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Detection of Merkel Cell Polyomavirus in the Human Tissues from 41 Japanese Autopsy Cases Using Polymerase Chain Reaction

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Key Words
Merkel cell polyomavirus · Human tissues · Autopsy · Polymerase chain reaction

Abstract
It has recently been shown that approximately 80% of Merkel cell carcinomas harbor a novel polyomavirus named Merkel cell polyomavirus (MCPyV). MCPyV has been detected in human tissue samples. However, detailed distribution of MCPyV in non-neoplastic Japanese human tissues remains unclear. To address this, we used single or real-time quantitative polymerase chain reaction (PCR) for 41 autopsy cases. PCR revealed MCPyV-DNA in non-neoplastic samples: total, 29/41 (71%); adult, 29/39 (74%); fetus or infant, 0/2; men, 24/28 (86%); women, 5/13 (38%); total human tissues, 66/572 (12%); skin, 8/15 (53%); adrenal gland, 9/33 (27%), and other 16 organs (4–25%). This study first reported the presence of MCPyV-DNA in non-neoplastic tissues of thyroid gland, adrenal gland, spleen, bone marrow, stomach, gallbladder, pancreas, heart, and aorta. PCR revealed that viral load ranged from 0.00026 to 0.22 in all MCPyV-positive tissues compared with Merkel cell carcinoma samples. These detailed PCR data showed higher prevalence of MCPyV infection in Japanese men than women ($p = 0.004$) and broad distribution of MCPyV with low viral load in more non-neoplastic human tissues than in the previous reports. These data provide valuable insights for further studies of MCPyV infection and MCPyV-related diseases.

Introduction

Merkel cell polyomavirus (MCPyV) was recently discovered to be associated with Merkel cell carcinoma (MCC), a rare and aggressive human skin cancer [1]. MCPyV-induced oncogenesis is thought to be involved in the transformative properties of the MCPyV large T antigen. MCPyV has been detected in MCC patients and appears to play the chief role in tumorigenesis; about 80% of MCCs harbor MCPyV [1]. Currently, the frequency of infection of MCPyV is not completely unclear. However, using real-time polymerase chain reaction (PCR), researchers have reported that MCPyV has been detected in normal human tissue samples such as the skin, liver, and respiratory secretion samples. Moreover, MCPyV has been detected in malignant and benign tis-

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Table 1. Detection of MCPyV-DNA in autopsy cases by single PCR

	Cases	MCPyV-positive cases	Frequency of positivity, %
Age			
Fetus or infant	2	0	0
23 years	1	1	100
33–88 years	38	28	74
Sex*			
Male	28	24	86
Female	13	5	38
Total	41	29	71

* p = 0.004.

sues [1–8]. However, previous reports on the presence of this virus in non-neoplastic human tissues have been partial and incomplete. The purpose of the present study was to elucidate the presence of MCPyV in every type of human tissue samples. Thus, we examined the samples from 41 autopsy cases. The presence or absence of MCPyV in each sample was evaluated by PCR and real-time PCR.

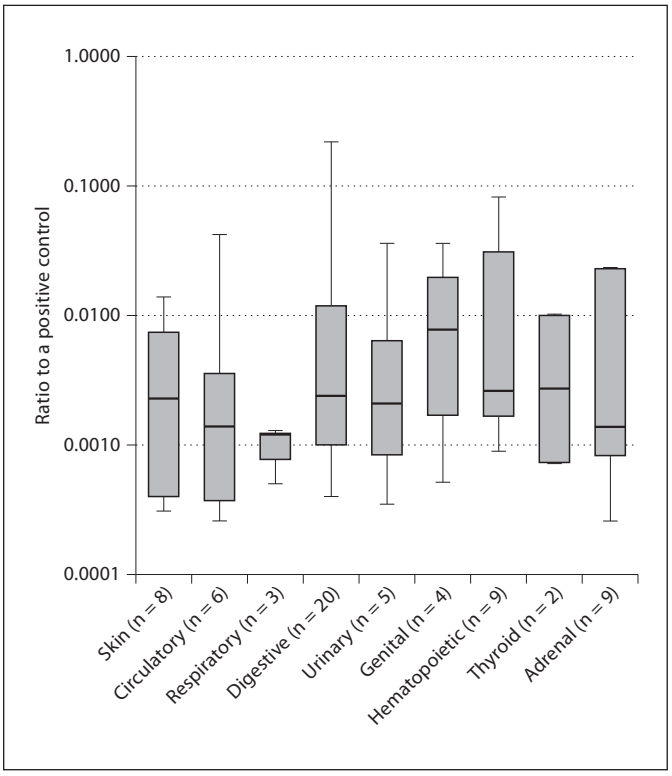

Fig. 1. Box plots show MCPyV levels relative to a reference MCC (1.0) through the different organ systems. Skin, circulatory, respiratory, digestive, genitourinary, hematopoietic and endocrine (thyroid gland, and adrenal gland) samples are shown. Box plots show the middle 50% of data, the line is the median, and the bars extend the median by 1.5 times the interquartile range. The median: skin, 0.0023; circulatory, 0.0014; respiratory, 0.0012; digestive, 0.0024; urinary, 0.0021; genital, 0.0078; hematopoietic, 0.0026; thyroid gland, 0.0027; adrenal gland, 0.0015.

Materials and Methods

Human Tissue Samples

The study was approved by the Institutional Review Board of the Faculty of Medicine, Tottori University. A total of 41 human autopsy samples from 39 adults, 1 fetus, and 1 infant as well as reference MCC samples were obtained from the Department of Pathology at Tottori University Hospital as excess pathological tissue was not required for diagnosis. The reference MCC samples were paraffin embedded. We tested all the tissue types from the 41 individuals. A total of 572 human tissue samples were examined, 117 of these were fresh frozen; the remaining 455 samples were paraffin embedded. Of the neoplastic tissues examined, 3 were benign, and 19 were malignant tumors. A sample of liver metastasis from a rectal carcinoid tumor was fresh frozen, but the remainder was paraffin embedded.

Polymerase Chain Reaction

To detect MCPyV by PCR, DNA was extracted from each sample using the QIAamp DNA FFPE Tissue Kit and Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocols. The presence of adequate DNA in samples was confirmed by measuring the internal control DNAs of RNase P. A primer pair targeting the position 859-934 on MCC350 (GenBank EU375803) [9] was used (MCPyVLT forward, 5′-AGGTTGACGAGGCCCCTAT-3′; reverse, 5′-TTCCCGAAGCTGAATCCTC-3′; amplicon size, 76 bp). PCR was performed using 1 U of TaKaRa Ex Taq HS (Takara Bio Inc., Otsu, Japan) with 30 ng of genomic DNA in 20 μl solutions for each sample and the controls. The positive control was MCCs, and water was used as the negative control. Amplifications consisted of an initial denaturation for 5 min at 94° with 40 cycles of denaturation for 30 s at 94°, annealing for 30 s at 59°, and extension for 1 min at 72°. PCR products were electrophoresed, stained with ethidium bromide, and visualized under UV light. Cases in which one primer set amplified visible DNA fragments of the expected length (76 bp) were considered MCPyV positive; all others were considered to be MCPyV negative.

Real-Time PCR

To determine the MCPyV-DNA ratio relative to MCPyV-DNA of the reference MCC (MCC = 1.0) for each case, real-time PCR was performed using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, Calif., USA). A total of 30 ng of each DNA sample was amplified with 5 μl of EXPRESS qPCR Supermix with Premixed ROX (Invitrogen, Carlsbad, Cal-

Table 2. MCPyV detection in each organ from autopsy cases by PCR

Organs	Sample number	Single PCR	Real-time PCR
Skin	15 [14]	8 (53) [8/14]	[0.00031–0.015]
Endocrine system			
Thyroid gland	36 [5]	2 (6) [0/5]	0.00074–0.0010
Adrenal gland	33 [5]	9 (27) [0/5]	0.00026–0.023
Hematopoietic system			
Lymph node	3	0 (0)	ND
Thymus	1	0 (0)	ND
Spleen	37 [9]	3 (8) [1/9]	0.00090–0.0062 [0.00090]
Bone marrow	36 [9]	6 (17) [0/9]	0.0017–0.083
Aerodigestive tract			
Salivary gland	2	0 (0)	ND
Tongue	10 [4]	0 (0) [0/4]	ND
Esophagus	33 [6]	5 (15) [1/6]	0.00047–0.029 [0.0021]
Stomach	32 [7]	3 (9) [1/7]	0.0015–0.039 [0.039]
Intestine	34 [9]	6 (18) [1/9]	0.00040–0.22 [0.011]
Liver	35 [9]	3 (9) [2/9]	0.0060 [0.00062–0.0010]
Gallbladder	4 [1]	1(25) [1/1]	[0.012]
Pancreas	37 [7]	2 (5) [0/7]	0.015–0.20
Circulatory system			
Heart	35 [3]	5 (14) [0/3]	0.00026–0.042
Aorta	26	1 (4)	0.0036
Respiratory tract			
Lungs	36 [7]	3 (8) [0/7]	0.00050–0.0013
Genitourinary			
Kidney	39 [11]	2 (5) [1/11]	0.00084–0.0064 [0.0063]
Bladder	28 [4]	3 (11) [1/4]	0.00035–0.036 [0.0013]
Ovary	8 [1]	0 (0) [0/1]	ND
Uterus	11	0 (0)	ND
Prostate	18	2 (11)	0.0056–0.011
Testis	21 [4]	2 (10) [0/4]	0.00052–0.036
Cerebrum	1 [1]	0 (0) [0/1]	ND
Skeletal muscle	1 [1]	0 (0) [0/1]	ND
Total	572 [117]	66 (12) [17/117]	0.00026–0.22 [0.00031–0.039]

ND = Not done. Figures in parentheses indicate percentages and figures in square brackets frozen sample.

if., USA), 240 nmol/l of fluorescein-labeled locked nucleic acid hydrolysis probe 22 (5′-TGGTGGAG-3′) from a Universal Probe Library (Roche Diagnostics, Basel, Switzerland), and 0.9 μmol/l of a primer in the final volume of 10 μl. The same primer sequences were used for PCR and real-time PCR. Locked nucleic acid probe 22 was used for viral DNA detection. Thermal cycling consisted of incubation for 2 min at 50°with an initial denaturation for 10 min at 95°followed by 40 cycles of denaturation for 15 s at 95°and annealing for 1 min at 60°, as previously described [9]. The ratio of the virus was determined using the virus signal in a positive MCC sample as the reference. Thresholds were plotted against each standard sample. All reactions of samples and the controls were performed in triplicate, and the average was reported. The MCPyV DNA ratio in each sample was determined based on the corresponding standard curves.

Results

Prevalence of MCPyV-DNA in Autopsy Cases

The results of the analyses of tissues from the autopsy cases are shown in table 1. MCPyV-DNA was present in more than one organ, and MCPyV infection was detected in more than 70% of the adults, but neither the fetus nor the infant appeared to be infected. The incidence of MCPyV infection was 2-fold higher in men compared with women, indicating a significant difference ($p = 0.004$).

MCPyV-DNA Detection in Non-Tumor Tissues

We tested the DNA from 572 human tissue samples (table 2). The sample set included samples of the normal

Table 3. MCPyV detection in benign and malignant tumors from autopsy cases by PCR

Tumor	Total	Single PCR	Real-time PCR
Hepatocellular carcinoma	4	0	ND
Lung cancer (SCC: 2, adenocarcinoma: 1)	3	0	ND
Papillary thyroid cancer	2	0	ND
SCC of tongue	1	0	ND
SCC of larynx	1	0	ND
Intrahepatic cholangiocarcinoma	1	0	ND
Pancreatic cancer	1	0	ND
Gallbladder cancer	1	0	ND
Rectal carcinoid	1	0	ND
Liver metastasis of rectal carcinoid	1	0	ND
Malignant lymphoma	1	0	ND
Ectopic pancreas of jejunum polyp	1	1	0.0018
Endometrial polyp	1	0	ND
Myoma uterus	1	0	ND
Colon adenoma	2	0	ND
Total	22	1	

ND = Not done; SCC = squamous cell carcinoma.

skin, the endocrine system (thyroid gland and adrenal grand), the hematopoietic system (lymph node, thymus, spleen and bone marrow), the aerodigestive tract (salivary gland, tongue, esophagus, stomach, intestine, liver, gallbladder, and pancreas), the circulatory system (heart and aorta), the respiratory tract (lungs), the genitourinary system (kidney, bladder, ovary, uterus, prostate and testis), the cerebrum, and the skeletal muscle. The ratio range of real-time PCR was 0.00026–0.22, and the median was 0.0021. Eight out of the 15 skin samples were positive for MCPyV, indicating the high prevalence of MCPyV infection in skin. However, the quantitative real-time PCR ratio was not significantly higher in the skin relative to the other tissues (fig. 1). Interestingly, the frequency of MCPyV infection of the adrenal gland was higher than that of any other tissue sample except for skin samples. More detailed information is provided in table 2.

MCPyV-DNA Detection in Benign and Malignant Tumor Tissues from Autopsy Cases

The ectopic pancreas of one jejunum polyp was positive for MCPyV-DNA, whereas the remainder of the tumor tissues was found to be negative for MCPyV-DNA (table 3).

Discussion

In this study, we used PCR to investigate whether MCPyV was present in a large variety of human tissue samples. The possibility of contamination is unlikely as the ultrapure water-negative controls were consistently negative. As previously reported [2–8], PCR revealed MCPyV-DNA in many types of human samples, particularly skin samples. Loyo et al. [7] reported real-time PCR data showing MCPyV-DNA in the liver and urinary bladder of more than 50% of their tested samples; our level of detection was much lower (liver, 9%; urinary bladder, 11%). However, the high prevalence of MCPyV-DNA in the skin samples (53%) in our study corresponds to the presence (48%) of MCPyV-DNA reported by Loyo et al. [7]. This appears to indicate that MCPyV has more affinity for the skin compared with other organs. Some reports [6, 7] have indicated the presence of MCPyV in non-neoplastic and neoplastic skin samples; we also detected MCPyV in many non-neoplastic skin samples. Therefore, we speculate that MCPyV is latent in the skin.

Whilst reference MCC samples had the highest levels of MCPyV, low levels of MCPyV were present in a wide variety of the human tissues. The MCPyV-DNA ratio of non-neoplastic human tissues relative to the reference MCC ranged from 0.0026 to 0.22, indicating that organs positive for MCPyV-DNA had lower viral loads than the MCC sample.

Moreover, we examined 22 benign and malignant tumors, but only the ectopic pancreas of one jejunum polyp was positive for MCPyV-DNA and had CM2B4 (MCPyV-LT)-positive lymphocytes. It is possible that MCPyV is not associated with tumors other than MCC tumors, and lymphocytes are latently infected with MCPyV. A recent study [10] reported a prevalence of 22% of MCPyV sequences in the buffy coats of healthy blood donors. Our study detected MCPyV in many organs, but some of these results may be because of MCPyV in blood vessels or infiltrating lymphocytes. Although only 1 fetus and 1 infant were included in this study, it appears that MCPyV infection may not occur during the fetal period or early infancy. Chen et al. [11] indicated a 9% prevalence of MCPyV-IgG among children aged 1–4 years; this was increased to 35% among those aged 4–13 years. They concluded that MCPyV primary infections occur during childhood. To confirm whether infants are infected, additional studies are required. Schrama et al. [12] reported that 249 (76.9%) of 324 male and 268 (85.3%) of 314 female MCC patients were MCPyV positive. The data from the present study indicate that the prevalence of MCPyV in-

fection is significantly higher among men (86%) compared with women (38%; p = 0.004). This association of MCPyV status with gender has not been reported previously, and more studies are required to confirm the higher prevalence of MCPyV among the men of Tottori Prefecture (West Japan).

The possibility of contamination is unlikely as the water controls were consistently negative, and the single PCR assay was reconfirmed by real-time PCR assay at two different time points.

The mechanism by which the infection is spread remains unknown. However, it is obvious that the mere presence of MCPyV-DNA is not sufficient for malignant transformation.

In conclusion, the presence of MCPyV-DNA in human non-neoplastic tissues such as the skin, esophagus, lung, liver, intestine, and urinary bladder samples was confirmed by PCR analyses performed. This study is the first to demonstrate the presence of MCPyV-DNA in the following organs and tissues: thyroid gland, adrenal gland, spleen, bone marrow, stomach, gallbladder, pancreas, heart, aorta, kidney, prostate, and testis. Furthermore, it is the first to report its absence in the lymph nodes, thymus, tongue, ovary, uterus, cerebrum, and skeletal muscle tissues. Real-time PCR revealed an extremely low viral load in MCPyV-positive human organs and tissues. These detailed data regarding the prevalence of MCPyV-DNA in non-neoplastic human tissues are important and provide valuable insights for further studies of MCPyV infection and MCPyV-related diseases.

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