ORIGINAL ARTICLE

Merkel Cell Polyomavirus in Cutaneous Squamous Cell Carcinoma of Immunocompetent Individuals

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Squamous cell carcinoma (SCC) is the second most frequently diagnosed skin cancer. It has a higher incidence in immunosuppressed individuals such as organ transplant recipients and human immunodeficiency virus (HIV) carriers. Recently, a newly described polyoma virus, Merkel cell polyomavirus (MCPyV), was found in Merkel cell carcinoma (MCC), a rare aggressive skin cancer also associated with immunosuppression. We hypothesized that MCPyV would be present in SCCs. To test for the presence of MCPyV in immunocompetent SCC patients, we used PCR primer sets directed against the large T (LT) antigen and VP1 gene of MCPyV. We detected MCPyV in 15% (26/177) of SCC DNA samples and 17% (11/63) of adjacent skin DNA samples from 21 of 58 (36%) individuals studied. We did not detect MCPyV in any matched normal blood DNA (0/57), but observed the presence of MCPyV DNA in 1 of 12 normal mouthwash DNAs. All sequenced SCC samples had a common mutation truncating the LT antigen that provides indirect evidence of viral integration. The presence of MCPyV in ~15% of SCCs from immunocompetent individuals warrants evaluation of MCPyV as an etiologic agent in the carcinogenesis of SCC.

INTRODUCTION

Non-melanoma skin cancers, including basal cell carcinoma and squamous cell carcinoma (SCC), are the most frequently diagnosed tumors. Their incidence continues to increase worldwide (Boukamp, 2005). SCC is the second most common skin cancer with over 200,000 cases diagnosed in the United States each year. Risk factors leading to SCC development include immunosuppression, UV light, soot, arsenic ingestion, and burns (Boukamp, 2005). Viruses, such as human papilloma virus (HPV), have also been suggested to have a causal role in cutaneous SCC (Hengge, 2008). Recent studies have searched for the presence of a newly described polyomavirus, the Merkel cell polyomavirus (MCPyV), in SCC cases (Kassem et al., 2009).

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Abbreviations: HPV, human papilloma virus; LT, large-T antigen; MCC, Merkel cell carcinoma; MCPyV, Merkel cell polyomavirus; SCC, squamous cell carcinoma; SD, standard deviation

Polyomaviruses can be divided into three subgroups: avian, mammalian related to murine polyomavirus (MuPyV), and mammalian related to SV40. In 2008, Feng et al. (2008) discovered a new polyomavirus, MCPyV, which occurs in a rare human cancer, Merkel cell carcinoma (MCC), and can integrate into cellular DNA. Before the discovery of MCPyV, all the known human polyomaviruses, such as BKV, JCV, KIV, and WUV, belonged to the mammalian related to the SV40 group. The recently discovered MCPyV shows close homology to the mammalian related to the MuPyV subgroup.

Owing to their ability to immortalize cells and cause cancer in animal models, polyomaviruses have been studied extensively for their role in tumorigenesis (zur Hausen, 2008). MCC, a rare neuroendocrine tumor of the skin, is observed in the elderly and immunosuppressed patients such as organ transplant recipients, chronic lymphocytic leukemia patients, and acquired immune deficiency syndrome patients (Heath et al., 2008). MCPyV has been detected in 77–84% of MCC tumors, but its potential contribution to MCC development is unknown (Feng et al., 2008; Kassem et al., 2008; Viscidi and Shah, 2008; Becker et al., 2009). MCPyV in SCC has been identified in 52% of SCCs from immunocompromised individuals and in 25% of SCCs from immunocompetent patients (Kassem et al., 2009). The aim of this study was to determine whether MCPyV was present in SCC and other tissues from immunocompetent patients in a large patient cohort.

RESULTS

MCPyV detection in SCCs and matched normal samples

On the basis of previous studies showing the presence of MCPyV in MCC samples, we hypothesized that the MCPyV would be present in SCCs of immunocompetent individuals.

In total, 21 of 58 (36%) individuals with SCC had one or more samples test positive for the MCPyV. These samples included 1 of 58 normal DNAs, 26 of 177 SCCs, and 11 of 63 adjacent skin samples (Table 1; Figure 1a and Supplementary

Table 1. Summary of all study samples

Individual	Sex	Age at first SCC (years)	SCC samples	SCCs VP1/ LT3	Adjacent skin samples	Adjacent skin VP1/LT3
111137	M	71	1	−	1	−
			1	+	−	
112866	M	78	5	−	2	−
116965	M	69	1	−	NA	NA
117185	M	82	6	−	4	−
			1	+		
120547	F	73	4	−	NA	NA
121885	M	80	1	−	NA	NA
124229	M	55	3	−	1	−
124385	F	71	1	−	1	−
124386	M	72	1	+	1	−
124387	M	62	5	−	6	−
			1	+		
126165	M	54	2	−	NA	NA
126286	M	63	1	−	1	−
128645	M	60	2	+	NA	NA
130785	F	56	5	−	1	−
			2	+	1	+
142585	M	72	6	−	4	−
			2	+	NA	NA
144345	M	66			1	−
			2	+	1	+
144365	M	59	2	−	NA	NA
146045	F	43	1	−	1	−
149827	M	70	1	−	2	−
			1	+	NA	NA
153065	M	88	1	+	1	+
153706	M	67	1	+	NA	NA
154107	F	59	2	−	NA	NA
154108	M	77	1	−	NA	NA
154566	M	53	1	−	1	−
155092	M	40	1	−	NA	NA
			3	+	2	+
155112	M	75	1	−	1	−
158251	M	56	6	−	1	−
			1		1	+
159438	F	59	3	−	4	−
			1	+	1	+
163489	M	83	1	−	1	−

Table 1. Continued

Individual	Sex	Age at first SCC (years)	SCC samples	SCCs VP1/ LT3	Adjacent skin samples	Adjacent skin VP1/LT3
166849	M	69	1	−	1	−
169773	F	55	1	−	NA	NA
173542	M	40	1	−	NA	NA
176171	M	69	1	−	1	−
176172	M	60	1	−	1	−
			1	+		
178542[1]	M	83	2	−	NA	NA
199527	M	UA	1	−	NA	NA
26143	F	67	1	−	NA	NA
27263	M	75	2	−	NA	NA
27622	M	57	8	−	NA	NA
28646	M	84	3	−	NA	NA
29003	F	53	1	−	NA	NA
29082	M	73	0	NA	NA	NA
29564	M	70	4	−	NA	NA
29942	M	80	2	−	NA	NA
30582	F	69	1	−	NA	NA
30606	M	55	14	−	1	−
30742	F	80	1	+	NA	NA
32183	M	71	1	−	NA	NA
32782	M	76	27	−	3	−
			1	+	1	+
34562	M	68	4	−	2	−
35243	F	78	2	−	1	−
35422	F	48	1	+	NA	NA
35462	F	44	1	−	2	−
36882	M	76	3	−	2	−
37022	M	56	7	−	4	−
38463	M	55	1	−	NA	NA
57565	M	65	1	+	1	+
69612	F	58	2	+	2	+
80306	F	53	1	−	NA	NA

Postive patients	21 (36%)			
Negative patients	37 (64%)			
Total patients	58			
Negative samples		151 (85%)		52 (83%)
Positive samples		26 (14%)		11 (17%)
Total samples		177		63

F, female; M, male; NA, nonapplicable; +, positive for virus; −, negative for virus; SCC, squamous cell carcinoma; UA, unavailable.
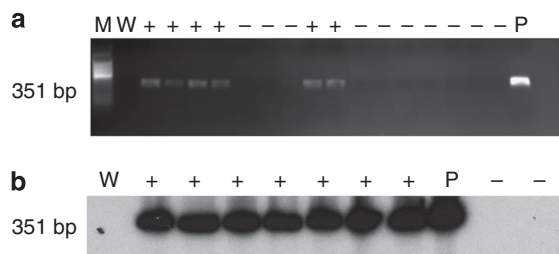[1]Positive for virus in mouthwash genomic DNA.

Figure 1. MCPyV detection. (**a**) The presence of MCPyV in SCCs, genomic normal DNA, and adjacent skin DNA was determined by PCR using VP1 primers. A representational result is shown with 6 of 16 samples tested showing a PCR product at 351 bp. All experiments included DNA from an MCPyV plasmid as a positive control (P) and a negative water control (W). M, molecular weight marker; +, positive for virus; −, negative for virus. (**b**) To increase sensitivity for detection of the MCPyV virus, radioactive PCR was conducted using VP1 primers. Samples include those that were positive for the LT3 primer set and negative for the VP1 primer set by regular PCR. All samples that were positive by one of the two primer sets by regular PCR were positive for the other primer set by radioactive PCR. Shown is a representational experiment that also includes 2 of 22 samples that were negative for both LT3 and VP1 primer sets by regular PCR. These were also negative by radioactive PCR. W, water control; +, positive for MCPyV; −, samples negative for MCPyV, and P, positive plasmid control.
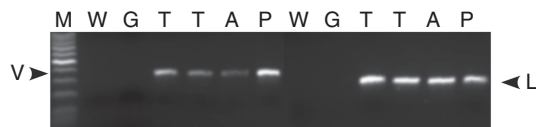

Figure 2. MCPyV detection in multiple samples from a single individual. Multiple samples including two SCCs, one adjacent skin DNA, and normal blood DNA from a single individual were amplified using MCPyV primers LT3 (308 bp) and VP1 (351 bp). Results show that both SCCs and the adjacent skin DNA samples tested positive for the presence of MCPyV on both primer sets, whereas the normal blood DNA was negative for the presence of the virus. M, molecular weight marker; W, water control; G, genomic normal DNA; T, SCC DNA; A, adjacent skin DNA; P, positive control; L, LT3 primer set; V, VP1 primer set.

Table S1) In our initial studies using published primer sets for both the large-T antigen (LT)3 and VP1 MCPyV primers (Feng *et al.*, 2008), 19 of 177 SCCs and 11 of 63 adjacent skin samples from 58 individuals were positive for one or more primer sets (Figure 1a). To obtain higher detection sensitivity, we analyzed 14 samples that were positive for only one primer set and 22 samples that were negative for both primer sets by radioactive PCR. All 14 samples that were positive for one primer set initially were positive by radioactive PCR for both primer sets, whereas none of the samples negative by PCR by both primer sets were positive by radioactive PCR (0/22; Figure 1b). As another means of increasing our detection sensitivity, we tested 21 samples that were negative for both primer sets at higher DNA input concentrations (50 ng). Of 22 samples, 7 (31%) that were negative by PCR at 15 ng input were positive at the higher input. No samples that were negative by radioactive PCR (0/5) were positive at the higher DNA input concentrations. At the end of these experiments, we had 100% concordance of calls of duplicate samples (47/47). No blood genomic DNA (0/57) tested positive by MCPyV DNA PCR; however, one normal mouthwash genomic DNA sample (1/1) from an SCC patient tested positive. In summary, 1.7% of normal control DNAs, 15% of SCCs, and 17% of adjacent skin samples from 21 of 58 individuals tested positive for MCPyV by PCR.

We hypothesized that individuals with one tumor positive for MCPyV would be more likely to have other SCCs positive for MCPyV. To test this hypothesis, we compared PCR results of the SCCs from 31 individuals who had more than one tumor analyzed for the presence of MCPyV. Of 31 patients, 6 (19%) with multiple tumors had more than one tumor positive for MCPyV (Table 1). Thus, within an individual, we observed variability in MCPyV positivity. We next correlated the results of viral status between normal adjacent and SCC DNAs in 32 patients for whom both types of samples were tested. From these patients, we had 46 pairs of matched tumor/adjacent skin samples (Supplementary Table S1). Of these, 33 pairs were negative for the presence of MCPyV in both tumor and adjacent skin samples, 6 were positive for only the tumor, and 7 were positive for both. Of the 46 paired sample sets, we had correlation for detection of the virus of 87% (Figure 2 and Supplementary Table S1).

MCPyV detection in mouthwash DNA samples
Initially, we tested one normal mouthwash DNA sample from an SCC patient for the presence of MCPyV. After this sample was found to contain MCPyV DNA, we tested 11 additional mouthwash DNA samples from individuals with non-oral, non-skin cancers to determine whether this was an unusual finding or whether MCPyV was present at a high frequency in apparently normal oral mucosa. Of the 12 total samples tested, only the initial sample tested positive for the virus, indicating that MCPyV is not found at a high frequency in normal oral muscosa.

The role of immunosuppression in MCPyV infectivity
As MCC is a disease of the elderly, which may correlate with decreased immune function in aging populations (Swann and Yoon, 2007), we wanted to determine whether the presence of MCPyV in SCC correlated with age of first SCC development. In our study population, the average age of SCC diagnosis in patients testing negative for MCPyV was 71 years (SD ± 38) and the average age of SCC diagnosis in patients testing positive for MCPyV was 65 years (SD ± 10). Of 42 male patients, 16 (38%) and of 16 female patients 5 (31%) tested positive for MCPyV. On the basis of these data, no definitive conclusions can be made regarding the role of age or gender in infection (Table 1).

All the samples included in the analysis were from individuals believed to have functioning immune systems. To determine whether individuals were taking medications that might depress their immune system, we analyzed the medical records and questionnaire information from a majority of study participants. Five individuals had a confirmed history of long-term use of medications that are known or suspected to affect the immune system. One of these individuals took Prednisone over a 13-year period and had no tumors test positive for MCPyV. The other four individuals all had tumors that tested positive for the polyomavirus. Two of these individuals had their SCC

diagnosed before the documented onset of immunosuppressive drugs, and thus their SCC diagnosis is not related to the use of these medications. Of the two other individuals, one took hydrocortisone for 3 months before the diagnosis of the MCPyV-positive tumor, and the second individual with two SCCs with MCPyV took Aldara (Graceway Pharmaceuticals, Bristol, TN) and Soriatane (Stiefel Laboratories, Coral Gables, FL) for at least a year before the time of diagnosis. These numbers are too small to reach any conclusions. However, it is interesting that of the 21 individuals who tested positive for polyomavirus, 2 (9%) were taking drugs affecting the immune system, whereas only 1 of 37 (2.7%) individuals who tested negative for MCPyV reported any long-term use of immunosuppressive agents.

HPV status

As HPV is postulated to have a role in SCC development and MCPyV is hypothesized to be important for the development of MCC, we speculated that if the MCC virus were having a role in SCC tumorigenesis, SCCs positive for MCPyV would be less likely to be positive for HPV. We tested 30 MCPyV-positive samples from 16 patients for the presence of HPV using HPV primer sets that detect over 25 HPV strains (Husnjak et al., 2000). In total, 20 of 30 (66%) polyomavirus-positive samples from 14 of 16 (87%) individuals tested positive for HPV. We compared these data with those from 21 MCPyV-negative samples from seven patients to determine whether the rates of HPV infection were similar in our sample sets. Of 21 samples, 9 (42%) from 4 of 7 (57%) MCPyV virus-negative patients tested positive for HPV.

MCPyV sequence analysis

A study by Shuda et al. (2008) showed that MCPyV T antigens derived from MCC acquire mutations ablating full-length LT expression and MCPyV replication capacity. To determine whether there were any VP1, LT, small T, or protein-binding domain mutations present in the samples containing MCPyV, we sequenced 31 positive samples. Not all samples were sequenced for all seven MCPyV primer sets because of insufficient DNA amounts or difficulty in amplifying specific regions, possibly because of mutations located at the sites of the primers. In total, 26 unique nucleotide changes were observed: 6 synonymous amino-acid changes, 17 non-synonymous amino-acid changes, 1 stop codon, and 2 changes of unknown significance (Table 2; Supplementary Table S2). The number of mutations per sample ranged from 1 to 10 substitutions in the regions sequenced (Supplementary Table S2). All samples except three adjacent skin samples and one mouthwash sample were found to have a common mutation at nucleotide 1401 (G1401>T) in exon 2, leading to a premature truncation in the LT helicase. In one paired normal adjacent/tumor sample set, the normal adjacent sample did not have the truncating mutation found in the tumor sample. This suggests that the mutation occurred post infection. The mouthwash sample contained no non-synonymous changes. The CR1, DNAJ and Rb-binding domains did not contain any non-synonymous or truncating mutations in any of the samples sequenced.

DISCUSSION

The recently identified MCPyV is the first human polyomavirus that shows clonal integration in a human malignant tumor (Feng et al., 2008). The finding of MCPyV in ∼80% of human MCCs suggests a role of this virus in the carcinogenesis of MCC (Feng et al., 2008; Kassem et al., 2008). The goal of this study was to test another malignant tumor of the skin, SCC, for the presence of MCPyV. Two large studies have looked for MCPyV in SCCs from immuncompromised organ transplant recipients. In one study, only 1 of 156 skin lesions was positive, whereas in the other study 52% of 56 SCCs were positive (Kassem et al., 2009; Ridd et al., 2009). Kassem et al. also looked for the presence of MCPyV in SCCs from immunocompetent individuals and found that 25% (7/28) of SCCs showed evidence of the virus. We detected MCPyV in 15% of SCCs. Our results are consistent with a study in which 15 SCCs were tested for MCPyV and 2 tumors (13%) were positive for the polyomavirus (Garneski et al., 2009). In the Garneski and Kassem studies, MCPyV was not detected in any of the normal skin samples. This differs from our results, in which we detected MCPyV in 17% of adjacent skin samples. It is possible that our normal adjacent samples, although histologically normal, were close enough to the tumor sites to house the virus and that the normal skin samples analyzed in the other studies were outside of a field of pre-malignant changes. The differences in detection rate between the studies and tissue types may be because of the differences in sensitivity for detection of different viral loads.

We may be underestimating the frequency of SCCs that are positive for the MCC virus. Initially, we tested 240 skin samples using 15 ng of input DNA, for which 14% were positive for one or more primers. We used two additional strategies for detection of virus in samples that were negative for both primer sets: radioactive PCR and higher input DNA (50 ng). We did not detect any additional positive samples (0/22) by radioactive PCR. However, using a higher input of DNA, 7 of 22 (32%) samples were positive. Five samples were tested by both methods; none of these were positive for the virus. These data suggest that the virus may be present in as many as 32% of samples and that we may be missing the virus in a subset of samples. These data also suggest that there may be differences in viral load between samples as samples with higher viral load may be more likely to test positive at lower DNA inputs.

In this study, multiple SCCs and matched normal samples from individuals were tested for the presence of MCPyV. We found an 87% concordance for the presence of MCPyV in 46 sets of matched SCC and adjacent skin samples (40/46). These data suggest a field effect of infection. We also showed that 6 of 31 (19%) individuals with multiple SCCs had more than one SCC test positive. These results are interesting, in that not all tumors arising in individuals with MCPyV are positive for the virus. This either suggests that there is no systemic effect of the virus or that we are not detecting the presence of the virus in some samples. Also interesting are two patients with large numbers of SCCs in which 0 of 14 and 1 of 28 tumors tested positive for the presence of the virus.

Table 2. Nucleotide substitutions detected by sequencing

EU375804 position	Large T	Small T	vp1	Mouthwash	Normal adjacent	SCC	Positive control[1]
c234g	leu13leu	leu13leu		1/1	0/9	1/14	1/1
a246g	leu17leu	leu17leu		1/1	0/9	0/14	0/1
a622g		thr143ala		0/1	0/9	1/14	0/1
t659c		leu155pro		0/1	1/9	2/15	1/1
g718a		glu175lys		0/1	1/10	1/17	0/1
a793g				0/1	2/10	1/18	0/1
c843g				1/1	1/10	0/18	1/1
g1242t	asp207tyr			0/1	0/10	7/18	0/1
c1360g	ser246cys			0/1	0/10	7/18	1/1
t1383c	ser263pro			0/1	0/10	1/18	0/1
g1401t	glu259stop			0/1	7/10	19/19	1/1
g1411t	arg263ile			0/1	0/10	1/19	1/1
a1428c	thr269pro			0/1	4/10	4/19	0/1
c1534t	pro304leu			0/1	3/4	4/8	0/1
g1541c	lys306asn			0/1	1/4	1/8	1/1
g1566a	asp315asn			0/1	0/4	1/8	1/1
a1575g	ile318val			0/1	2/4	4/8	1/1
c1581g	leu321val			0/1	0/4	1/8	1/1
c1771t	ser383phe			0/1	1/4	1/8	1/1
c3634t			leu201leu	0/1	1/2	7/9	1/1
g3691a			tyr182tyr	1/1	0/2	0/9	0/1
t3709c			gln176gln	0/1	0/2	4/9	0/1
c3712t			leu175leu	1/1	0/2	0/9	1/1
t3725c			glu171gly	0/1	0/2	1/9	0/1
t3797c			lys147arg	0/1	0/2	1/9	0/1
c3943a			leu108phe	0/1	0/2	1/9	0/1

SCC, squamous cell carcinoma.
[1]Mutations observed only in the positive control sample are not reported.

Although apparently normal skin samples can test positive for the polyomavirus, we, similar to other studies, did not identify any blood DNAs that were positive (Feng *et al.*, 2008; Kassem *et al.*, 2008).

Our results suggest that the polyomavirus may be present in apparently normal epithelial tissues. Consistent with this finding is that 1 of 12 normal mouthwash DNAs was positive for MCPyV. It is difficult to say from this study whether we are detecting the background rate of MCPyV infection of approximately 10–15% of samples or whether there is a link between the polyomavirus and the development of SCC. Although we cannot rule out a similar frequency of infection in individuals with no SCCs, previous studies assessing nine normal skin samples isolated from surgical patients observed no evidence of MCPyV (Feng *et al.*, 2008). Other studies have looked for MCPyV in non-skin tissues. Feng *et al.* (2008) found MCPyV in a small subset of control samples including the small bowel, hemorrhoids, appendix, and gall bladder. Another study looked for the presence of MCPyV in cancerous prostate epithelia, matched benign epithelia, and tumor-adjacent samples and found no evidence of the virus (Bluemn *et al.*, 2009). Two studies have examined nasopharyngeal aspirate samples for the presence of MCPyV and had detection rates of 1.3–4.3% (Bialasiewicz *et al.*, 2009; Goh *et al.*, 2009). Sastre-Garau *et al.* (2009) failed to detect MCPyV in 1,241 non-MCC tumor specimens of various types.

Previous studies for viral infectivity in cutaneous SCCs have focused on HPV. We tested for HPV status in MCPyV-positive and MCPyV-negative samples from 23 individuals. Although the overall rate of samples with detectable HPV was similar between the groups (66 versus 42%), there was a higher proportion of individuals in the MCPyV-positive group (87%) than the MCPyV-negative group (57%), which had one or more samples test positive for HPV. From these data, we are unable to establish a clear correlation between MCPyV and HPV infectivity. Our data are consistent with previous studies reporting that 30–92% of SCCs are positive for the presence of HPV (Berkhout *et al.*, 1995; Hopfl *et al.*, 1997; Pfister, 2003).

We sequenced portions of the MCPyV LT and VP1 genes. All but four of the MCPyV-positive samples shared a common mutation ablating full-length LT expression, which may abolish replication capacity. These results are intriguing in the context of the findings of Shuda *et al.* (2008), whose results suggested that truncating mutations in LT in tumors are not an experimental artifact but are a general feature of polyomavirus-mediated carcinogenesis. Their data suggest that MCPyV-associated MCC is a two-step process: first with viral genome integration followed by LT antigen mutation to prevent autonomous viral genome replication. Full-length LT is important for virus replication and cell lysis. Intact LT could also lead to DNA damage response or immune recognition hindering new tumor cell survival. The samples with intact LT antigen include the normal mouthwash sample and three normal adjacent skin samples, whereas all the tumors and the majority of adjacent skin samples had the truncated version. It remains to be seen whether the skin samples with truncating mutations show integration of the virus; this is an important question on which to follow up. As we did not sequence the complete MCPyV genome, it is possible that we missed other truncating mutations in these samples. Nonetheless, truncating mutations may serve as markers to distinguish tumorigenic versus non-tumorigenic MCPyV viruses. MCPyV has conserved features of other polyomaviruses including the CR1, DNAJ, and Rb-binding domains. These regions were intact in all of our sequenced MCPyV-positive samples and are thus predicted to be functional.

The majority of samples testing positive in our series contained unique sequence alterations, reducing the likelihood of PCR contamination. There were shared mutations in MCPyV-positive samples and the polyomavirus DNA was used as a positive control (MCC350). However, no sample matched the control in its entirety. Interestingly, the single truncating mutation observed in this study was identical to the one found in MCC350; however, the same amplicon in which the truncating mutation was observed had a number of sequence differences between MCC350 and other positive samples. We do not know the biological relevance of a common truncating mutation found in SCCs or if our results are because of some experimental artifact. Nonetheless, these results are intriguing, as MCCs are reported to have many different truncating mutations. These data may suggest a different mechanism of action between the tumor types.

We sequenced four tumor and normal adjacent matched sets and five pairs of independent tumors from the same person. Three of the four tumor/normal pairs and three of the five tumor/tumor pairs showed the exact nucleotide substitutions for sequenced regions. The remaining tumor/normal pair shared three of nine sequence changes and the two other tumor/tumor pairs shared one of the two and one of the seven changes, respectively. Combining the data, about half of the changes seen in multiple samples from an individual are shared, suggesting that individuals may either be infected with a single virus at various sites and that additional mutations occur subsequently or they

may be infected multiple times with different versions of the virus.

Results from this study show that MCPyV is found in SCCs arising in immunocompetent individuals and there is indirect evidence consistent with genomic integration in tumors and normally appearing skin. In our study, 36% of individuals had one or more samples test positive for the presence of MCPyV, indicating that the frequency of infectivity in the immunocompetent population may be higher than initially suspected. Despite the relatively low frequency of positivity in SCCs overall (15%), the pathogenic relevance of polyomavirus in SCC is unknown and warrants investigation.

MATERIALS AND METHODS
Patients and tissues
This study was approved by The Ohio State University (OSU) Cancer Institutional Review Board according to the Declaration of Helsinki Principles. All study participants signed informed consent. Patients were ascertained through OSU Medical Center dermatology clinics. Study criteria included a source of normal genomic DNA (blood, mouthwash, or saliva), available tissue from at least one SCC, a questionnaire on sun exposures and other medical history, and a signed medical record release form. Tissue samples included 240 formalin-fixed and paraffin-embedded biopsy specimens, 57 blood specimens, and 1 normal mouthwash specimen from a total of 58 individuals. Eleven additional mouthwash samples were obtained from individuals with other cancers to determine the presence of the polyomavirus in DNA from oral mucosa. Hematoxylin and eosin slides from tumors were reviewed by two pathologists, and areas comprising >70% of tumor cells were microdissected or cored from tumor blocks. Cores were also obtained from formalin-fixed and paraffin-embedded histologically normal adjacent skin when available.

Genomic DNA from normal adjacent tissue and SCCs was isolated from fixed archival tissue by the removal of paraffin using xylene and ethanol washes. Protein was removed by proteinase K treatment for 48 hours at 55 °C in nucleic acid lysis buffer. Samples were phenol/chloroform-extracted and ethanol-precipitated. DNA from blood and mouthwash samples was extracted by the Human Cancer Genetics Sample Bank.

Plasmid DNA containing MCPyV DNA for use as a positive control was obtained from Patrick S. Moore. pcDNA.MCV350(144-3696) and pcR.MCV(3508-1796) were transformed into competent cells using the pTargeT Vector protocol (Promega, Madison, WI). DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) as per the manufacturer's conditions. Caski cell line DNA containing hundreds of copies of HPV for use as a positive control was obtained from Gerard Nuovo.

MCPyV detection by PCR
Before MCPyV detection studies, the quality of the DNA was tested by a control PCR primer set mapping to chromosome 17. Only samples positive for the control PCR primers were included in further analyses. We tested 298 DNAs isolated from 177 SCCs, 63 adjacent histologically normal skin samples, 57 blood samples, and 1 mouthwash specimen from 58 study participants for MCPyV DNA using published primer sets LT3 and VP1 (Feng *et al.*, 2008). We included 47 duplicate samples for which we had multiple DNA extractions for the same tumor. PCR was carried out using 15 ng of

input DNA using Taq DNA polymerase (Qiagen), 1 μM forward and reverse primers, 1 × Q-Solution (Qiagen), 1 × PCR buffer (Qiagen), and 500 μM dNTPs (Denville Scientific, Metuchen, NJ) in a final volume of 20 μl. Experiments included pcDNA.MCV350(144-3696) or pcR.MCV(3508-1796) DNA as a positive control and water as a negative control. PCR products were run on a 2% agarose gel and visualized by ethidium bromide staining. The presence or absence of PCR products was determined by three independent researchers. For all samples testing positive in only one of two primer sets and an additional 22 samples that were negative for both primer sets, we ran radioactive PCR to increase the sensitivity of viral detection. Radioactive PCRs were carried out in 10 μl volume using 1 μM forward and reverse primers, 15 ng input DNA, and 0.1 μl of alphaP^{32} dCTP. After amplification, products were run on a 6% acrylamide gel, vacuum dried, and exposed to Kodak max film for 2–24 hours. For 22 samples negative by radioactive PCR and/or regular PCR, we increased DNA input to 50 ng.

HPV detection by PCR

PCR was performed on 30 samples positive for MCPyV and 21 samples negative for the virus using 15 ng of input DNA as described (Husnjak *et al.*, 2000). Experiments included Caski cell line DNA as a positive control and water as a negative control. For HPV detection, we used MY11/MY09 (MY primers) and HPV consensus primer sets that detect common HPV strains found in skin including 4, 5, 8, and 16.

Sequence analysis

We sequenced PCR products from 31 of the 38 samples that were positive for the MCPyV virus and the positive control sample. PCR products were obtained using nine different primer sets across the viral genome. PCR products were treated with Exo/SAP-iT to remove single-stranded DNA (USB, Cleveland, OH). Primers used for PCR were also used for sequencing. LT3 and VP1 primers are as published (Feng *et al.*, 2008). Additional primers are listed in Supplementary Table S3.

Automated sequencing of PCR products was conducted on an ABI 3700 (Applied Biosystems, Foster City, CA) by standard methods. Forward and reverse sequences were analyzed using DNAstar 3.0 (http://www.dnastar.com) and were compared with the reference sequences of the National Center for Biotechnology Information (NCBI) Entrez Nucleotide database gb|EU375803.1 MCPyV isolate MCC350 or gb|EU375804.1 MCPyV isolate MCC339, using the NCBI Blast program to identify mutations.

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