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Frequent detection of Merkel cell polyomavirus DNA in tissues from 10 consecutive autopsies

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Abstract

Merkel cell polyomavirus (MCPyV) has been identified in samples of Merkel cell carcinoma (MCC), an aggressive skin cancer. Seroepidemiologic studies indicated a high frequency of MCPyV infection in humans, suggesting respiratory and faecal–oral routes, or transmission by skin contact. Since MCC is more frequent in immunocompromised patients, a reactivation of MCPyV latently infecting target cells has been proposed. However, neither definite ways of transmission nor specific target organs have been identified with certainty. Ten autopsies with an extensive organ sampling for a total of 121 specimens (tissue and blood samples) were collected. All tissue specimens were fixed in formalin and embedded in paraffin. Real-time PCR was performed to quantify the copy number of the large T antigen (LT) gene and the capsid VP1 gene of MCPyV. MCPyV LT and/or VP genes were detected in all of the collected specimens. A high prevalence of MCPyV was found in the blood (six cases) and lung (five cases); the brain was positive in three cases. The highest viral copy number was detected in blood from two autopsies (21 610 570.09 copies per 10⁵ cells and 380 413.25 copies per 10⁵ cells), whereas the viral copy number in the other organs was low. Our data confirm the high frequency of MCPyV infection in the general population, which seems to indicate that the respiratory tract is a possible route for viral transmission and viral persistence in the brain. The frequent detection of MCPyV DNA in blood suggests that circulating leukocytes could be one of the reservoirs of MCPyV, whereas the high viral copy number also seems to indicate the possibility of viral reactivation in immunocompetent adults.

INTRODUCTION

Merkel cell polyomavirus (MCPyV) is a recently discovered human polyomavirus, identified by digital transcriptome subtraction in samples of Merkel cell carcinoma (MCC) [1]. MCC is a rare and very aggressive human skin cancer; its incidence is higher among immunocompromised patients and elderly people than in the general population, suggesting a possible mechanism of reactivation of MCPyV latently infected target cells, favoured by an impairment of immune surveillance [2]. A study by Nicol et al. [3] showed that the seroprevalence of MCPyV was 85% in the adult healthy population and 58% in children less than 10 years of age, indicating that primary exposure to MCPyV infection occurs frequently in early childhood.

Possible sites of viral persistence have been investigated in body fluids [4, 5], tissue biopsies [6] and surgical specimens from several organs [7], but to the best of our knowledge, only one study has investigated the distribution of MCPyV in autopsy cases in Japan [8]. Therefore, more systematic

studies searching for MCPyV DNA in human tissues are needed to elucidate the possible anatomical sites of viral persistence.

Our study aimed to investigate the presence of MCPyV DNA in several organs from 10 complete autopsies, in order to identify the prevailing sites of viral persistence and contribute to comprehension of the mechanisms of MCPyV transmission.

RESULTS

The main demographic data and causes of death are shown in Table 1. There were four females and six males, with nine Caucasians and one South American. The mean age was 67.9 years (range, 30–90 years) and the causes of death included cardiovascular or respiratory diseases (four cases) and septic or haemorrhagic shock (six cases).

A total of 121 specimens (111 tissue and 10 whole blood samples) taken during the autopsies were obtained from an extensive sampling (Table 2).

Table 1. Main demographic data and causes of death

Autopsy no./sex/age (years)	Country	Interval death–autopsy (hours)	Cause of death	Main pathologies
1/M/77	Italy	72	Heart failure	Cardiovascular disease
2/F/70	Albania	48	Haemorrhagic shock	None
3/F/72	Italy	48	Septic shock	Diverticular disease, with perforation
4/F/70	Italy	48	Septic shock	Cytomegalovirus colitis
5/M/71	Italy	48	Respiratory and heart failure	Coronary artery disease
6/M/66	Italy	48	Cardiogenic shock	Cardiovascular disease
7/M/56	Italy	72	Acute respiratory distress syndrome	Aortic aneurysm
8/M/30	Brazil	72	Haemorrhagic shock	None
9/F/90	Italy	48	Pulmonary thromboembolism; heart failure	Generalized vasculopathy
10/M/77	Italy	72	Septic shock	Chronic leishmaniasis

M, male; F, female

Genomic sequences of LT, VP or both regions of MCPyV were detected in all cases in at least one sample. In detail, viral DNA was detected in samples from five organs in two cases (in autopsies nos7 and #10), from four organs in three cases (in autopsies #2, #4 and #9) and from one organ in four cases (in autopsies #1, #5 and #6) (see Table 3).

A high prevalence of MCPyV DNA was found in the blood (six cases) and lung (five cases), and low prevalence was found in the small bowel, liver and ovary (one case each). Viral DNA was also detected in the stomach (three cases), heart (three cases), skin (three cases), brain (three cases) and bladder (two cases). Uterus and prostate specimens were always negative.

The copy number of the MCPyV LT and VP1 was calculated among different organs and within a single autopsy case (Table 3). The highest copy number was detected in autopsies #7 and #9, in which blood samples had

21610570.09 copies per 10^5 cells and 380 413.25 copies per 10^5 cells, respectively. Interestingly, autopsy #7 had a high MCPyV copy number in the skin, suggesting the possibility of viral replication in skin, followed by a viraemic phase. Moreover, autopsy #9 had a high copy number in the brain sample (5051.32 copies per 10^5 cells) and in the ovary (369.81 copies per 10^5 cells), but the skin sample was negative. The copy number of MCPyV was low in eight autopsies and in most of the organs examined (Table 3), suggesting a high frequency of viral persistence.

Tissue samples with MCPyV LT DNA that was detectable by molecular analysis were submitted to immunohistochemistry for MCPyV T antigen detection. None of the samples tested positive.

DISCUSSION

Epidemiological studies have shown the high frequency of MCPyV exposure in the human population [9–11], similar to other members of the Polyomaviridae family [12, 13]. In most of the cases, the primary infection occurs during childhood and reaches 85% in adulthood [10], as demonstrated by the presence of antibodies against MCPyV antigens in serum samples of healthy adults [3]. Moreover, sustained MCPyV-specific antibody titres may indicate a persistent infection or viral latency within several target organs and tissues.

A few works have reported the detection of genomic sequences of MCPyV in the respiratory tract and saliva [7, 14, 15], the gastrointestinal tract [16], lymphoid tissue [17] and skin [1].

Several means of transmission have been hypothesized, such as a respiratory route [18], a faecal–oral route [7, 16] and shedding of viral particles from the skin [19], although none of these has been confirmed with certainty.

In this work, we evaluated the presence and quantity of MCPyV LT and VP1 DNA sequences in different organs and whole blood from 10 autopsies. The causes of death were cardiovascular/respiratory diseases or septic and haemorrhagic

Table 2. Distribution of MCPyV in organs/whole blood

Organ (no.)	VP1 (%)	LT (%)	VP1 and LT (%)	MCPyV (%)
Brain (n=10)	1 (10)	2 (20)	0	3 (30)
Heart (n=10)	1 (10)	2 (20)	0	3 (30)
Liver (n=10)	0	1 (10)	0	1 (10)
Small bowel (n=10)	1 (10)	0	0	1 (10)
Spleen (n=10)	0	0	0	0
Lung (n=10)	3 (30)	2 (20)	0	5 (50)
Kidney (n=10)	2 (20)	1 (10)	1 (10)	4 (40)
Bladder (n=10)	1 (10)	0	1 (10)	2 (20)
Skin (n=10)	3 (30)	0	0	3 (30)
Stomach (n=10)	3 (30)	0	0	3 (30)
Uterus (n=3)	0	0	0	0
Ovary (n=3)	0	1 (33.3)	0	1 (33.3)
Prostate (n=5)	0	0	0	0
Whole blood (n=10)	1 (10)	3 (30)	2 (20)	6 (60)

Table 3. MCPyV copy number (copies per 10⁵ cells) in organs and whole blood from 10 autopsies

Autopsy no.	Organs and Blood										
	Brain	Heart	Liver	Small bowel	Lung	Kidney	Bladder	Skin	Stomach	Ovary	Whole blood
1	0	0	0	0	0	3.94 ^L 0.0044 ^V	0	0	0	0	0
2	42.92 ^L	134.84 ^L	0	0	12.9 ^L	0	0	0	0.28 ^V	0	0
3	0	0	0	0	0	0	0	0	0	0	1.82 ^L 80.79 ^V
4	0.07 ^V	0	0	0	0	0.08 ^V	50.4 ^L 0.087 ^V	0	0.05 ^V	0	2124.63 ^L
5	0	0	0	0	0	0	0	57.63 ^V	0	0	0
6	0	0	0	0	0.37 ^V	0	0	0	0	0	0.00089 ^V
7	0	1.26 ^V	0	10.46 ^V	1.53 ^V	2.1 ^V	0	192 251 784.0 ^V	0	0	64.58 ^L 21 610 570.09 ^V
8	0	0	0	0	0	0	0	12.06 ^V	0	0	1433.3 ^L
9	5051.32 ^L	0	0	0	99.0 ^L	0	10.41 ^V	0	0	369.81 ^L	380 413.25 ^L
10	0	4.22 ^L	10.71 ^L	0	0.01 ^V	17.1 ^L	0	0	0.09 ^V	0	0

^L, MCPyV LT; ^V, MCPyV VP1

shock. None of the subjects had known causes of immunosuppression. An extensive sampling of organs and tissues was performed for a total of 121 specimens.

Our results confirm the high frequency of MCPyV exposure in the general population. Indeed, LT and/or VP1 genomic sequences were detected at least in one sample of all the autopsies examined. It is worth underlining that in most of the positive cases we only detected LT or VP1 genomic sequences, and we rarely detected both; this could be explained by viral DNA degradation in autopsy specimens occurring in the interval between death and autopsy and by the different amplicon lengths (59 bp for VP1, 146 bp for LT).

MCPyV DNA was found in a wide variety of organs and apparatus; notably, lung tissues disclosed the highest prevalence of viral DNA (5 of 10 cases). Since in two of these cases matched blood samples were MCPyV DNA-negative, the possibility of blood contamination by circulating viral DNA could be excluded. Babakir-Mina et al. [18], identified MCPyV DNA in 5 (17.24%) out of 87 lower respiratory tract samples, which suggests that the virus may be transmitted through the respiratory route and then, after spreading in the body, may undergo low-level replication and persistence or may establish latency in other anatomical sites. The results of our study seem to support Babakir-Mina's hypothesis: viral DNA was detected in lung samples from five autopsies, and all cases showed viral DNA in several organs or in matched blood samples.

Another interesting result of our study was the detection of MCPyV DNA in brain samples from three autopsies. To the best of our knowledge, this is the first study to report this finding. Lam et al. [20] studied 300 brain samples from 30 autopsies of unselected individuals to search for the human polyomaviruses BK, JC, KI, WU and MCPyV by means of nested PCR, and no samples tested positive for MCPyV DNA [20]. Methodological differences and pre-analytical

processing of the samples may explain the fact that the results for the rate of detection of MCPyV DNA obtained in the study by Lam et al. [20] and the present study differ. Indeed, the quality and method of fixation may influence the rate of viral detection, while low levels of viral DNA can be undetectable when using lower sensitivity DNA amplification methods. Matsushita et al. [8] performed an analysis of 41 autopsies and did not find genomic sequences of MCPyV in brain tissue. Notably, in our study, viral DNA was detected in brain autopsy specimens with positive and negative matched blood samples, avoiding the possibility of cross-contamination with blood included in paraffin blocks. Similar to other members of the Polyomaviridae family, such as JC virus and BK virus, that have been reported to infect brain tissue at some frequency, it is conceivable that MCPyV could also target brain cells, remaining in a latent state during the lifetime of the host.

In our study, skin samples were positive in three cases. This finding is slightly different from the results of Matsushita et al. [8] and Loyo et al. [7], which reported a prevalence of MCPyV DNA of about 50 % in autopsies and surgical samples of skin, including non-neoplastic skin biopsies. Moreover, the detection of MCPyV on environmental surfaces in contact with human skin [7] suggests that this virus is part of the normal microflora of the skin and viral particles could be transmitted by direct or indirect contact with human skin. Oh et al. [21], by using a metagenomic sequencing approach, demonstrated that MVPyV, like other microorganisms, tended to be stable over time on skin surface. In our study, the relatively low prevalence of MCPyV detection could be tentatively explained by the degradation of viral genome or the detachment of viral particles from skin after death.

MCPyV DNA was detected in whole blood from six autopsies. All the samples were taken during autopsy, and separation of plasma or serum from whole blood was technically

not possible, therefore we were not able to identify the source of the viral DNA. Pancaldi et al. [22] identified MCPyV DNA in the buffy coats of healthy donors, indicating a possible infection of leukocytes and suggesting that these cells are a possible viral reservoir in a fraction of healthy subjects. Interestingly, in two blood samples we found a very high copy number of MCPyV, indicating viral reactivation followed by a viremia. The two subjects were dead from cardiovascular diseases and no causes of immunosuppression were reported by clinicians. We speculated that MCPyV could also reactivate in conditions of stress, or in minor immunosuppressive states. Alternatively, the higher viral copy number detected in blood, compared to the formalin-fixed paraffin-embedded (FFPE) tissue specimens from autopsies, could also be due to the better preservation of DNA in blood samples.

Our quantitative data obtained by real-time PCR indicated that MCPyV DNA was present at a low copy number in most of the tissues, excluding blood samples and the skin sample in autopsy #7. These data suggest that MCPyV DNA may persist in several human tissues during life. Different positivity cut-offs, either for MCPyV or for other members of the polyoma virus family, have been reported in the literature, depending on the methodology used. According to Bhatia et al. [23], who used qRT-PCR, our samples were considered positive for MCPyV DNA detection when amplicons were generated within the range of 40 cycles. Accordingly, a very small amount of viral DNA was expected in cases with low levels of viral replication or tissue persistence.

The main limit was the low number of cases included in this study, which did not allow definite conclusions about the tissue distribution of MCPyV in adult populations to be drawn. However, these observational data seem to indicate a high prevalence of MCPyV DNA in the respiratory tract and demonstrate the possibility of viral persistence in circulating leukocytes and the central nervous system. Further research in larger autopsy series is needed to confirm our findings.

METHODS

Clinical specimens

Tissue samples were obtained from 10 autopsies, performed consecutively from April 2015 to September 2015. The interval between death and the execution of the autopsy was 48–72 h. The samples were obtained from brain, lung, kidney, heart, liver, spleen, bladder, stomach, small intestine, skin, prostate, ovary and uterus for a total of 111 samples. In all 10 autopsy cases, whole blood samples were collected into EDTA tubes. Autopsy specimens were immediately fixed in 10% neutral buffered formalin for a minimum of 48 h and embedded in paraffin. Three-micrometre-thick sections of FFPE tissues were stained with haematoxylin and eosin (H and E) and examined by light microscopy.

DNA preparation

Genomic DNA was isolated from three to five (5-µm thick) sequential sections of FFPE tissues using a QIAamp DNA

FFPE kit (Qiagen, Germany), and extraction was performed by QIACUBE (Qiagen). To avoid cross-contamination of samples, the microtome blade was cleaned with xylene between each block. The DNA extraction from 200 µl whole blood was performed using NucleoSpin Blood (Machery-Nagel, Germany) according to the manufacturer's protocol.

The b-actin gene was amplified in all cases and used as a positive control for DNA extraction by qualitative PCR. The b-actin primers were forward primer 5'-CCTTCC TGGGCATGGAGTCCTG-3' and reverse primer 5'-GGAGCAATGATCTTGATCTTC-3' [amplification product of 202 base pairs (bp)]. PCR products were analysed using agarose gel electrophoresis (2%).

Detection and quantification of MCPyV DNA

The primers targeting the LT and the VP1 genes of MCPyV (Table 4) were used according to the method described by Goh et al. [14], with modifications. Briefly, each 10 µl reaction mixture contained 1.6 µl (100 ng µl⁻¹) of genomic DNA, 6 µl of TaqMan Universal PCR Master mix (Applied Biosystems, USA), 800 nM of LT primers or 450 nM of VP1 primers and 320 nM of LT probe or 450 nM of VP1 probe. The thermal cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 5 s and 60°C (LT assay) or 58°C (VP1 assay) for 1 min. Real-time PCR was performed on the 7500 Fast Real-Time PCR system (Applied Biosystems, USA).

The pCR.MCV350 plasmid described by Feng et al. [1] was used as the template to prepare the standard curve for the real-time PCR assay and analyse assay sensitivity. The standard curves used to quantify the MCPyV LT or VP1 copy number were made with five serial dilutions (1:10) of the plasmid, ranging from 300 000 to 30 copies. MCPyV LT or VP1 copy numbers were normalized to the amount of cellular DNA by the quantification of human RNase P copies and were expressed as number of copies per 10⁵ cells. The standard curve used for the human RNase P gene quantification was made according to the manufacturer's instructions with five serial dilutions (1:10) of calibrated human genomic DNA, ranging from 100 000 to 10 copies ml⁻¹ of RNase P (TaqMan RNase P Detection Reagents Kit, Applied Biosystems, USA). The standard curve, with no template control (nuclease-free water), and samples were all tested in triplicate. Samples were considered positive for MCPyV DNA detection when amplicons were generated within the range of 40 cycles.

Immunohistochemistry

To estimate the expression of MCPyV T antigen protein, 3 µm-thick FFPE tissue sections were stained with a monoclonal antibody, CM2B4 (Santa Cruz Biotechnology, USA). Immunohistochemical staining was performed on a Dako Autostainer (Dako, Germany) following the manufacturer's instructions. Briefly, the slides were deparaffinized and hydrated, and antigen retrieval was performed using Tris-EDTA buffer (pH 8) in a microwave oven for 14 min (750 W). Endogenous peroxidase activity was blocked using

Table 4. Primers and probes used for the LT and VP1 regions of MCPyV, real-time PCR

Primer/probe	Sequence (5' to 3')	Amplicon size (bp)
MCPyV LT		146
LT forward	CCACAGCCAGAGCTCTTCCT	
LT reverse	TGGTGGTCTCCTCTCTGCTACTG	
LT probe	FAM-TCCTTCTCAGCGTCCCAGGC TTCA-TAMRA	
MCPyV VP1		59
VP1 forward	TGCCTCCACATCTGCAAT	
VP1 reverse	GTGTCTCTGCCAATGCTAAATGA	
VP1 probe	6FAM-TGTCACAGGTAATATC- MGBNFQ	

hydrogen peroxide 3% for 10 min. Specimens were then incubated with primary antibody (at a dilution of 1 : 50) for 30 min at room temperature. After washing, specimens were incubated for 30 min with Dako Envision + Dual Link at room temperature for subsequent deaminobenzidine (DAB) reaction. Positive controls from previously tested MCPyV samples, consisting of Merkel cell carcinoma tissue, were used in every immunohistochemistry session. All slides were counterstained with haematoxylin and protected with a coverslip for microscopic examination.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Ethical statement

All the patients' relatives gave informed consent for the diagnostic and scientific use of tissue (and blood) samples taken during autopsies. This study was approved by the Medical Ethical Committee of the Hospital 'Maggiore della Carità' of Novara.

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