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The prevalence of Merkel cell polyomavirus in Japanese patients with Merkel cell carcinoma

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ABSTRACT

Background: A novel polyomavirus, the Merkel cell polyomavirus (MCPyV) has been implicated in the pathogenesis of Merkel cell carcinoma (MCC); however, the prevalence of MCPyV in Japan has not been extensively investigated.
Objective: To clarify the prevalence of MCPyV in Japanese patients with MCC.
Methods: MCPyV DNA was examined by polymerase chain reaction (PCR) in formalin-fixed paraffin-embedded (FFPE) or frozen tissue samples from 26 patients with MCC diagnosed in four medical centers in Japan. Immunohistochemistry was simultaneously performed using a monoclonal antibody against the viral large T (LT) antigen. FFPE samples from basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) were also analyzed as controls.
Results: Twenty-three out of 26 cases (88.5%) were positive for MCPyV DNA by PCR. The amplified products harbored 4 patterns of mutations. Phylogenetic analysis demonstrated that one of our strains was closely related to the other Japanese strains previously reported. The LT antigen was expressed in various degrees in 20 of 26 cases (76.9%) by immunohistochemistry. Histological type had little relation to CM2B4 positivity, whereas 3 of 5 trabecular-type tumors showed no staining. The immunoreactivity for CM2B4 did not correlate with the relative viral DNA load. In BCC and SCC, the LT antigen was immunohistochemically positive, but MCPyV DNA was not detected by PCR. The cells around some MCC and non-MCC tumors were stained with CM2B4 with a distribution similar to CD20- and CD45RO-(especially CD8-) positive lymphocytes.
Conclusion: MCPyV was highly positive in Japanese patients with MCC. It is of note that the positive rate differs depending upon the detection method.

1. Introduction

Merkel cell carcinoma (MCC) is an aggressive neuroendocrine skin cancer which primarily affects elderly and immune-suppressed individuals. Although MCC is rare, its incidence is increasing [1–6]. Recently, Feng et al. demonstrated that a new human polyomavirus, designated as Merkel cell polyomavirus (MCPyV), is frequently detected in patients with MCC [7]. Integration of MCPyV DNA within the tumor genome in a clonal pattern suggests that MCPyV is a causative agent in MCC. The MCPyV genome encodes a large T (LT) antigen, which contains some conserved domains shown to play roles in cell transformation [1,5,6,8,9].

MCPyV was discovered using digital transcriptome subtraction of MCC [7]. MCPyV is a 5.4-kbp long, double-stranded DNA virus with a genome that contains early and late regions [7,10]. The former encodes nonstructural proteins, small and large T antigens that are responsible for viral replication. The latter encodes viral proteins (VPs) that constitute viral particles. LT antigen may play an essential role in MCC tumorigenesis by inhibiting the cell-cycle-regulating function of retinoblastoma protein [5,8,9], whereas integrated LT antigen harbors truncating mutations upstream of

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Table 1
Primers used for PCR analysis.

Experiment	Primer name		Primer sequence	Position[a]	Size (bp)[a]	Reference
MCPyV detection	LT1	Forward	5′-TACAAGCACTCCACCAAAGC-3′	1514–1953	440	[7]
		Reverse	5′-TCCAATTACAGCTGGCCTCT-3′			
	LT3	Forward	5′-TTGTCTCGCCAGCATTGTAG-3′	571–879	309	
		Reverse	5′-ATATAGGGGCCTCGTCAACC-3′			
	VP1	Forward	5′-TTTGCCAGCTTACAGTGTGG-3′	4137–3786	352	
		Reverse	5′-TGGATCTAGGCCCTGATTTT-3′			
MCPyV quantificaion	LT_SYBR	Forward	5′-TCTTCCTCTGGGTATGGGGTC-3′	1047–1208	162	[29]
		Reverse	5′-ATTGGGTGTGCTGGATTCTC-3′			
Control	GAPDH	Forward	5′-GGTCTCCTCTGACTTCAACA-3′		116	[28]
		Reverse	5′-AGCCAAATTCGTTGTCATAC-3′			
LT sequencing	LTagEx2-1	Forward	5′-TACTGCTTACTGCATCTGCACC-3′	727–1345	619	[30]
		Reverse	5′-GGCGAGCTTCTTGAGGAG-3′			
	LTagEx2-2	Forward	5′-GCGATGAATCACTTTCCTCC-3′	1267–1952	686	
		Reverse	5′-CCAATTACAGCTGGCCTCTT-3′			
	LTagEx2-3	Forward	5′-CCCCTTACAAATTACTGCAAGAG-3′	1870–2488	619	
		Reverse	5′-GATGGACAGTTTATATTCAAGGCC-3′			
	LTagEx2-4	Forward	5′-GCTTTGCTGCAGCCTTAATAG-3′	2428–3109	682	
		Reverse	5′-CAAACACAGGAAATATGAAGCAG-3′			

[a] Position and size in GenBank NC_010277.1.

the helicase domains, rendering the viral replication function inactive [11]. Small T antigen of MCPyV has also been shown to have cell transformation ability by dysregulating cap-dependent translation [12]. However, there has not yet been any definitive proof that polyomavirus plays a relevant role in human carcinogenesis.

The frequency of MCPyV-positive MCC is generally high, ranging from 75% to 90% in Europe and North America [7,13–19], while it is less prevalent in Australian patients (18.3–24%) [20,21]. This difference may be due to the increased sun exposure in Australia, making a possible viral contribution less frequent; however, another group has recently reported a high prevalence rate of the virus in Australian MCC patients comparable to those from Germany [22]. In contrast there is relatively limited information available on the prevalence of MCPyV in Asian MCC patients [1,23–27], and it is unknown whether or not geographical differences exist.

In this study, we investigated the positive rate of MCPyV DNA in Japanese MCC patients in 4 medical centers. To evaluate the polymerase chain reaction (PCR) results, we also performed immunohistochemistry using the identical MCC tissue samples. Samples from basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) were also analyzed to confirm the association of the virus in non-MCC skin cancers.

2. Materials and methods

2.1. Samples

The formalin-fixed paraffin-embedded (FFPE) tissue specimens from patients with MCC (11 samples from 7 patients), BCC (10 samples from 10 patients) and SCC (10 samples from 9 patients) were retrieved from the archives of the Department of Dermatology, Gunma University Hospital. Blood samples were collected from 19 healthy volunteers and 2 of the 7 patients with MCC. FFPE samples of MCC tumors were also collected from 3 medical centers: Saitama Medical University Hospital (6 samples from 6 patients); Niigata University Hospital (3 samples from 3 patients); and Niigata Cancer Center Hospital (12 samples from 10 patients). All 3 centers are located approximately 57, 168, and 167 km away from Gunma. Fresh tumor tissue samples were obtained from 2 of the 26 patients with MCC. The average age of the MCC patients at the time of resection was 80 ± 13 years (range 48–96 years). Of the 26 MCC cases, 16 (62%) were women and 10 (38%) were men. This study was conducted according to the Declaration of Helsinki Principles and was approved by the Ethics Committee of Gunma University. All samples were obtained upon informed consent or in compliance with the institutional review board for human studies.

2.2. Preparation of DNA and standard PCR analysis

Sections of 5 μm were obtained from the FFPE tissue specimens from the patients. DNA was extracted by DEXPAT (TaKaRa, Otsu, Japan) according to the manufacturer's protocol, followed by ethanol precipitation. The precipitate was dissolved in TE buffer and used for PCR analysis. Genomic DNA was isolated from whole blood or frozen tissue using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI) according to the manufacturer's protocol.

The presence of MCPyV was detected by primer-directed amplification with PCR. Specific primer pairs, described previously [7] were used to detect the viral LT antigen (LT1 and LT3) and the viral capsid protein (VP1) (Table 1). For the PCR amplification, EX Taq DNA polymerase system (TaKaRa) was used with 3 μl of genomic DNA and 0.4 μM of each primer (30 μl per reaction). A 116-bp segment of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene [28] was also amplified to demonstrate the quality and quantity of the DNA samples (Table 1). The cycling condition consisted of an initial incubation at 95 °C for 1 min, followed by 45 cycles with denaturation at 95 °C for 15 s, annealing at 60 °C for 15 s, and elongation at 72 °C for 30 s. The final elongation step was performed at 72 °C for 10 min. The PCR products were purified and sequenced (Bio Matrix Research, Nagareyama, Japan).

2.3. Quantitative real-time PCR

FFPE tissue specimens from either Gunma University or Niigata Cancer Center were subjected to real-time PCR analysis. DNA was extracted by NucleoSpin FFPE DNA (Macherey-Nagel, Düren, Germany), followed by measurement of DNA concentration by a NanoDrop spectrophotometer (ND-1000, Thermo Scientific, Wilmington, DE). The relative viral load was obtained by amplifying the equal volume of DNA with 0.6 μM of each primer (LT_SYBR, Table 1) [29] in the THUNDERBIRD SYBR qPCR Mix (Toyobo, Osaka, Japan) using the 7300 Real Time PCR system (Applied Biosystems, Foster City, CA). The cycling conditions