Frequent Detection of Merkel Cell Polyomavirus in Human Merkel Cell Carcinomas and Identification of a Unique Deletion in the *VP1* Gene

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Abstract

Merkel cell carcinoma (MCC) is a rare but very aggressive human malignancy of the elderly or immunosuppressed patients. Recently, the clonal integration of a new human polyoma virus, which was termed Merkel cell polyomavirus (MCPyV), has been reported in 8 of 10 MCC patients. In the present study, we studied the formalin-fixed and paraffin-embedded tissue specimens of 39 MCC for the presence of MCPyV by PCR. We applied four different primer sets directed against the large T antigen and the *VP1* gene of MCPyV. We were able to detect MCPyV in 77% (n = 30) of MCC as confirmed by sequence analyses of the PCR products. Sequence analyses showed only minor nucleotide changes compared with the previously published MCC sequences. In addition, one patient revealed the amplification of two PCR products using PCR primers directed against the *VP1* gene. Sequence analyses confirmed the presence of the expected 351-bp PCR product and in addition a second PCR product of 261 bp containing a unique 90-bp deletion in the *VP1* gene, which will lead to a predicted loss of 28 amino acids. The unique 90-bp deletion within the *VP1* gene possibly is a result of incomplete viral integration of MCPyV in the MCC. The presence of MCPyV in the majority of MCC tissue specimens in our study strongly underlines a possible role for MCPyV as an etiologic agent in the carcinogenesis of MCC. [Cancer Res 2008;68(13):5009–13]

Introduction

Polyomaviruses are small double-stranded DNA viruses that are suspected as etiologic agents of human cancer. Currently, five different human polyoma viruses are known (1–5). Four of these (i.e., BKV, JCV, KIV, and WUV) belong to the SV40 subgroup. Although some of these human polyomaviruses have been shown to be tumorigenic in experimental animals, there is currently no convincing evidence that these viruses are associated causally with malignant tumors in humans (6). BKV and JCV have been initially described in 1971; KIV and WUV were identified in 2007 (1, 2). Recently, Feng and colleagues reported the identification of a fifth human polyoma virus that was designated Merkel cell polyomavirus (MCPyV) based on its detection in Merkel cell carcinomas (MCC) by digital transcriptome subtraction technique (5). They reported the presence of MCPyV in 8 of 10 human MCC and also clonal integration of the viral DNA. According to its nucleotide sequence, MCPyV shows close homologies to the murine polyomavirus subgroup (5).

MCC is a rare and aggressive malignant skin cancer of the elderly. It is thought to arise from the Merkel cell in the epidermis and has been described relatively recently (7, 8). It is a primary neuroendocrine carcinoma consisting of malignant small blue cells typically expressing neuroendocrine markers and revealing a characteristically paranuclear dot-like expression pattern of cytokeratin 20 (9). MCC has a high tendency for local recurrences, lymph node metastasis, and, finally, hematogenous spread (10). Thus, its mortality rate is high and its overall survival ranges were between 30% and 75% (7). The incidence of MCC has been reported to increase steadily during recent years (11, 12). MCC occurs more often in Caucasian males and is associated with sun exposure and immunosuppression (7, 13).

In the present study, we investigated a relatively large patient cohort of MCC for the presence of specific MCPyV DNA sequences. In addition, we tested 45 healthy blood donors for the presence of MCPyV. PCR products were confirmed by sequence analyses.

Materials and Methods

Patients and tissues. The study included representative formalin-fixed and paraffin-embedded (FFPE) resection and biopsy specimens of 41 MCC of which 21 have been selected from the archives of the Institute of Pathology, University Hospital Freiburg, of the years 1991 to 2007. The other 20 MCC were selected from the archives of the Center for Dermatopathology, Freiburg, Germany. All tissue samples were collected for diagnostic purposes and studied in accordance with national ethical principles. Details of clinicopathologic variables are included in Table 1. The investigation protocol was approved by the review board of the institute. Serial sections of all specimens were used for H&E staining and DNA preparation. In addition, DNA of randomly selected whole blood of 45 healthy blood donors was tested for the presence of MCPyV. The blood donors gave informed consent to participate in this study.

DNA preparation. First, an H&E stain of the selected specimens was reviewed by four experienced pathologists (A.z.H., C.D., W.W., and M.W.) to select paraffin material containing >95% tumor tissue. Two consecutive 5-μm paraffin sections from each specimen were subjected to DNA extraction. In brief, after deparaffinization, the tissues were lysed by proteinase K overnight (56°C) until complete tissue lysis, and DNA was extracted using the DNeasy Tissue kit (Qiagen). Purified DNA was measured in a spectrophotometer (Nano-drop, ND1000; PeqLab) and directly used for PCR.

MCPyV detection by PCR. DNA quality was confirmed by β-globin PCR using the GH20 (5′-GAAGAGCCAAGGACAGGTAC-3′) and PCO4 (5′-CAACTTCATCCACGTTCACC-3′) primer set.

PCR was performed with 120 ng of genomic DNA using the AmpliTaq Gold (Roche) DNA polymerase in a final volume of 50 μL. For MCPyV detection, we used the LT1, LT3, VP1, and M1/2 primer sets as published

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Table 1. Characteristics of Merkel cell carcinoma cases and results of MCPyV PCR

	Laboratory ID	Gender	Age (d)	Tissue specimen	β-Globin	MCV primers				
						LT1 (440 bp)	VP1 (351 bp)	LT3 (308 bp)	M1/2 (178 bp)	Nested (LT1/M) combination
UHF Pathology	1	M	81	R	+	−	+	+	−	−
	2	M	71	R	+	−	−	+	+	+
	3	F	81	R	+	−	+	+	−	+
	4	F	60	R	+	−	−	+	−	−
	5	F	80	R	+	−	−	+	−	−
	6	M	81	R	+	+	+	+	−	+
	7	F	72	R	+	−	+	+	+	+
	8	F	84	R	+	−	−	−	−	−
	9	F	92	B	+	+	+	+	+	+
	10	F	71	R	+	−	+	+	+	+
	11	M	76	R	+	+	+	+	+	+
	12	F	90	B	+	−	−	−	−	−
	13	M	94	R	+	+	−	+	+	+
	14	F	85	B	+	−	+	+	−	+
	15	M	84	R	+	−	−	−	−	−
	16	F	61	R	+	+	+	+	+	+
	17	M	86	R	+	−	−	−	−	−
	18	M	78	R	+	−	−	−	−	−
	19	M	72	R	+	−	+	+	−	−
	20	M	81	RR	+	−	+	+	−	−
Center for Dermatopathology	21	M	87	B	+	−	−	−	−	−
	22	F	90	R	+	−	−	−	−	−
	23	M	73	R	+	−	−	+	−	−
	24	M	78	R	+	−	−	−	−	−
	25	F	74	R	+	−	−	+	+	−
	26	F	86	R	+	−	−	+	+	+
	27	F	76	R	+	−	−	+	−	−
	28	M	86	R	+	+	+	+	+	+
	29	F	80	R	+	+	−	+	+	+
	30	M	77	R	+	+	+	+	+	+
	31	F	96	B	+	−	+	−	+	−
	32	F	68	B	+	−	−	+	−	−
	33	F	86	R	+	−	−	+	+	+
	34	M	69	B	+	−	−	−	−	−
	35	M	72	R	+	+	−	−	−	−
	36	M	80	R	+	−	−	+	−	−
	37	F	86	R	+	−	−	+	+	+
	38	M	93	R	+	+	−	+	−	+
	39	M	78	R	+	+	−	+	−	+
Pos/tot						11/39	14/39	28/39	15/39	18/39
%						28.2%	35.9%	71.8%	38.5%	46.2%
								30/39 = 77%		

Abbreviations: UHF, University Hospital Freiburg; M, male; F, female; R, resection; B, biopsy.

(5). In addition, we evaluated the use of the LT1 and M1/2 primer sets for nested PCR purposes using 31 cycles for each primer set. DNA and PCR mixtures were prepared and kept in separate rooms. Water instead of DNA template was used for PCR-negative controls containing all other PCR components.

Sequence analyses. PCR products were submitted to automated nucleotide sequencing in an ABI 3130XL genetic analyser (ABI). 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 or gb|EU375804.1 Merkel cell polyomavirus isolate MCC339, using the NCBI Blast program.

Results

MCPyV detection in MCC. Before MCPyV detection by PCR, the integrity and quality of the DNA was tested by β-globin DNA PCR. Of the 41 MCC specimens, 39 revealed a β-globin PCR product (Table 1). The mean age of these 39 MCC patients was 79.9 years and 51.3% (n = 20) were male and 48.7% (n = 19) were female

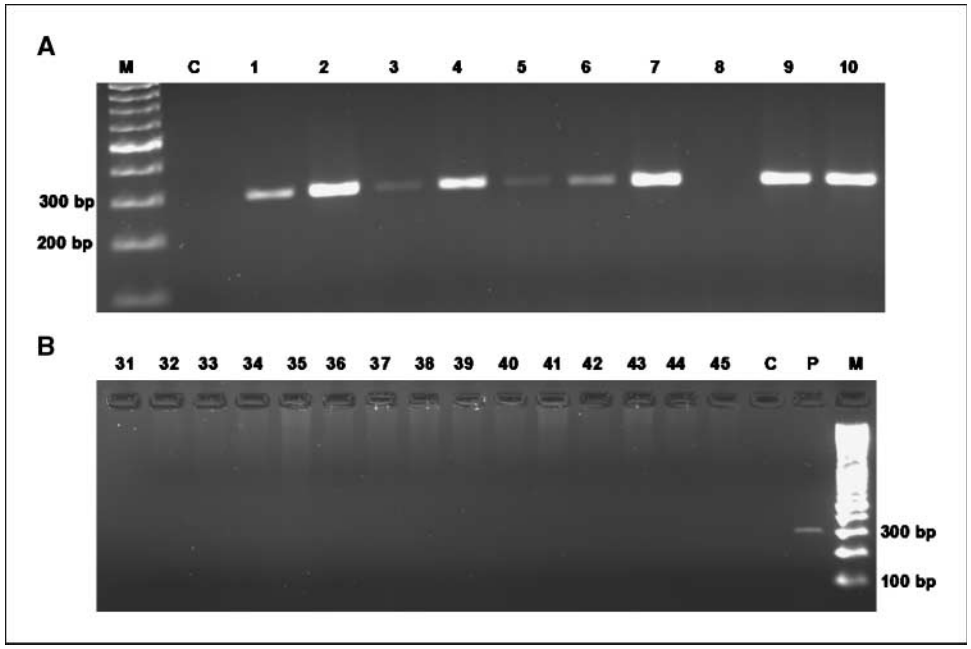

Figure 1. *A,* representative results of the PCR products (308 bp) using MCC DNA with LT3 primers. *M,* molecular weight marker; *C,* water control. The numbers indicate the cases as presented in Table 1. *B,* representative results of the PCR using the DNA of 45 healthy blood donors. The numbers above the lanes indicate the individual case. *C,* water control; *P,* positive control; *M,* molecular weight marker.

patients. These 39 MCC were finally included for further DNA PCR testing. Next, the MCPyV primer sets LT1, LT3, VP1, and M1/2 were used to amplify MCC DNA. All results for the individual primer sets are shown in Table 1.

In total, 30 of 39 MCC (77%) were tested positive by MCPyV DNA PCR. Of these, 5 MCC were tested positive in all four MCPyV primer sets used, 5 MCC in three MCPyV primer sets, 13 MCC in two MCPyV primer sets, and 7 MCC in one primer set. In contrast, 9 MCC remained negative in all MCPyV primer sets. Of the 30 MCPyV-positive MCC, 28 were detected by the LT3 primer set. Only 2 of the MCC that were tested negative by the LT3 primer set were tested positive by the LT1 or VP1 primer sets.

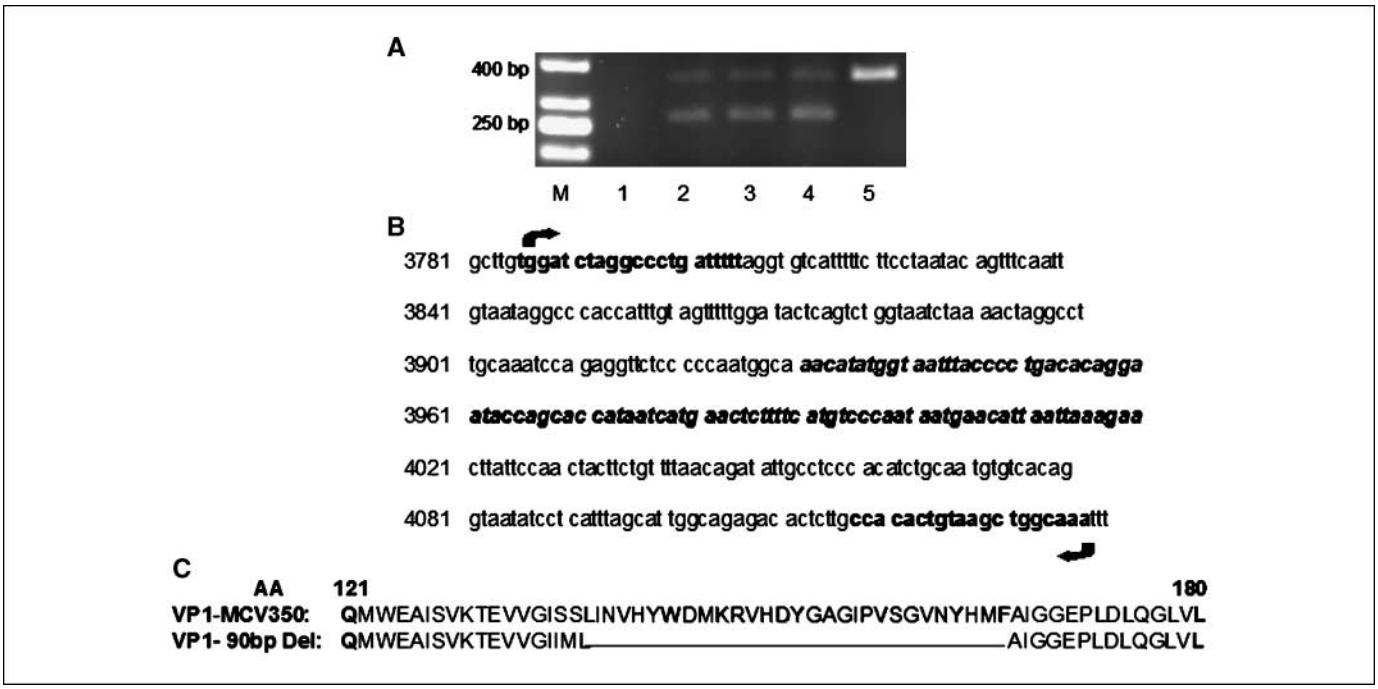

Figure 2. *A,* agarose gel (ethidium bromide) of the amplification products using the VP1 primers. *M,* molecular weight marker. *Lane 1,* water control; *lanes 2* to *4,* PCR products of three independent amplifications of DNA of tumor no. 31 (Table 1) using VP1 primers. The upper PCR product corresponds to the expected 351-bp PCR product. The lower 261-bp PCR product was only observed in tumor no. 33. *Lane 5,* positive control that is derived from tumor no. 10 (Table 1). *B,* nucleotide sequence of the *VP1* gene (based on the nucleotide sequence of the MCV350 genome) indicating, in bold letters, the sense and antisense primers. The part of the sequence in bold italic letters indicates the 90-bp sequence that is deleted in the 261-bp PCR product. *C,* predicted changes of the amino acid (*AA*) sequence of the deleted (VP1 90 bp Del) and MCV350 genotype. The underlined sequence shows the altered amino acid sequence.

Of interest, one MCC (no. 31; Table 1) revealed two PCR products, one of 351 bp and another of 261 bp, using the VP1 primer set. This MCC is the only one that was tested positive by VP1 and M1/M2 primer sets and was negative using LT3 or LT1 primer sets. The PCR-negative controls containing all other PCR components except DNA were negative in all experiments (Fig. 1*A*).

MCPyV nested PCR. To evaluate the use of a nested PCR approach for the detection of MCPyV sequences in archival FFPE tissue specimens, we used the LT1 and M1/2 as nested primer set. This approach detected only 18 MCPyV-positive MCC out of the 30 MCPyV-positive cases, which were detected by the use of the LT3 primer set.

MCPyV detection in healthy blood donors. Based on the results of MCPyV detection in MCC, we decided to test the blood of 45 healthy blood donors for the presence of MCPyV sequences by the LT3 primer set. Before the LT3 PCR, the DNA quality was confirmed by β-globin DNA PCR. No MCPyV sequences were detected in any of the 45 DNAs isolated from whole blood in contrast to the selected positive control of a MCPyV-positive MCC (case no. 3; Fig. 1*B*).

MCPyV sequence analyses. Sequence analyses of the PCR products identified all PCR products obtained with the MCPyV primers as MCPyV sequences. Next to minor changes in the nucleotide order, one MCC revealed a 90-bp deletion of the *VP1* gene (Fig. 2*A*–*C*). The 90-bp deletion leads to a predicted loss of 28 amino acids with alteration of the open reading frame.

Discussion

The recently identified MCPyV is the first human polyomavirus that reveals clonal integration in a human malignant tumor (5). The finding of MCPyV in human MCC, which is a rare and aggressive malignant skin cancer of the elderly and of immunosuppressed patients, certainly suggest a role of this virus in the carcinogenesis of MCC. Feng and colleagues showed the presence of MCPyV in 8 of 10 human MCC (5). In the present study, we investigated the presence of MCPyV in a large number of human MCC specimens. Although we used DNA extracted from FFPE MCC specimens, 77% of the tested MCC specimens revealed MCPyV DNA in our study cohort. These data are the first to test the findings of Feng and colleagues and indeed we are able to show a comparable number of MCPyV-positive MCC. In order not to miss MCPyV-positive MCC, we decided to use all of the previously published primer sets by Feng and colleagues, including the M1/M2 primers, which were originally designed for the generation of a Southern blot probe. In our laboratory, the primer set LT3 proved to be most useful for a reliable detection of MCPyV DNA in FFPE tissues as confirmed by sequence analyses. It is well known that the use of formalin chops nucleic acids to small-sized fragments (14). Thus, the low frequency of MCPyV DNA detection in MCC by LT1 (440-bp PCR product) and VP1 (351-bp PCR product) primers, 28.2% and 35.9%, respectively, seems to be obvious. However, we were surprised that the LT3 (308 bp) primer set turned out to be superior to the M1/M2 primer set, which yields in a 178-bp PCR product. Although speculative, the differences in detection frequency might be due to changes introduced in the process of viral DNA integration. The LT3 primer set (71.8% MCPyV MCC) even remained superior to the LT1 and M1/M2 primer set in a nested PCR protocol (48.7% MCPyV MCC), most likely due to the length of the LT1 PCR product. However, 9 (23%) MCC were tested negative in all four primer sets. This might point to a heterogeneous etiology of MCC. However, this interpretation should be done with caution because it might also be that the copy number of MCPyV DNA in these 9 MCC was below the detection limit for DNA PCR derived from FFPE tissues. Further studies on the presence of MCPyV in MCC will certainly help to answer this question.

We have sequenced the PCR products and all of these revealed a 97% or 98% homology to the previously published MCC sequences. Thus, only minor nucleotide changes were found. In addition, we were able to show an amplification of two PCR products using the VP1 primer set in one MCC (no. 31). To exclude an amplification artifact introduced by PCR, the amplification in this case was performed repeatedly (six times) by three different laboratory coworkers using the appropriate controls, and the PCR products were submitted to sequence analyses. This case revealed amplification of the expected 351-bp VP1 PCR product and, in addition, a PCR product that is characterized by a 90-bp deletion within the *VP1* gene as confirmed by sequence analysis (Fig. 2). The VP1 protein is a structural protein of MCPyV. Deletions or mutations of the *VP1* gene of other human polyoma viruses, such as JCV and BKV, and also other human DNA viruses, e.g., human papilloma viruses, are well known (15–18). These deletions are most likely to occur within the process of viral DNA integration or as a consequence of viral DNA integration. Although mutation or deletions in structural genes of other human polyoma viruses have been described, this is the first finding of a VP1 deletion of MCPyV. Previous findings have implicated deletions in *VP1* gene of JCV with altered DNA-binding functions and putative loss of VP1 nuclear transport. The VP1 of SV40 controls the functions of VP2 and VP3 by directing their localization between the particle and the endoplasmic reticulum membrane (19). The extent to which these alterations also apply to functions of MCPyV VP1 needs to be established. In addition, the finding of this 90-bp deletion might indirectly point to viral DNA integration in the tumor DNA. Although our study analyzed a large number of the rare MCC entity, it is hampered by the use of FFPE tissues, which yet does not allow an adequate testing of whether the viral DNA is integrated. However, our findings of the presence of MCPyV DNA in 77% of human MCC, including the identification of a unique *VP1* gene deletion, together with the recently reported finding of 80% (8 of 10) MCPyV DNA–positive MCC with clonal integration, closely links MCPyV as a putative etiologic agent to the carcinogenesis of MCC. Further studies on the presence of MCPyV in other human tumors are warranted. One of the most interesting tumor entities to test for the presence of MCPyV certainly will be small cell lung cancer, which is also a neuroendocrine human malignancy and is, at least with regard to histopathologic findings, nearly indistinguishable from MCC (20).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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