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Detection of Merkel cell polyomavirus in Merkel cell carcinomas and small cell carcinomas by PCR and immunohistochemistry

Hun Soon Jung[1*], Yoon-La Choi[2,3*], Jong-Sun Choi[4], Ji Hyeon Roh[4], Jai-Kyong Pyon[5], Kyong-Je Woo[5], Eun Hee Lee[6], Kee-Taek Jang[3], Joungho Han[3], Chan-Sik Park[7], Young Soo Park[7] and Young Kee Shin[1]

[1]Research Institute of Pharmaceutical Science, Department of Pharmacy, Seoul National University College of Pharmacy, Seoul, Korea, [2]Laboratory of Cancer Genomics and Molecular Pathology, Samsung Medical Center, Seoul, Korea, [3]Department of Pathology, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea, [4]Department of Pathology, Dongguk University-Seoul, Graduate School of Medicine, Seoul, Korea, [5]Department of Plastic Surgery, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea, [6]Department of Pathology, Samsung Changwon Hospital, Sungkyunkwan University School of Medicine, Changwon, Korea and [7]Department of Pathology, University of Ulsan College of Medicine, Asan Medical Center, Seoul, Korea

* Authors contributed equally to the work

Summary. Recently, the clonal integration of a new human polyomavirus (Merkel cell polyomavirus or MCPyV) has been reported in Merkel cell carcinoma (MCC). In order to investigate the presence of MCPyV in small cell carcinomas (SCCs) and small round cell tumors (SRCTs), we collected formalin-fixed paraffin-embedded tissue specimens including 14 MCCs, 24 SCCs, 7 Ewing sarcoma/primitive neuroectodermal tumors (ES/PNETs) and 5 neuroblastomas. We also collected specimens of other cancers including 12 malignant melanomas, 10 breast, 10 ovarian and 20 gastric cancers.

We used 3 primer sets for which the sequences were previously published (LT1, LT3, and VP1) and 3 newly designed primer sets (LT1-1, LT1-1a, and LT3a). Quantitative real-time PCR was also performed with the LTq primer set. Nested PCR using the LT3a primer set detected more cases of MCPyV infection in MCC. In total, 12 of 14 (85.7%) MCC cases were positive for MCPyV by PCR, which was consistent with published data. Some SCC specimens were also positive for MCPyV (37.5%) by PCR. PCR products from MCC and SCC cases showed premature truncation and frameshift mutation. Furthermore, one case of ES/PNET and one gastric carcinoma showed MCPyV DNA. However, MCPyV DNA and transcript were only detected in MCCs with quantitative real-time PCR analysis. In addition, 11 of 13 (84.6%) MCC cases and 6 of 23 (26.1%) SCC cases showed immunoreactivity with monoclonal antibodies against MCPyV large T-antigen. Considering both PCR and IHC results, MCPyV was detected in all MCCs tested.

The presence of MCPyV in all MCC cases tested and in some SCC cases suggests that MCPyV may be involved in the malignant transformation.

Key words: Merkel cell carcinoma, Merkel cell polyomavirus, Small cell carcinoma

Offprint requests to: Young Kee Shin, M.D., Ph.D., Molecular Pathology Laboratory, Department of Pharmacy, Seoul National University College of Pharmacy, San 56-1. Sillim-dong, Gwanak-gu, Seoul, 151-742, Korea. e-mail: ykeeshin@snu.ac.kr

Introduction

Merkel cell carcinoma (MCC) is a primary cutaneous neuroendocrine carcinoma. MCC has a poor outcome with a high risk of lymphatic metastasis (Hodgson, 2005; Knobloch et al., 2009). The overall incidence of MCC is estimated to be 0.44 cases per 100,000. The incidence of MCC is reported to have risen substantially over time, with an 8% increase annually between 1986 and 2001; however, few cases have been reported in Asian patients (Hodgson, 2005). The tumors

are composed of malignant small blue cells typically expressing neuroendocrine markers and revealing a characteristically paranuclear dot-like expression pattern of cytokeratin 20 (Bobos et al., 2006). Although sun exposure is thought to be one of the risk factors of MCC, the histogenesis of MCC is still under debate (Cerroni and Kerl, 1997; Iacocca et al., 1998; Brenner et al., 2001; Aydin et al., 2003). Patients undergoing immunosuppressive therapy or patients with HIV infection are at high risk for MCC, which supports the involvement of viral infection in the pathogenesis of MCC (Swann and Yoon, 2007). Recently, a new polyomavirus was reported in MCC tumors. This virus, named Merkel cell polyomavirus (abbreviated MCV or MCPyV), was discovered by using the digital transcriptome subtraction technique (Feng et al., 2008). Feng et al. (2008) reported clonal integration of a polyomavirus in human MCC, implying that MCPyV may be a contributing factor in the pathogenesis of MCC. They also reported the presence of MCPyV in 8 of 10 human MCCs and also clonal integration of the viral DNA.

Polyomaviruses are small double-stranded DNA viruses that are suspected to be etiologic agents of human cancer. Currently, 5 different human polyomaviruses are known. Of these, 4 (i.e., BKV, JCV, KIV, and WUV) belong to the SV40 subgroup. Although some of these human polyomaviruses are tumorigenic in experimental animals, there is currently no convincing evidence that these viruses are associated causally with malignant tumors in humans. Recently, MCPyV was detected in 75–89% of MCCs and was found to be strongly and selectively associated with MCC (Foulongne et al., 2008; Kassem et al., 2008; Becker et al., 2009; Garneski et al., 2009). MCPyV is a 5.4 kbp DNA virus that expresses tumor (T) antigen mRNAs in tumor tissue. The MCPyV large T (LT) gene sequences obtained from tumor-derived viruses have truncating mutations that are not present in the wild-type virus (Shuda et al., 2008).

While the association between MCC and MCPyV has been made clear in several reports (Buck and Lowy, 2009), the frequency of other tumors, including skin tumors or other organ tumors, is not known. Several controversial data have been published. The availability of more sensitive methods has allowed detection of MCPyV in normal skin, normal blood and other tumor types (Loyo et al., 2010). Recently, Tolstov et al. (2009) reported that MCPyV is a common human infection that can be detected by conformational capsid epitope immunoassays. They found that the prevalence of MCPyV increases from 50% among children 15 years of age or younger to 80% among persons older than 50 years of age. They demonstrated that MCPyV is a widespread but previously unrecognized human infection (Tolstov et al., 2009). However, Loyo et al. (2010) reported that the levels of MCPyV in MCCs were over 60 times higher than the highest values in all other tissues.

MCC and small cell carcinoma (SCC) are aggressive neuroendocrine carcinomas and are histologically composed of morphologically identical small cells. Small round cell tumors (SRCTs), including neuroblastoma, lymphoma and Ewing sarcoma/primitive neuroectodermal tumor (ES/PNET), have a similar appearance under light microscopy, and are often indistinguishable using common immunocytochemical makers (Gregorio et al., 2008). SRCTs also display similar histological morphologies to MCC, but their oncogenetic etiology is not yet understood. Several studies have reported the presence of MCPyV in SCC, SRCTs, and also in other carcinomas; however, the results have been controversial (Bluemn et al., 2009; Busam et al., 2009; Duncavage et al., 2009b; Helmbold et al., 2009).

We investigated 14 cases of MCC from 11 patients evaluated between January 2000 and December 2008 in Korea. Furthermore, we collected 36 cases of SCC, ES/PNET and neuroblastoma to investigate the presence of MCPyV by PCR, real-time PCR, sequencing and immunohistochemistry (IHC).

Materials and methods

Patient samples and nucleic acid purification

The present study included the formalin-fixed paraffin-embedded (FFPE) resection and biopsy specimens of 11 MCC patients, 9 of whom were selected from the archives of the Department of Pathology of the Samsung Medical Center. The samples were collected between 1994 and 2008. Of these 9, 6 cases (MCCA1, 2, 5, 6, 7, and 8) had been included in a previous study (Woo et al., 2010). The other 2 specimens were selected from the archives of the Department of Pathology of the Asan Medical Center. SCC samples from 24 patients were used, representing 16 primary lung tumors, 6 lymph node metastases, 1 brain metastasis, 2 bladder tumors and 1 uterine cervical tumor. Details of the clinicopathologic variables of MCC and SCC patients are included in Table 1. In total, 7 ES/PNETs, 5 neuroblastomas, 12 malignant melanomas, 20 gastric cancers, 10 breast cancers and 10 colon cancers were collected from the archives of the Department of Pathology of the Samsung Medical Center. The investigation protocol was approved by the institutional review board. Serial sections of all specimens were used for hematoxylin and eosin (H&E) staining and nucleic acid preparation. The H&E staining of the selected specimens was reviewed by two pathologists (YLC, KTJ) to select paraffin material containing more than 50% tumor tissue.

DNA extraction was performed on 2 to 10 consecutive 4-μm paraffin sections from each specimen. In brief, after deparaffinization, tissues were lysed by proteinase K overnight (56°C) until complete tissue lysis was achieved and DNA was extracted using the DNeasy Tissue kit (Qiagen, Valencia, CA, USA). Purified DNA

was measured using a spectrophotometer (Nano-drop, ND1000; PeqLab, Germany) and directly used for PCR. RNA was extracted from formalin-fixed paraffin-embedded (FFPE) tissues using the RNeasy FFPE kit (Qiagen).

MCPyV PCR and sequence analyses

PCR was performed with 50 ng of genomic DNA using the h-Taq DNA polymerase (SolGent, Korea) in a final volume of 25 μL. For MCPyV detection, we used the LT1, LT3, and VP1 primer sets as published (Feng et al., 2008) and newly designed LT1-1, LT1-1a, and LT3a primer sets (Table 4). Cycling conditions for the first PCR were 15 min at 95°C, followed by 35 cycles each at 94°C for 20 sec, 57.5°C for 40 sec and 72°C for 1 min, with a final elongation cycle at 72°C for 5 min. The annealing temperature of LT1-1a was 58.5°C. In addition, we evaluated the use of the LT1-1a and LT3a primer sets for nested PCR purposes using 30 cycles for each primer set. The PCR products were analyzed by gel electrophoresis and by sequence analysis. The integrity and quality of the DNA was tested by ß-globin DNA PCR. PCR was performed with 100 ng of genomic DNA in a final volume of 25 μL. Water instead of DNA template was used for PCR-negative controls containing all other PCR components. Case MCCA1 tested positive with all 5 MCPyV primer sets (Table 1). DNA and RNA were extracted from FFPE and frozen tissues of MCCA1, and were used as positive controls. The

Table 1. Characteristics of Merkel cell carcinomas and small cell carcinomas, and the results of MCPyV PCR and IHC.

ID	Site	Sex	Age (yr)	Dx	ß-Globin	MCPyV Primer Sets							IHC	
						LT1	LT 1-1a	LT3	LT3a	VP1	LTq (gDNA)	LTq (cDNA)	T	L
MCCA1	Neck	F	72	MCC	+	+	+	+	+	+	+	+	++	-
MCCA2-1	Arm	M	71	MCC	+	-	+	+	+	-	+	+	+++	-
MCCA2-2	Arm		71	MCC	+	-	+	+	+	-	NT	NT	+++	+
MCCA3	Arm	F	63	MCC	+	+	+	-	+	+	+	+	+++	-
MCCA4	Eyelid	F	75	MCC	+	-	+	-	+	-	+	+	+	++
MCCA5	Eyelid	F	58	MCC	+	-	+	+	+	-	-	-	NT	NT
MCCA6	Eyelid	F	74	MCC	+	+	+	+	+	-	NT	NT	++	-
MCCA7-1	Leg	M	68	MCC	+	-	-	+	+	-	+	IN	++	-
MCCA7-2	LN*		69	MCC	+	-	-	ND	+	-	+	IN	+	-
MCAC8-1	Scalp	F	33	MCC	+	-	-	+	+	-	-	-	-	-
MCCA8-2	LN**		34	MCC	+	-	-	ND	+	-	-	-	-	-
MCCA9	Chin	F	72	MCC	+	-	-	+	+	-	NT	NT	+++	-
MCCA10	NA	F	66	MCC	+	-	-	-	-	-	NT	NT	+	-
MCCA11	NA	F	47	MCC	+	-	-	-	-	-	NT	NT	+++	-
SCC1	Lung	M	64	SCC	+	-	-	+	+	-	-	NT	+	-
SCC2	Lung→LN	M	67	SCC	+	-	-	-	+	-	-	-	-	+
SCC3	Uterine cervix	F	30	SCC	+	-	-	+	+	-	-	IN	+	-
SCC4	Lung→LN	M	53	SCC	+	-	-	-	+	-	-	-	+	++
SCC5	Lung→LN	M	66	SCC	+	-	-	-	+	-	-	-	+	+
SCC6	Lung	F	77	SCC	+	-	-	-	+	-	-	NT	NT	NT
SCC7	Lung→LN	M	62	SCC	+	-	-	-	+	-	-	-	-	+
SCC8	Lung	M	57	SCC	+	-	-	+	+	-	-	-	+	-
SCC9	Lung→LN	M	58	SCC	+	-	-	-	+	-	-	NT	+	++
SCC10	Lung	M	66	SCC	+	-	-	-	-	-	ND	ND	-	-
SCC11	Lung	M	82	SCC	+	-	-	-	-	-	ND	ND	-	-
SCC12	Lung	M	60	SCC	+	-	-	-	-	-	ND	ND	-	-
SCC13	Lung	M	67	CSL	+	-	-	-	-	-	ND	ND	-	-
SCC14	Lung	M	71	SCC	+	ND	-	ND	-	-	ND	ND	-	-
SCC15	Bladder	M	61	SCC	+	ND	-	ND	-	-	ND	ND	-	-
SCC16	Lung	M	83	SCC	+	ND	-	ND	-	-	ND	ND	-	-
SCC17	Bladder	M	60	SCC	+	ND	-	ND	-	-	ND	ND	-	-
SCC18	Lung	M	56	CSL	+	ND	-	ND	-	-	ND	ND	-	-
SCC19	Lung→Brain	M	56	SCC	+	ND	-	ND	-	-	ND	ND	-	-
SCC20	Lung→LN	M	58	SCC	+	ND	-	ND	-	-	ND	ND	-	-
SCC21	Lung	M	57	CSL	+	ND	-	ND	-	-	ND	ND	-	-
SCC22	Lung	M	75	CSL	+	ND	-	ND	-	-	ND	ND	-	-
SCC23	Lung	M	54	SCC	+	ND	-	ND	-	-	ND	ND	-	-
SCC24	Lung	M	66	SCC	+	ND	-	ND	-	-	ND	ND	-	-

NA: Not available; ND: Not determined; NT: Not tested; IN: Invalid result; IHC: immunohistochemistry; MCC: Merkel cell carcinoma; LN: Lymph node; CSL: Combined small cell carcinoma with large neuroendocrine cell carcinoma; SCC: Small cell carcinoma; LN: lymph node; *: inguinal; **: postauricular; T: tumor; L: lymphocyte

detection sensitivity of the PCR was investigated (data not shown). DNA sequences were compared with the reference sequences of the National Center for Biotechnology Information (NCBI) Entrez Nucleotide database gb|EU375803.1 Merkel cell polyomavirus isolate MCC350, gb|EU375804.1 Merkel cell polyomavirus isolate MCC339, gb|FJ464337.1 Merkel cell polyomavirus strain TKS, and gb|FJ173815.1 Merkel cell polyomavirus isolate MKL-1, using the NCBI BLAST program.

Quantitative Real-time PCR (qPCR) and RT-PCR (qRT-PCR) analysis

RNA was reverse transcribed using oligo-dT primers and SuperScript III reverse transcriptase (Invitrogen, CA, USA). PCR primers and dual color TaqMan probes were designed by TIB MOLBIOL (Berlin, Germany). The LTq probe was selected from the Universal Probe Library (UPL) system (Roche). The Primers and probe sequences of LTq and the endogenous reference genes ACTB and HPRT are shown in Table 4. ACTB was used as an internal control in qPCR using genomic DNA as the template and HPRT was used in qRT-PCR using cDNA as previously described (Kwon et al., 2009). qRT-PCR was carried out in the LightCycler Real-time PCR Detection system (Roche Diagnostics, Basel, Switzerland). The mRNA levels were detected at 530 and 560 nm, respectively. The conditions for PCR were as follows: 95°C for 10 min (1 cycle), 95°C for 10 s, 55°C for 30 s (single; 45-cycle quantification), and 40°C for 30 s (1 cycle). Relative expression quantification was performed using the 2(-Delta Delta C(T)) Method.

IHC

Tissue sections of 5 μm thickness were applied to glass slides for IHC and heated overnight at 60°C to ensure proper adherence. The sections were deparaffinized with xylene, hydrated in serial dilutions of alcohol, and then immersed in 3% hydrogen peroxide solution to neutralize endogenous peroxidase activity. Next, sections were microwaved in 10 mM citrate buffer for antigen retrieval. Slides were incubated with monoclonal antibodies (mAb) against MCPyV large T-antigen (CM2B4, sc-136172, 1:50 dilution, Santa Cruz Biotechnology, CA, USA) for 1 h at room temperature. After washing, the slides were incubated for 30 min at room temperature with anti-mouse dextran polymer (Envision K4001, DAKO, Glostrup, Denmark) as the secondary antibody. After 3 rinses with distilled water with 0.1% Tween 20, the slides were washed and the chromogen was developed for 5 min with liquid 3,3'-diaminobenzidine (DAKO). Immunostaining of the tissues was semi-quantified based on the number of immunopositive tumor cells and graded as follows: negative (-) no immunostaining; + less than 50% tumor cells with weak intensity; ++ more than 50% tumor cells with weak to moderate intensity; and +++ more than 50% tumor cells with strong intensity.

Results

MCPyV detection in MCC

The mean age of the MCC patients tested was 63.5 years (range 33-74 years) and 18.2% (n=2) were male and 81.8% (n=9) were female. Head and neck were the most common sites and there were 3 cases with MCC of the eyelid (Fig. 1A). The representative histology of case MCCA2 shows the presence of a monotonous small round cells that was positive for CK20 with dot-patterned and diffuse chromogranin (Fig. 1B). The regions amplified by PCR, qPCR, and qRT-PCR are indicated on the genomic sequence of MCPyV (Fig. 2A). The positive rate of MCPyV detection in FFPE tissues varied with the primer set used. In total, 9 of 11 MCC cases (81.8%) tested positive for MCPyV DNA by PCR using the LT3a primer set. The detection rates of MCPyV in MCC by LT3, LT1, and VP1 primers were 63.6%, 27.3%, and 18.2%, respectively. Of these, only one MCC case tested positive in all 5 MCPyV primer sets. In total, 3 metastatic or recurrent cases were included, and these yielded consistently positive results (MCCA2, 7 and 8). Sequence analyses of the PCR products identified all PCR products obtained with the MCPyV primers as MCPyV sequences. Representative cases of LT1-1a positive MCC resulted in the generation of 136 bp PCR products (Fig. 2B).

MCPyV detection in other tumors

The MCPyV detection test was applied to other tumors, including SCC, which is also a neuroendocrine carcinoma that is often confused with MCC because of its similar histology. No MCPyV sequences were detected in any of the DNAs isolated from neuroblastomas, malignant melanomas, breast cancers, or ovarian cancers (Tables 2, 3). However MCPyV DNA was detected in 9 of 24 SCCs and particularly in 6 cases of lymph node metastases from the lung, 2 primary lung tumors and 1 uterine cervical tumor. With the exception of 3 cases that were detected by the LT3 primer set, only the LT3a primer set detected MCPyV DNA.

Among the SRCTs included in this study, only 1 case of ES/PNET (EW4) was found to be positive for MCPyV. The patient was an 11 year-old boy with a brain mass. From the similar studies, 1 case of gastric cancer (GC1), corresponding a signet ring cell carcinoma from a 77 year-old female, had MCPyV DNA. Both of these PCR positive cases were detected specifically with the LT3a primer set.

MCPyV detection using quantitative real-time PCR (qPCR)

To further detect MCPyV at the genome level, we performed qPCR using genomic DNA from FFPE tissue

as template. Cases of MCC, SCC, ES/PNET and gastric cancer that were positive for MCPyV DNA by PCR were tested with the LTq primer set, although some cases were not tested because there was not enough paraffin-embedded tissue (Tables 1-4). MCPyV DNA was detected in only 6 cases of MCC. A similar result was also obtained by qRT-PCR using cDNA from FFPE tissue.

Alignment of the Sequence of MCPyV

When all of the MCPyV DNA sequences detected were compared with the reference sequences of the National Center for Biotechnology Information (NCBI) Entrez Nucleotide database, most of them revealed a 97% or 98% homology to previously published MCC sequences. In the case of LT1-1a, MCCA1 showed a truncated stop sequence and several mutations (MCCA2 and MCCA4) (Fig. 3A). MCCA1 and MCCA4 mutations resulted in premature truncation and frameshift of the Rb-binding domain, respectively. Sequences amplified using the LT3 and LT3a primer set were aligned and case SCC8 showed truncation in LT3 region (Fig. 3B). Case GC1 showed a completely different sequence from the LT3a region of the reference sequence.

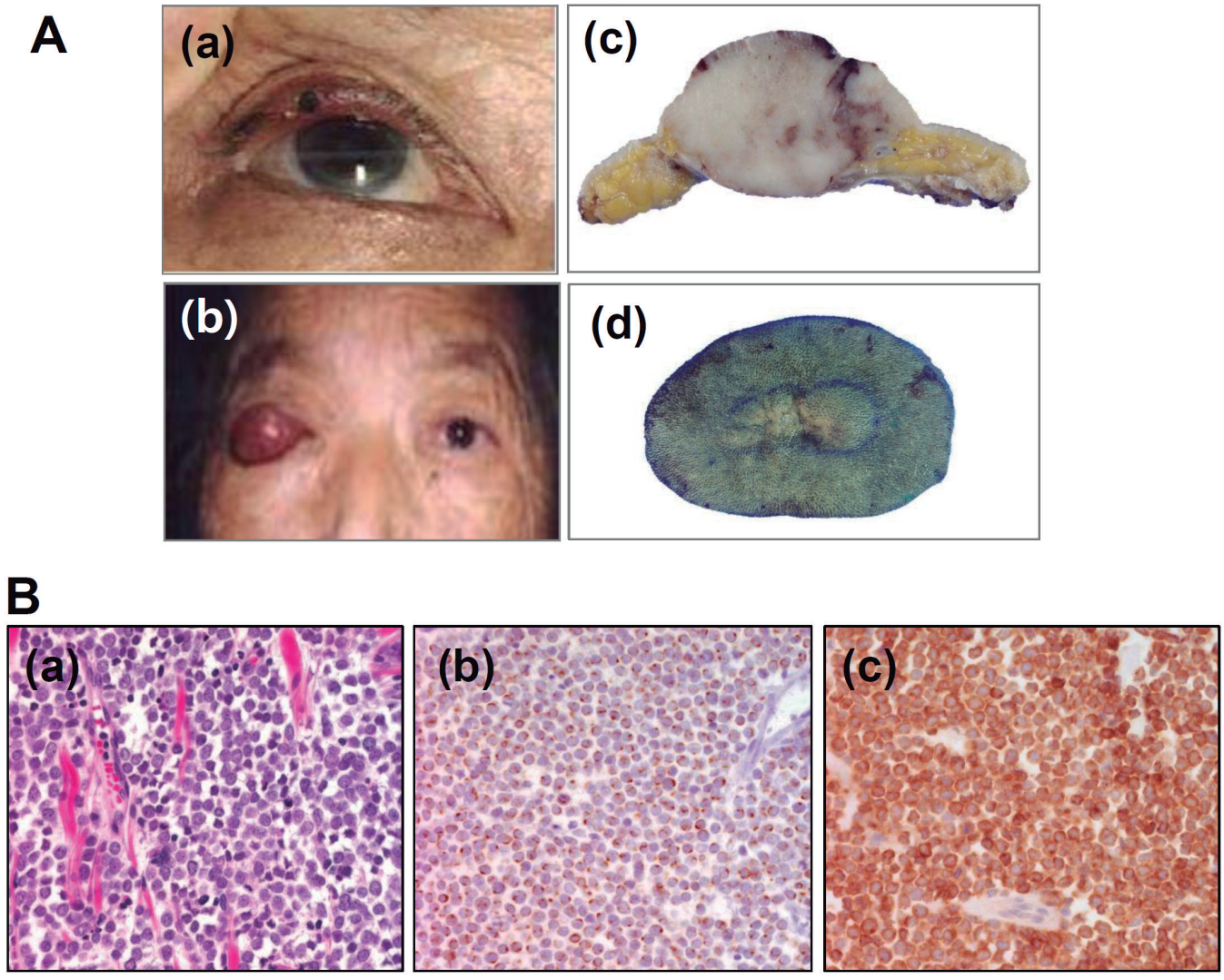

Fig. 1. A. Representative gross appearances of MCCs. **(a)** Small nodule in upper eyelid of MCCA5. **(b)** Protruding mass of upper eyelid of MCCA6. **(c)** The cut surface of the mass of MCCA2 is solid and white. **(d)** Ill-defined mass in scalp from MCCA8. **B.** Histologic features of Merkel cell carcinoma. **(a)** The monotonous population of neoplastic cells with round nuclei, fine chromatin, and inconspicuous nucleoli. H&E stain. **(b)** CK20 immunostaining shows characteristic punctate perinuclear staining. **(c)** Chromogranin immunostaining shows a diffuse cytoplasmic staining. x 40

IHC

The epitope site of mAb CM2B4 occurs in a unique span of exon 2 of MCPyV T antigen (Fig. 2 and 3). Overall, 11 (84.6%) of the 13 IHC-available MCCs were immunoreactive for CM2B4. Nine of 11 positive cases were positive for MCPyV by PCR (Table 1). Two cases of MCC (MCCA10 and MCCA11) in which MCPyV was not detected by PCR showed immunoreactivity. The majority of the tumors showed strong and diffuse

Table 2. Characteristics of small round cell tumors (SRCTs) and results of MCPyV PCR.

ID	Site	Sex	Age (yr)	Dx	ß-Globin	MCPyV Primer Sets				
						LT1-1a	LT3a	VP1	LTq (gDNA)	LTq (cDNA)
EW1	Soft tissue, Arm	F	42	ES/PNET	+	-	-	-	ND	ND
EW2	Nasal cavity	M	42	ES/PNET	+	-	-	-	ND	ND
EW3	Soft tissue, Buttock	F	60	ES/PNET	+	-	-	-	ND	ND
EW4	Brain	M	11	ES/PNET	+	-	+	-	-	-
EW5	Soft tissue, Chest wall	F	18	ES/PNET	+	-	-	-	ND	ND
EW6	Soft tissue, Popliteal	M	7	ES/PNET	+	-	-	-	ND	ND
EW7	Bone, Humerus	M	20	ES/PNET	+	-	-	-	ND	ND
NB1	Brain	M	48	ONB	+	-	-	-	ND	ND
NB2	Nasal cavity	F	70	ONB	+	-	-	-	ND	ND
NB3	Oral cavity	M	74	ONB	+	-	-	-	ND	ND
NB4	Nasal cavity	M	71	ONB	+	-	-	-	ND	ND
NB5	Nasal cavity	M	60	ONB	+	-	-	-	ND	ND
MM1	Soft tissue, flank	M	47	MM	+	-	-	-	ND	ND
MM2	Chest wall	F	55	MM	+	-	-	-	ND	ND
MM3	Soft tissue, axilla	M	50	MM	+	-	-	-	ND	ND
MM4	Skin	M	66	MM	+	-	-	-	ND	ND
MM5	Colon	M	50	MM	+	-	-	-	ND	ND
MM6	Colon	M	73	MM	+	-	-	-	ND	ND
MM7	Oral cavity	F	53	MM	+	-	-	-	ND	ND
MM8	Femur	F	64	MM	+	-	-	-	ND	ND
MM9	Axilla	M	50	MM	+	-	-	-	ND	ND
MM10	Oral cavity	F	46	MM	+	-	-	-	ND	ND
MM11	Rectum	F	53	MM	+	-	-	-	ND	ND
MM12	Skin->Pleura	M	57	MM	+	-	-	-	ND	ND

ES: Ewing sarcoma; PNET: primitive neuroectodermal tumor; ONB: olfactory neuroblastoma; MM: malignant melanoma; ND: Not determined

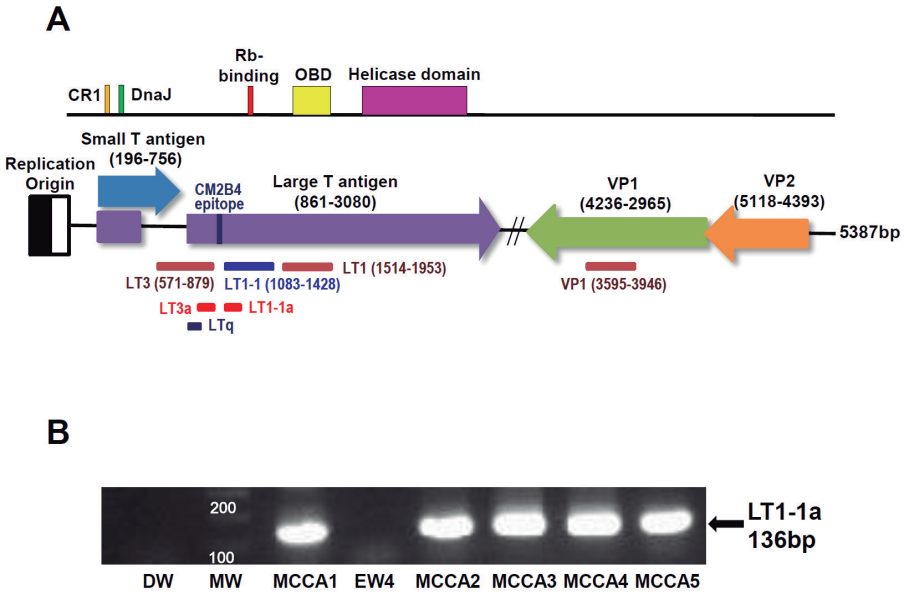

Fig. 2. A. Map of the 5387 bp Merkel cell polyomavirus genome. The locations of primer sets used to detect the virus are shown. LT1, LT3, and VP1 are from published reports. LT1-1, LT1-1a, LT3a and LTq are unique to this study. Large T antigen protein contains CR1 (orange), DnaJ (green), Rb-binding (red), origin binding (yellow) and helicase domains (pink). **B.** Representative results of the PCR using LT1-1a primer to detect Merkel cell polyomavirus. The nested LT1-1a products (136 bp) are detected in Merkel cell carcinomas but not in Ewing sarcoma (EW4).

nuclear staining (Fig. 4A,F,H). In contrast, primary and metastatic tumors from MCCA8 were immunonegative for CM2B4 but showed the presence of MCPyV by PCR using the LT3 or LT3a primer set (Fig. 4G).

Six (26.1%) of 23 SCCs (SCC1, SCC3, SCC4, SCC5, SCC8, and SCC9) showed weak (grade +) immunoreactivity in tumor cells (Fig. 4J-N). Two cases of SCC that showed MCPyV PCR positivity (SCC2 and SCC7) had CM2B4-positive lymphocytes but no CM2B4-positive tumor cells (Fig. 4I). None of the MCPyV PCR-negative SCCs showed CM2B4 immunoreactivity in either tumor cells or lymphocytes (Table 1 and Fig. 4O). None of the cases of the MCPyV PCR-positive SRCT (EW4) or carcinoma (GC1) showed any nuclear staining of either tumor cells or lymphocytes, indicating low MCPyV copy numbers (data not shown).

Discussion

MCPyV DNA was detected in 81.8% of FFPE MCC specimens, a comparable number to previous reports,

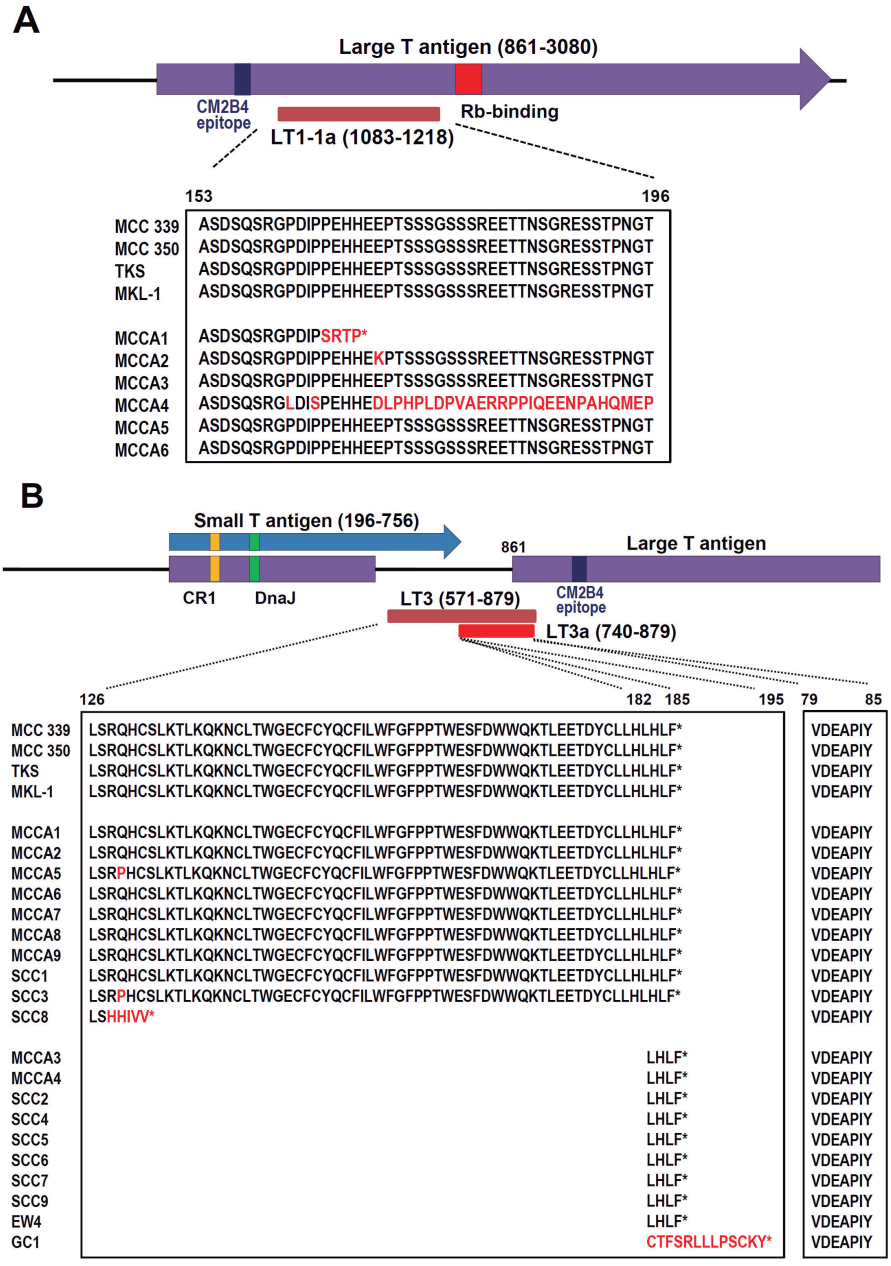

Fig. 3. A. Amino acid alignment of the LT1-1a product from MCC with reference sequence. MCCA1 has a premature stop codon mutation. **B.** Amino acid alignment of LT3 and LT3a products from MCCs, SCCs, and each of EW4 and GC1 with the reference sequence. SCC8 has a premature stop codon mutation.

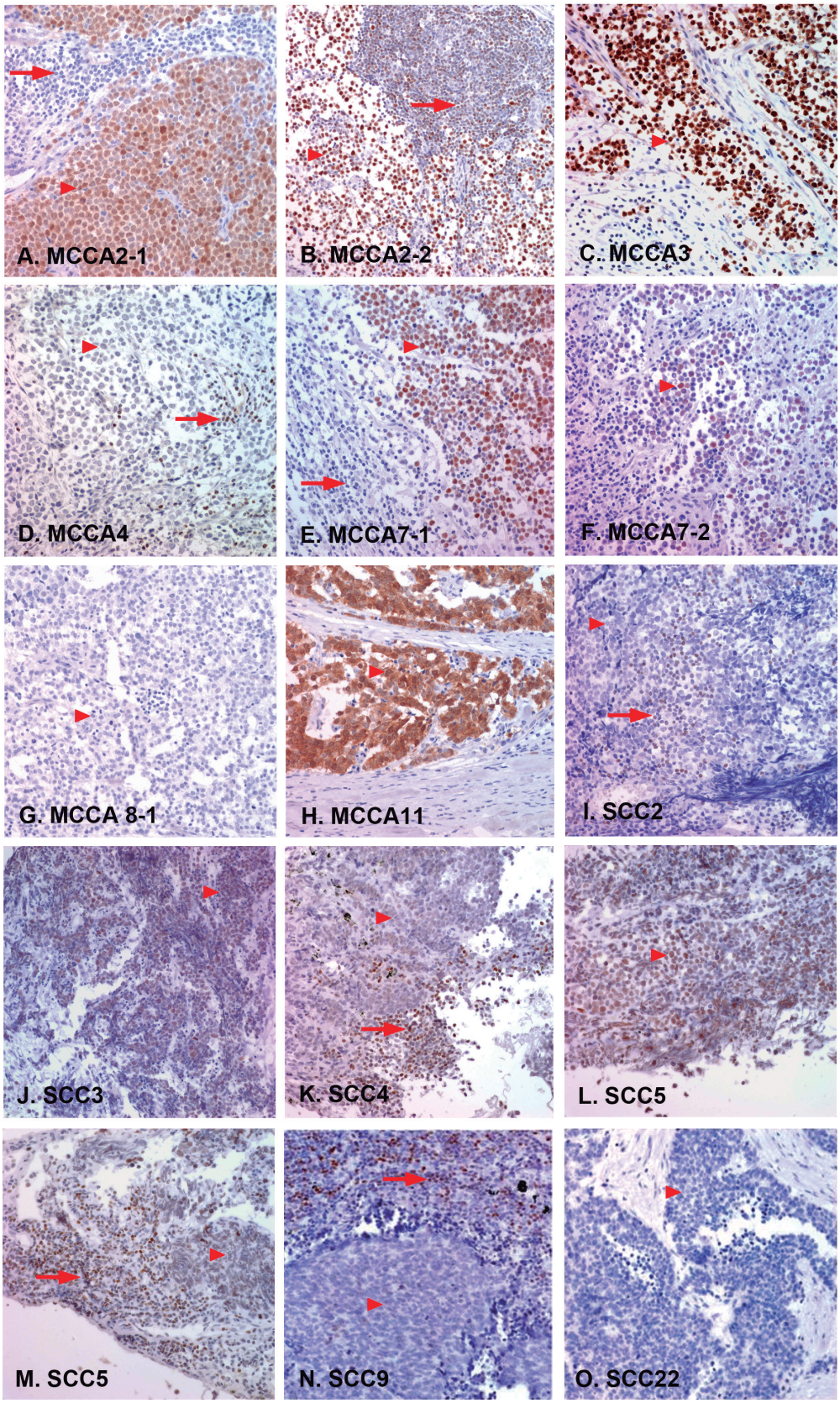

Fig. 4. Nuclear labeling with CM2B4. **A.** MCCA2 shows strong (+++) immunoreactivity in tumor cells and no reactivity in lymphocytes. **B.** Recurrent mass of MCCA2 shows +++ immunoreactivity in tumor cells and weak (+) immunoreactivity in lymphocytes. **C.** MCCA3 shows +++ immunoreactivity in tumor cells. **D.** MCCA4 shows + immunoreactivity in tumor cells and moderate (++) immunoreactivity in lymphocytes. **E.** Primary tumor of MCCA7 shows ++ immunoreactivity in tumor cells and no reactivity in lymphocytes. **F.** Metastatic lymph node of MCCA7 shows + staining in tumor cells. **G.** MCCA8 does not show immunoreactivity in either primary tumor or metastatic lymph node. **H.** MCCA11 shows strong immunoreactivity in nucleus and cytoplasm. **I.** SCC2 shows immunoreactivity in lymphocytes but not in tumor cells. **J.** SCC3 shows weak positivity in small cell carcinoma cells. **K.** SCC4 shows weak positivity in tumor cells and also immunoreactivity in lymphocytes. **L-N.** SCC5 and SCC9 show immunoreactivity in both tumor cells and lymphocytes. **O.** SCC22 shows no immunoreactivity in either tumor cells or lymphocytes (arrow head: tumor cells; arrow: lymphocytes). x 400

implying that the prevalence of MCPyV in MCC is similar among Asians and Caucasians, although MCC is very rare in Asians. This result is consistent with an initial report according to which 80% of MCCs were positive for MCPyV (Feng et al., 2008). The primer sets named LT1, LT3, and VP1 previously published by Feng and colleagues (2008) were used in our study. However, it is well known that the use of formalin results in the fragmentation of nucleic acids into small-sized segments. Thus, a low frequency of MCPyV DNA detection in MCC by the LT1 (440 bp PCR product) and VP1 (351 bp) primers was observed in both the previous reports and our results as expected. In fact, the LT3 (308 bp) primer set was established as a superior primer set for the detection of MCPyV in several papers (Kassem et al., 2008; Duncavage et al., 2009a). MCPyV was detected in 30 of 39 cases (77%) of MCC from FFPE tissue using the LT3, LT1, VP1, and M1/M2 primer sets (Kassem et al., 2008). In our study, with the LT3 primer set, 71% of the MCC specimens were positive for MCPyV. In order not to miss any MCPyV-positive MCC specimens, a new primer set (LT3a) for nested PCR of shorter sequences (140 bp) was designed. As expected, the positive rate increased to 81.8% by nested PCR using the LT3a primer set. The primer set LT3a proved to be most sensitive for detecting MCPyV DNA in FFPE tissues when compared with previously reported primer sets. Given the decreased amplification efficiency of

Table 3. Characteristics of carcinomas and results of MCPyV PCR.

ID	Sex	Age (yr)	Dx	ß-Globin	MCPyV Primer Sets				
					LT1-1a	LT3a	VP1	LTq (gDNA)	LTq (cDNA)
BC1	F	55	ILC	+	-	-	-	ND	ND
BC2	F	44	IDC	+	-	-	-	ND	ND
BC3	F	48	ILC	+	-	-	-	ND	ND
BC4	F	66	IDC	+	-	-	-	ND	ND
BC5	F	50	IDC	+	-	-	-	ND	ND
BC6	F	34	IDC	+	-	-	-	ND	ND
BC7	F	56	IDC	+	-	-	-	ND	ND
BC8	F	39	IDC	+	-	-	-	ND	ND
BC9	F	59	IDC	+	-	-	-	ND	ND
BC10	F	64	IDC	+	-	-	-	ND	ND
OC1	F	71	SCa	+	-	-	-	ND	ND
OC2	F	43	Papillary SCa	+	-	-	-	ND	ND
OC3	F	59	SCa	+	-	-	-	ND	ND
OC4	F	56	SCa	+	-	-	-	ND	ND
OC5	F	53	Papillary SCa	+	-	-	-	ND	ND
OC6	F	63	SCa	+	-	-	-	ND	ND
OC7	F	63	SCa	+	-	-	-	ND	ND
OC8	F	42	TCC	+	-	-	-	ND	ND
OC9	F	59	Papillary SCa	+	-	-	-	ND	ND
OC10	F	54	Papillary SCa	+	-	-	-	ND	ND
GC1	F	77	SRCC	+	-	+	-	-	NT
GC2	M	69	ACa pd	+	-	-	-	ND	ND
GC3	M	88	ACa md	+	-	-	-	ND	ND
GC4	F	75	ACa md	+	-	-	-	ND	ND
GC5	M	79	ACa md	+	-	-	-	ND	ND
GC6	M	70	ACa pd	+	-	-	-	ND	ND
GC7	M	51	ACa md	+	-	-	-	ND	ND
GC8	F	39	ACa md	+	-	-	-	ND	ND
GC9	F	48	ACa pd	+	-	-	-	ND	ND
GC10	M	48	ACa pd	+	-	-	-	ND	ND
GC11	M	53	SRCC	+	-	-	-	ND	ND
GC12	M	52	ACa pd	+	-	-	-	ND	ND
GC13	M	76	ACa wd	+	-	-	-	ND	ND
GC14	M	46	SRCC	+	-	-	-	ND	ND
GC15	M	55	ACa md	+	-	-	-	ND	ND
GC16	M	38	SRCC	+	-	-	-	ND	ND
GC17	M	63	ACa pd	+	-	-	-	ND	ND
GC18	F	48	ACa md	+	-	-	-	ND	ND
GC19	M	56	ACa pd	+	-	-	-	ND	ND
GC20	F	45	ACa pd	+	-	-	-	ND	ND

ILC: invasive lobular carcinoma; IDC: invasive ductal carcinoma; SCa: Serous carcinoma; TCC: transitional cell carcinoma; ACa: adenocarcinoma; SRCC: signet ring cell carcinoma; md: moderately differentiated; wd: well differentiated; pd: poorly differentiated; ND: Not determined; NT: Not tested

larger amplicons, the increased efficacy of nested PCR, and the well-known consequence of formalin fixation, it is not surprising that the LT3a primer set detected MCPyV in more cases of MCC than previous primer sets.

Three patients (MCCA2, MCCA7, and MCCA8) underwent separate excisions for metastases. Of these, MCPyV was detected in both the primary and metastatic samples, which showed the same detection frequency. This result is consistent with the idea that MCC is a clonal disease. In the case of MCCA3 and MCCA4, only the LT3a primer set, but not the LT3 primer set detected MCPyV. The failure to detect MCPyV was most likely due to the length of the longer PCR product. However, two MCCs (MCCA10 and MCCA11) tested negative with all 5 primer sets but showed immunoreactivity to MCPyV T antigen by IHC, suggesting that all cases of MCC may result from clonal integration of MCPyV. The PCR-negative data might be related to MCPyV variance.

Shuda et al. (2008) recently showed that MCPyV is integrated into the host genome and acquires a large T antigen truncating mutation in the replication origin-binding domain (OBD) and the viral DNA helicase domain, leading to tumorigenesis. However the CR1, DnaJ, and Rb-binding domains remain intact. The truncation mutation may explain why the LT1 and VP1 primers had a low frequency of MCPyV DNA detection. Only 3 cases of MCC showed MCPyV DNA with the LT1 primer set. Thus, we designed new primer sets (LT1-1 for the first PCR and LT1-1a for the nested PCR) that annealed to the 5' side of LT1. The LT1-1a primer set detected 4 additional cases. Among these, one case showed a premature truncation stop codon in MCCA1, which was the only case that tested positive with all 5 primer sets, and another case showed a frame-shift mutation in MCC4A. In particular, both mutations ablated the Rb-binding domain, which is associated with tumorigenesis in other polyomavirus models of in large T antigen. Further studies are required to understand the role of the Rb-binding domain in polyomavirus-induced merkel cell transformation.

For the SCCs, none of the cases showed MCPyV DNA with the LT1, LT1-1a, or VP1 primer sets. However, 3 cases with LT3 and 9 cases with LT3a (37.5%) showed MCPyV DNA. Similarly, other recent work (Helmbold et al., 2009) demonstrated that MCPyV is found in 39% of small cell lung cancers using PCR. However, qPCR with the LTq primer set did not detect MCPyV DNA. This might be due to the low copy number of the virus. The cases of the MCPyV PCR-positive ES/PNET or gastric cancer might also be explained by the low copy number of the virus. However, the MCPyV transcript was detected only in MCCs by qRT-PCR analysis. Consistently, some studies have shown that MCPyV is not detected in other neuroendocrine tumors or other carcinomas (Bluemn et al., 2009; Duncavage et al., 2009b).

IHC of all the cases analyzed by PCR was performed using mAb CM2B4. Most MCCs positive for MCPyV by PCR were also immunoreactive for CM2B4. Only 1 MCC case (MCCA8), in which MCPyV was detected by LT3a but not by LT1-1a, failed to show MCPyV immunoreactivity. It is possible that the CM2B4 epitope, which is located between LT3a and LT1-1a, is removed by the truncation mutation .

Busam et al. (2009) showed that 79% (37 of 53) of MCCs and 76.9% (10 of 13) of MCPyV-positive cases detected by PCR were immunoreactive for CM2B4. In their report, the 16 SCCs tested did not show any nuclear staining with CM2B4. However, we detected MCPyV DNA in 6 (26.1%) of 23 SCC cases and the immunohistochemical analysis clearly distinguished MCPyV in non-tumor cells from tumoral MCPyV. In fact, in 2 cases of SCC (SCC2 and 7) that were positive for MCPyV by PCR, the tumor cells were immunonegative for CM2B4 staining, despite the immunoreactivity of the surrounding lymphocytes. These results highlight the possibility that circulating lymphocytes might act as a putative reservoir of MCPyV infection. Interestingly, none of the cases that were negative for MCPyV by PCR showed any nuclear

Table 4. PCR primers and Probes for MCPyV detection.

Name	Forward (F) and reverse (R) primer sequences	Product size (bp)
Primers for first PCR		
LT1	F:5'-TACAAGCACTCCACCAAAGC-3' R:5'-TCCAATTACAGCTGGCCTCT-3'	440
LT3	F:5'-TTGTCTCGCCAGCATTGTAG-3' R:5'-ATATAGGGGCCTCGTCAACC-3'	308
LT 1-1	F:5'-GCTTCAGACTCCCAGTCCAG-3' R:5'-TGGTGAAGGAGGAGGATCTG-3'	346
VP 1	F:5'-TTTGCCAGCTTACAGTGTGG-3' R:5'-TGGATCTAGGCCCTGATTTTT-3'	351
ß-Globin	F:5'-CAACTTCATCCACGTTCACC-3' R:5'-GGTTGGCCAATCTACTCCCAGG-3'	200
Primers for nested PCR		
LT 1-1a	F:5'-GCTTCAGACTCCCAGTCCAG-3' R:5'-CACTGGTTCCATTTGGTGTG-3'	136
LT 3a	F:5'-ATCTGCACCTTTTCTAGACTCC-3' R:5'-ATATAGGGGCCTCGTCAACC-3'	140
Primers and Probes for qPCR or qRT-PCR		
LTq	F:5'-GGTTGACGAGGCCCCTAT-3' R:5'-TTCCCGAAGCTGAATCCTC-3' Probe: FAM-TGGTGGAG-Dark quencher	75
ACTB	F:5'-CCAACCGCGAGAAGATGA-3' R:5'-GAGAAAGGGCGCAGCTCC-3' Probe: DYXL-AGGTGAGTGGCCCGCTACCT-BBQ	94
*HPRT	F:CTCAACTTTAACTGGAAAGAATGTC R:TCCTTTTCACCAGCAAGCT Probe: YAK-TTGCTTTCCTTGGTCAGGCAGTATAATC–BBQ	231

*HPRT: hypoxanthine-guanine phosphoribosyl transferase.

staining in either tumor cells or lymphocytes with CM2B4. Therefore, the immunohistochemistry data strongly support the MCPyV- positive results in SCC.

The results show that MCPyV may be a putative etiologic agent in all MCC and some SCC cases. However, MCPyV does not seem to play any role in the tumorigenesis of other tumor types, although we cannot rule out the possibility that MCPyV levels in these tumors may be below the limit of detection of our assays, due to the low copy number of the virus.

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