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Human Polyomavirus 6 and 7 Are Associated with a Pruritic and Dyskeratotic Dermatosis

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Abstract

Background—Human Polyomavirus 6 (HPyV6) and Human Polyomavirus 7 (HPyV7) are shed chronically from human skin. HPyV7, but not HPyV6, has been linked to a pruritic skin eruption of immunosuppression.

Objective—We determined whether biopsies showing a characteristic pattern of dyskeratosis and parakeratosis might be associated with polyomavirus infection.

Methods—We screened biopsies showing "peacock plumage" histology by PCR for human polyomaviruses. Cases positive for HPyV 6 or 7 were then analyzed by immunohistochemistry, electron microscopy (EM), immunofluorescence, quantitative PCR, and complete sequencing, including unbiased, next generation sequencing (NGS).

Results—We identified three additional cases of HPyV6 or 7 skin infections. Expression of T antigen and viral capsid was abundant in lesional skin. Dual immunofluorescence staining

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experiments confirmed that HPyV7 primarily infects keratinocytes. High viral loads in lesional skin compared to normal skin and the identification of intact virions by both EM and NGS support a role for active viral infections in these skin diseases.

Limitation—This was a small case-series of archived materials.

Conclusion—We have found that HPyV6 and HPyV7 are associated with rare, pruritic skin eruptions with a unique histologic pattern and describe this entity as "HPyV6- and HPyV7-associated pruritic and dyskeratotic dermatosis (H6PD and H7PD)."

INTRODUCTION

Human polyomaviruses (HPyVs) were first described in 1971, when JC polyomavirus and BK polyomavirus were identified in immunosuppressed individuals with progressive multifocal leukoencephalopathy and nephropathy, respectively [1, 2]. In the past decade, an additional 11 human polyomaviruses have been described [3]. Of these, several appear to reside chronically in human skin—Merkel cell polyomavirus (MCV), trichodysplasia spinulosa polyomavirus (TSPyV), HPyV6, and HPyV7. MCV was discovered within, and has been strongly linked to the pathogenesis of, a rare but deadly skin malignancy, Merkel cell carcinoma (MCC) [4]. Trichodysplasia spinulosa polyomavirus has been linked to a folliculocentric eruption first described in an immunosuppressed individual [5, 6].

HPyVs 6 and 7 are closely related polyomavirus species first identified through rolling circle amplification (RCA) of DNA isolated from swabs of healthy human skin [7]. They are thought to infect the skin in a latent or subclinical manner in the majority of people. In healthy individuals with clinically normal skin, previous studies have detected HPyV6 and HPyV7 sequences from skin swabs in 14–28% and 11–13% of samples, respectively [7, 8]. Less is known about skin diseases associated with HPyV6 and 7. Recent studies have revealed that HPyV7 could infect and actively replicate in biopsies taken from immunosuppressed, lung transplant recipients. In these patients, the skin infection presented as pruritic, scaly, brown plaques. Biopsies from lesional skin showed a characteristic pattern of parakeratosis described as "peacock plumage" [9]. In contrast to HPyV7, HPyV6 has not yet been linked with specific skin disease. Low levels of HPyV6 DNA have been detected in several types of epithelial neoplasms and a contribution of HPyV6 to these neoplasms has not been excluded [10, 11].

We identified biopsies showing a characteristic pattern of dyskeratosis and parakeratosis, previously described as "peacock plumage" for HPyV7 skin infections and investigated whether polyomavirus infections might be associated with these eruptions. We identify HPyV6 and 7 infection in three additional patients with pruritic dermatoses and provide evidence for the involvement of these viruses in the pathogenesis of the eruptions [12, 13]. Our work expands the spectrum of skin diseases associated with HPyV6 and 7 and yields novel insights into the biology of these ubiquitous skin polyomaviruses.

METHODS

This was a retrospective case series of archived skin biopsies. Histologically normal skin and archived biopsy samples were obtained through an IRB-exempt protocol. For Patient B, written, informed consent was obtained for collecting skin swabs for diagnostic and research purposes.

Human polyomavirus PCR

Formalin-fixed, paraffin-embedded (FFPE) sections were deparaffinized with xylene (Sigma, St. Louis, MO, USA) and DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). Typically, PCR was performed on 20ng of genomic DNA with polyomavirus screening primers (Table S3). Quantitative PCR (qPCR) was used to determine the copy number of HPyV6, HPyV7, and MCV using SYBR green and primers targeting the small T antigen region. LINE1 primers were used as a normalization reference. Anonymized skin biopsies from 8 patients with histologically normal skin were assessed by qPCR as a control.

Histology, immunohistochemistry, and immunofluorescence studies

5 μm FFPE sections underwent xylene deparaffinization, rehydration, antigen retrieval, and blocking. These slides were stained overnight at 4°C with 6V32 antibody (Buck lab, 1:100) to detect HPyV6 and 7 viral capsid protein, 2t10t (Buck lab, 1:100) for HPyV7 small T antigen, or 1t1 (Buck lab, 1:200) for HPyV6 small T antigen. Slides were then stained with appropriate secondary antibody conjugated to HRP (Santa Cruz Biotechnology, Dallas, TX). Lastly, the slides were developed with the VECTOR VIP Peroxidase Substrate Kit (Vector Laboratories, Burlingame, CA). For co-staining experiments, slides were incubated with HPyV7 antibodies and rabbit anti-cytokeratin 10 (clone EP1607IHCY, 1:10,000, Abcam), rabbit anti-cytokeratin 14 (catlog#PA5-28002, 1:5,000, Thermo Scientific), or rabbit anti-vimentin (clone D21H3, 1:400, Cell Signaling Technology). Goat anti-mouse Alexa Fluor 546 and goat anti-rabbit Alexa Fluor 647 were used as secondary antibodies. Confocal images were taken using a Zeiss LSM 880.

Electron microscopy

Biopsies were deparaffinized in xylene, dehydrated, and placed in Trump's fixative. The tissue was treated with osmium tetroxide, 2% uranyl acetate, rinsed, and dehydrated in alcohol. The tissue was embedded in epoxy resin, and thick and thin sections were generated and stained with toluidine blue (with sodium borate).

HPyV6 and HPyV7 Genome Sequencing

PCR was performed in a total volume of 50μl with 20ng of DNA using Sapphire Amp Fast PCR Master Mix (Takara Bio Inc, Shiga, Japan) and 0.3μM of sequence-specific primers (Table S3). Additional HPyV6 sequencing primers were designed with Primer3 to ensure overlapping coverage of the genome. Reactions were denatured at 95°C for 5 m, followed by 35 cycles with denaturation at 95°C for 1 m, annealing at 56°C for 1 m, and extension at 72°C for 2 m. Amplification products were agarose gel-purified with the NucleoSpin Gel and PCR Clean-up kits (Machery-Nagel, Düren, Germany) and sent for bidirectional Sanger

(GeneWiz, South Plainfield, NJ, USA). Methods for unbiased sequencing of circular DNA viruses were previously described [7, 14]. Briefly, skin swabs were layered onto an Optiprep (Sigma) gradient, centrifuged to purify encapsidated virions, Benzonase Nuclease (Sigma) digested, and amplified by RCA prior to whole genome sequencing (Illumina MiSeq).

RESULTS

The clinical characteristics of the patients, which have been previously reported, are summarized in Table I [12, 13]. All three patients presented with generalized, scaly, hyperpigmented papules coalescing into plaques, which had all been present for at least 12 months. All dermatoses were associated with some degree of pruritus (Fig 1A, Table I). While Patient A was immunosuppressed from HIV that had progressed to AIDS, Patient C was immunosuppressed from a kidney/pancreas transplant. Curiously, Patient B did not report any known immunosuppression at the time of initial diagnosis and biopsy in 2008. Importantly, all cases were first identified based on biopsies that showed a characteristic histologic pattern—scattered dyskeratotic cells throughout the epidermis along with irregular columns of parakeratosis in the stratum corneum (Fig 1B). This pattern has been described in the literature as "columnar dyskeratosis," "tiered parakeratosis with dyskeratosis," or "peacock plumage" [9, 12, 13]. FFPE lesional skin biopsy samples from these patients were screened for known human polyomaviruses by endpoint PCR. Sample A amplified HPyV7, while samples B and C amplified HPyV6 (Fig 1C). Weaker bands consistent with MCV were detected in samples A and C in replicate experiments; however, MCV was not detected by quantitative PCR (data not shown). We did identify three additional cases that showed "peacock plumage" on histology. However, none of those cases showed evidence of HPyV6 or 7 by PCR (data not shown) suggesting that this histologic pattern is not pathognomonic for polyomavirus infection. Classically, electron microscopy (EM) has been used to identify virions in tissues with suspected polyomavirus infections. EM sections were prepared from samples A and B; insufficient tissue remained from sample C for processing. While no viral particles could be identified from sample A, the biopsy from patient B revealed cytoplasmic arrays of icosahedral structures approximately 40nm in size, consistent with the reported size of polyomavirus virions (Fig 1D). The viral particles in sample B were identified in cells without recognizable features.

To confirm the expression of viral proteins in lesional skin, sections were stained with antibodies recognizing HPyV6 and HPyV7 capsid and small T antigen proteins. T antigen expression was detected in scattered cells throughout all levels of the epidermis. In contrast, capsid protein expression appeared to be enriched in the upper layers of the epidermis including within parakeratotic cells in the stratum corneum (Fig 2A, B). Viral capsid could be detected in both the nucleus and cytoplasm of infected cells (Fig 2C), while T antigen expression was largely limited to the nucleus of infected cells (Fig 2D). At higher magnification, the clear presence of desmosomes between infected cells provided histologic confirmation that keratinocytes could support active HPyV7 replication (Fig 2D).

To better characterize the skin infections caused by HPyV6 and 7, sections from patient A were co-stained with antibodies against viral antigens—capsid or small T—and cell-type specific proteins—cytokeratin 10 (CK10, a marker of more differentiated keratinocytes),

cytokeratin 14 (CK14, a marker of basal keratinocytes), or vimentin (a mesenchymal/ fibroblast marker). The majority of viral antigen positive cells co-stained for CK10 (66.7%) (Fig 3A) or CK14 (91.6%) (Fig 3B), while a very small number of cells also appeared to stain positively for vimentin (6.4%) (Fig 3C). These findings confirm that HPyV7 productively infects keratinocytes. Interestingly, we frequently noted the presence of CK10 negative, capsid positive cells in the upper layers of the epidermis (Fig 3A, arrowheads).

Because HPyV6 and 7 can be detected at low levels in clinically normal skin from healthy individuals [8], we performed quantitative PCR (qPCR) and sequencing to better characterize the HPyV6 and 7 infections. DNA extracted from two distinct lesional biopsies from Patient A revealed an average HPyV7 load of ~2904 copies/LINE repeat (a normalization control). In contrast, a collection of skin biopsies from asymptomatic skin donors (n=8) showed an average of ~14.3 copies/LINE repeat. Patient B was calculated to have a mean HPyV6 load of ~1.44×10^6 copies/LINE repeat, and Patient C, ~2.37×10^6 copies/LINE repeat. In contrast, control samples showed an average of ~31.1 copies/LINE repeat (Fig 4A). Thus, the levels of HPyV6 and 7 detected in multiple independent lesional biopsies were found to be several orders of magnitude more abundant than normal skin controls, suggesting a possible pathogenic role for the virus in the described rashes. The complete genomes of HPyV7 and 6 (GenBank KX771234-5), respectively, were successfully sequenced through primer walking. These sequences clearly diverged from published genomes (Fig 4B, Table S1). Specifically, the HPyV6 strain present in patient B demonstrated a complex deletion/ insertion in the non-coding control region (NCCR) along with multiple nucleotide substitutions in the NCCR, major capsid protein VP1, and large T (LT) antigen regions. The HPyV7 strain from patient A had a deletion in the NCCR and nucleotide substitutions in major capsid proteins VP1/2/3 and LT antigen regions. The possible effects of the mutations in the non-coding control region, capsid proteins, and large T antigens on viral replication and virulence are yet to be determined. Moreover, swabs collected from Patient B in July 2016, after the dyskeratotic dermatosis had resolved, were analyzed through unbiased deep sequencing of encapsidated, circular DNA viruses [7, 14]. In addition to abundant reads representing the complete genomes of nine human papillomaviruses and a MCV strain (GenBank KX781279-88), 121 reads matching HPyV6 isolate UTSW6.1 were observed (Fig 4C). The results show that patient B continued to shed HPyV6 (alongside other typical members of the human skin virome) after resolution of symptoms.

DISCUSSION

Ho et al[9] first associated HPyV7 infection with pruritic rashes in immunosuppressed transplant patients with a characteristic "peacock plumage" histology. Our study confirms and extends these seminal findings by linking HPyV6 with similar rashes. This study also broadens the range of immunosuppressed states that may allow for HPyV7 infection from iatrogenic immunosuppression from organ transplantation to the immunocompromised state of HIV infection. Of note, the HIV patient described here was noted to have several types of HPV by PCR and has been diagnosed with a variant of epidermodysplasia verruciformis[13]. Although the data here suggest that HPyV7 infection contributed significantly to the patient's disease, HPV co-infection could also contribute to the patient's clinical and histologic findings. Curiously, Patient B in this study was not reported to be

immunosuppressed at the time of the original biopsies. However, we speculate that the patient may have an uncharacterized, acquired immunosuppression based on his recent, frequent hospitalizations for severe infections.

These studies also expand our understanding of the biology of HPyV7. First, we definitively identify keratinocytes as the primary targets of HPyV7 infection in the skin. While MCV, TSV, HPyV9, and HPyV10 DNA can be detected on the skin[8, 15], no other polyomaviruses have been demonstrated to infect non-follicular keratinocytes. Given the detection of HPyV6 and 7 in other tissues[16, 17], it is likely that HPyV6 and 7 can infect or remain latent in non-epidermal tissues, as is the case for other polyomaviruses. Our studies also suggest that like other human polyomaviruses, HPyV6 may persist as a latent infection in patients without clinical evidence of disease. Specifically, Patient B possessed detectable levels of HPyV6 on his skin despite the fact that his pruritic dermatosis had resolved. How polyomaviruses, including HPyV6 and 7, maintain latency remains unclear. The decreased expression of CK10 in some HPyV7-infected keratinocytes, in concert with the notable dyskeratosis in these infections, suggests that latent infections might be maintained, in part, through the inhibition of normal keratinocyte differentiation. Moreover, the numerous sequence changes in the strains identified in our work could also contribute to the virulence and/or persistence of these strains and deserve further investigation.

Because our case series is very small and largely retrospective in nature, additional cases showing HPyV6 and 7 infection are necessary to confirm our findings. Moreover, the limited number of cases prevents us from definitively assigning causation to HPyV6 and 7. We speculate that additional cases of H6PD and H7PD will be identified based on the characteristic "peacock plumage" histologic pattern, in which nucleated, eosinophilic keratinocytes are present within the stratum corneum. In future studies, it will be interesting to investigate whether patients previously diagnosed with epidermodysplasia verruciformis show clinical or histologic changes consistent with the polyomavirus infections described here. Cases showing the characteristic histology could then be further screened for the presence of human polyomaviruses, especially HPyV6 and 7.

In summary, we provide multiple lines of evidence suggest that highly active HPyV6 and 7 infections are associated with skin disease, including high viral copy number in lesional skin, the expression of viral proteins by IHC in dyskeratotic keratinocytes, and the detection of encapsidated virions by both electron microscopy and unbiased sequencing. Given the characteristic clinical and histologic presentation of this rash, we propose that this skin disease be described as HPyV6- and HPyV7-associated pruritic and dyskeratotic dermatosis (H6PD and H7PD). This rare condition could be included in the differential diagnosis of immunosuppressed patients with recalcitrant, pruritic rashes. The identification of additional cases would be an important step toward improving our understanding of human polyomavirus biology and ultimately developing better treatments for their associated diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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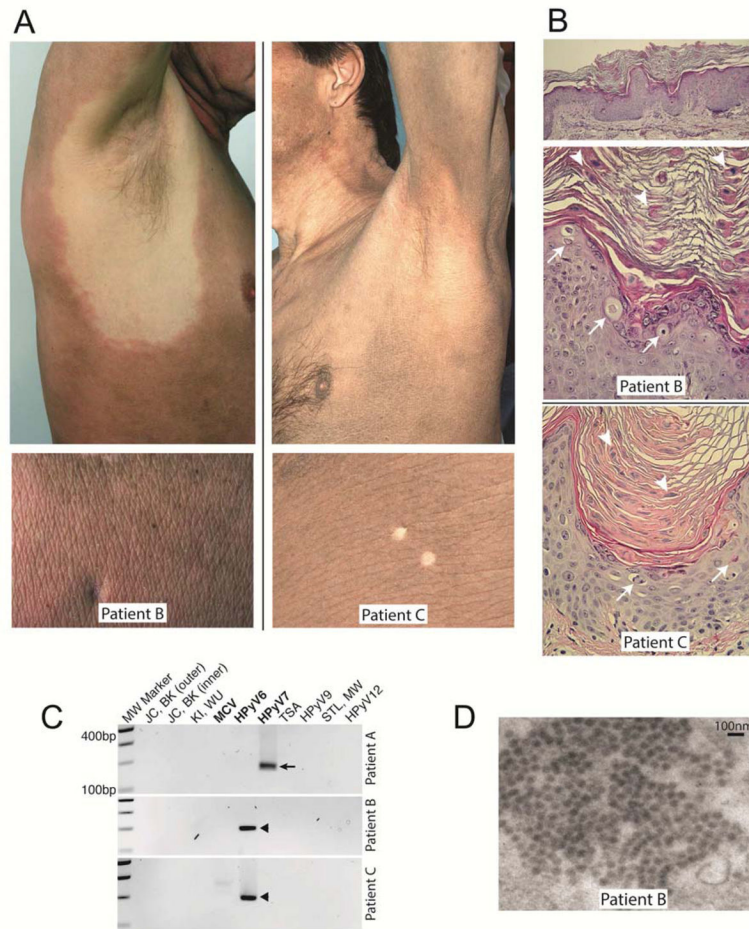
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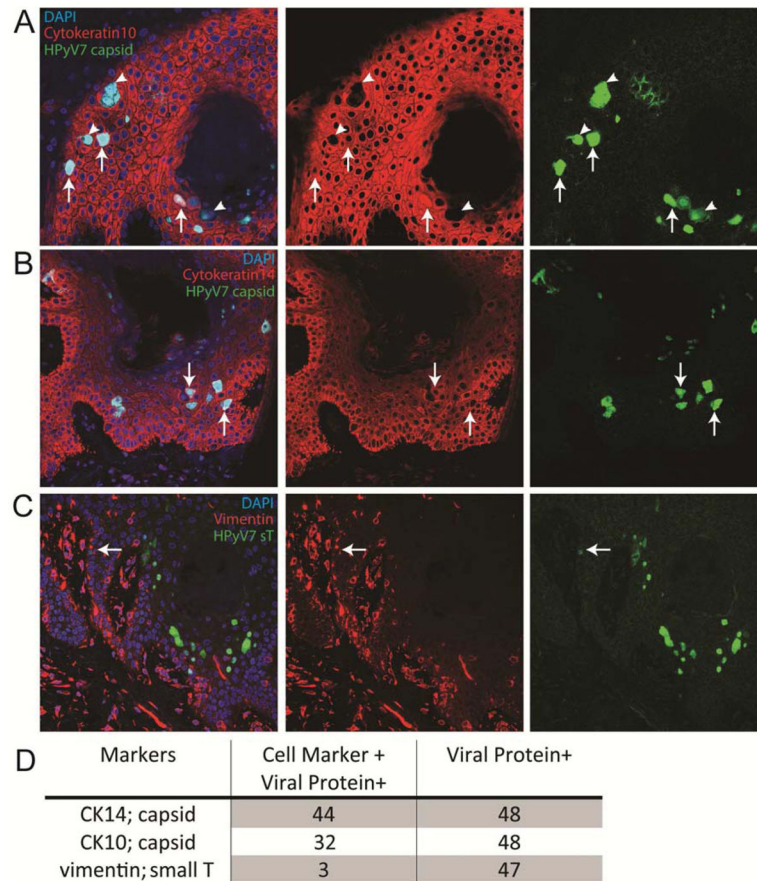
Figure 1. HPyV6 and 7 are associated with a pruritic and dyskeratotic dermatosis
(A) Generalized, hyperpigmented, scaly eruptions in Patient B, with no known immunosuppression, and Patient C, a kidney/pancreas transplant patient, (Pock and Stork 2010). Images from an HIV/AIDS patient (Patient A) have previously been published (Champagne 2015); **(B)** From Patient B, on routine H&E sections, there is mild acanthosis and papillomatosis (top, H&E, 40X total magnification); several dyskeratotic cells are noted in the superficial epidermis (arrows), with eosinophilic, nucleated keratinocytes forming irregular columns of parakeratosis (arrowheads) in the stratum corneum (middle, H&E, 200X). From Patient C, an area with prominent columnar dyskeratosis showing dyskeratotic cells (arrows) and nucleated keratinocytes in the stratum corneum (arrowheads) (bottom, H&E, 200X) **(C)** PCR for human polyomaviruses yielded specific bands for HPyV7 (arrow) and HPyV6 (black arrowhead). **(D)** Electron microscopy of affected cells revealed numerous cytoplasmic ~40-nm icosahedral virions from Patient B (18000X, original direct magnification).
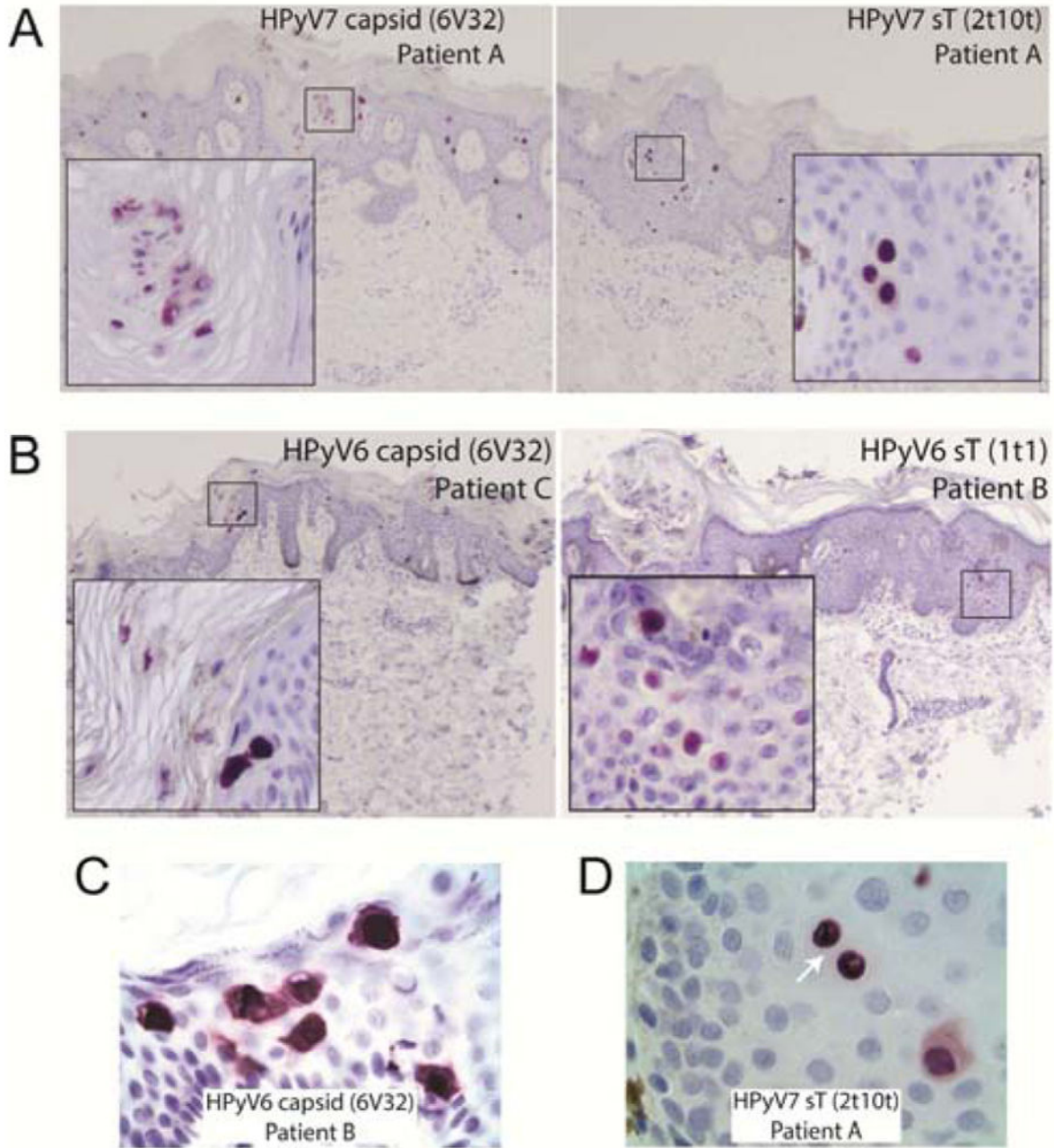
Figure 2. Immunohistochemistry against HPyV6 and 7 viral proteins
(**A**) Sample A shows capsid (6V32) expression in the nucleus and cytoplasm of affected cells. HPyV7 small T antigen (2t10t) is detected more strongly in the nuclei of affected cells. Samples B and C show capsid and HPyV6 small T antigen (1t1) in affected cells (insets, 200X). (**B**) High power images of the immunohistochemical stains show capsid protein expression in the nucleus and cytoplasm of the keratinocytes with viral cytopathic changes (left, oil immersion, 1000X). (**C**) Small T antigen is detectable in adjacent cells attached by desmosomes (right, arrow, oil immersion, 1000X) confirming their identity as keratinocytes.

Markers	Cell Marker + Viral Protein+	Viral Protein+
CK14; capsid	44	48
CK10; capsid	32	48
vimentin; small T	3	47

Figure 3. HPyV7 infects keratinocytes

(A) Dual immunofluorescence (IF) staining for Cytokeratin 10 (CK10) and capsid (6V32) confirms that most capsid expressing cells also express CK10 (arrows, 400X). However, there are several cells in which the expression of CK10 is undetectable (arrowheads). **(B)** Dual IF staining for Cytokeratin 14 (CK10) and capsid (6V32) confirms that almost all capsid expressing cells also express CK14 (arrows, 400X). **(C)** Dual IF staining for Vimentin and small T antigen (2t10t) identifies rare T antigen expressing cells that also express vimentin (arrow, 400X). **(D)** Quantitation of dual IF staining images.
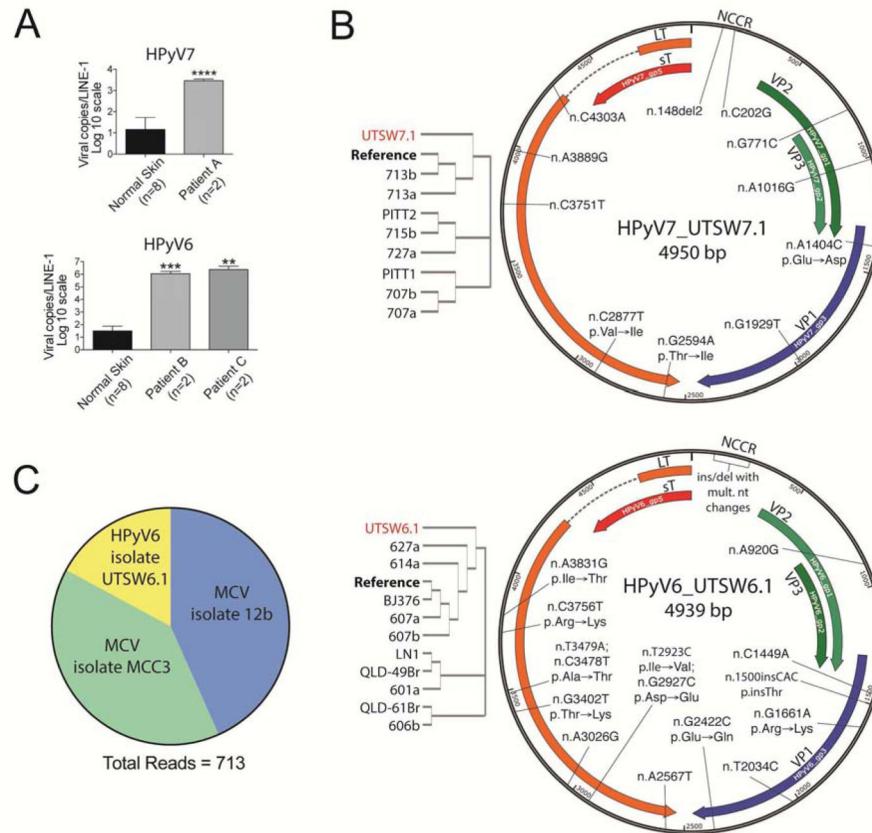
Figure 4. Novel strains of HPyV6 and 7 are abundant in lesional skin but can still be detected in asymptomatic patients

(A) Quantitative PCR comparing levels of HPyV6 and 7 detected in normal skin compared to lesional skin normalized to LINE-1 repeats. (n=independent biopsy; error bars=SD; t-test; **p 0.01, ***p 0.001, ****p 0.0001). **(B)** Schematic of HPyV7 isolate from Patient A (HPyV7_UTSW7.1) and HPyV6 isolate from Patient B (HPyV6_UTSW6.1) with labels indicating the approximate position of nucleotide and AA changes. Cladograms (left) indicate the phylogenetic relationship of the current strains with previously sequenced strains. **(C)** Patient B possessed subclinical infections of HPyV6 isolate UTSW6.1 and MCV isolates after clinical symptoms had resolved in 2016. Pie chart indicates the proportion of reads (300bp or longer) corresponding to the indicated virus.

Table I

Clinical characteristics of patients with HPyV6 and 7 associated dermatoses.

Patient	Age/Sex	Immunosuppresion	Pruritus	Clinical History
A	36/F	HIV/AIDS	severe	Worsening pruritic rash over 12 months; HPV-5, HPV-111, HPV-120, HPV-124, HPV isolate FA-88 positive; patient deceased
B	54/M	None at the time of diagnosis	present	Initial worsening over 2yrs followed by resolution over several years; HPV- by PCR; HIV negative; prominent palmar involvement; recent hospitalizations for sepsis, parapharyngeal abscess, pneumonia
C	52/M	Kidney/pancreas transplant (tacrolimus/rapamycin)	present	Pruritic rash over >2yrs; HPV-; prominent palmar involvement; patient deceased