pathology create challenges for the assembly of large case series with fresh tissue. In our study, we explored the feasibility of detecting MCPyV fragments in FFPE tissue from MCC tumors. The study demonstrated that detecting the MCPyV by PCR from archival (FFPE) tissue of MCC is indeed feasible and reproducible.

Approximately half of the cases in our series showed MCPyV involvement, lower than the 80% rate reported in the original paper by Feng *et al.* [11]. Prior studies investigating the frequency of MCPyV detection by PCR have reported rates of about 75–85% [21–24]. A somewhat wider detection rate in the range of 63–89% is reported by IHC techniques using an antibody against the large T antigen (CM2M4) [14, 25–27]. Recently, a detection rate of 97% was reported by Rodig *et al.* using a new antibody against LT (AB3) [28]. The reasons for the lower frequency of association with MCPyV in our study compared to some of the prior reports are unclear. One explanation could be that our series included a high proportion of MCC tumors from the head and neck area (79%). A prior study by Paik *et al.* corroborates our results by showing that head and neck MCCs demonstrate a lower frequency of MCPyV-positive tumors compared to other sites [29]. The authors also reported a MCPyV frequency of only 18% in MCCs from a cohort of patients from Australia [29]. Together these results suggest that at specific sun-exposed sites and in specific populations, there may be a more prevalent role for UV exposure in MCC carcinogenesis that is not dependent on MCPyV genomic integration. We cannot exclude however, that some of the differences in virus detection are caused by impaired sensitivity of the assay due to the DNA degradation associated with the processing of FFPE tissue. We observed in our data that lower size amplicons had a higher rate of successful PCR amplification and detection. For example, LT1 (440 bp) showed no PCR products after a single round of PCR, while LT3 (309 bp) and M1–M2 (179 bp) amplicons were found in five of the eight (63%) samples positive for the virus. Using a nested PCR approach by re-amplifying the M1/M2 portion (179 bp) within the LT1 amplicon increased the LT1 detection rate from 0% to 17%. Therefore, it is likely that the use of larger amplicon size results in more false negative results due to DNA fragmentation during processing. Selection of PCR products with a size under 200 bp and a nested PCR approach is clearly desirable for FFPE tissue.

The lifecycle of MCPyV is not yet known and there is limited information regarding the cell type that supports the growth of this virus [17, 30]. A few studies attempted transfection of various human cells with MCPyV with variable and modest results [31, 32]. In addition, robust propagation of virions in culture systems has, to date, not been successful [18]. Several studies have detected the presence of MCPyV in normal skin, which may suggest that virus growth is dependent on the process of differentiation of the stratified epidermis [33–35].

Epidemiological studies have determined that up to 80% of the adult population tests positive for serum MCPyV suggesting that asymptomatic infection is a common occurrence [36]. Moreover, MCPyV has been detected at multiple anatomic sites besides skin, including the respiratory tract, gastrointestinal tract, lymphoid tissues, urine, saliva and multiple other organs; however, in these sites the viral load is relatively low compared to skin and MCC tumors [14, 35, 37–40].

Analysis of the clinical implications of MCPyV status in our series showed no significant differences in clinical outcome between the positive and negative cases; however, we observed a slight trend for more aggressive behavior in viral-positive *versus* negative tumors. These results are similar to other studies that found no difference in outcome related to viral integration or found a non-significant trend for worst behavior in MCPyV-positive MCCs [22, 24]. However, other investigators found that MCPyV-positive status is associated with a favorable outcome [41]. It appears that studies with more statistical power are needed to elucidate the impact of viral integration on MCC behavior. We found a trend for a lower frequency of MCPyV-positive MCC tumors originating from the head and neck compared to those arising in the extremities (45.5% *versus* 100%, respectively). These findings corroborate other studies and may suggest that UV exposure may drive MCC oncogenesis through a mechanism independent from MCPyV [29].

The precise mechanism of MCPyV oncogenesis is still unclear; however, the high occurrence of MCPyV incorporation in the MCC cells genome suggests a function of the virus in the pathogenesis and/or maintenance of the tumor. Previously it was found that other polyoma viruses such as Simian virus 40 (SV40) were likely to cause cancers in humans *via* integration of viral DNA into the human genome [42, 43]. In this context, MCPyV could initiate tumor formation, particularly in immunocompromised patients or augment tumor severity by maintaining its growth. Another element supporting an active role of the virus is the expression of viral T antigen transcripts and proteins by the tumor cells in which the virus in integrated. Recent research has found that knockdown of LT and sT in MCC cell lines infected with MCPyV inhibits cell growth supporting a role for these factors in oncogenesis [19, 44]. The significance of LT may be related to its role in promoting cell entry into the S phase by inactivating p53 and pRb *via* the LxCxE motif in addition to promoting topoisomerase I, primase, and DNA polymerase [16]. Another finding by Shuda *et al.* was that in MCC tumors the integrated MCPyV carries mutations of the T antigen locus. These mutations result in expression of a truncated form of the LT that lacks the origin binding and helicase domains of the wild LT, which eliminates the replication capability of the virus but retains the pRb binding motifs, which is thought to contribute to uncontrolled cellular proliferation [15]. This combination of events that prevents viral replication and preserves the stimulation of cellular proliferation are common in viral oncogenesis [45]. One explanation advanced by the authors was that there is a selection pressure for the virus to undergo a mutation, which prevents viral replication that could be detrimental to the tumor cell survival while maintaining the cell proliferation activity [15].

Another interesting observation was the discrepancy between the MCPyV status in primary and metastatic sites. In two out of three cases in which both primary and metastatic foci were investigated, the MCPyV was detected only in the primary tumor. While we cannot exclude that this discrepancy is due to false negative results due to DNA degradation, the findings open the possibility that in some cases the metastatic foci may lose the MCPyV. This observation lends support to the "hit-and-run" theory presented by Houben *et al.* in a recent letter to the editor [46]. The authors advanced the idea that in some MCCs the virus is only necessary for tumor initiation and that further mutations may render them independent from the virus. Since there is no more selection pressure for maintaining the virus integrated in the genome, new tumor clones may become MCPyV-negative [46].

Conclusions

We have detected in our study the presence of MCPyV in about half of the MCC cases tested using FFPE tissue from archival specimens. We found no significant correlation between the presence of the virus and clinical outcome or stage at presentation; however, a trend was noted for a worst outcome in virus-positive cases. Also, the lower frequency of MCPyV-positive tumors in head & neck areas suggests that a proportion of MCC tumors arise through an alternative pathway, possible triggered by UV exposure. While there is significant evidence to suggest that the MCPyV has an active role in MCC oncogenesis the exact mechanism by which incorporation of the virus into the tumor genome contributes to oncogenic transformation still needs further research.

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