Improved Detection Reveals Active β-Papillomavirus Infection in Skin Lesions from Kidney Transplant Recipients

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Running title: Active β-HPV Infection in Skin Lesions from KTRs

Abstract

The goal of this study was to determine whether detection of β-HPV gene products, as defined in epidermodysplasia verruciformis skin cancer, could also be observed in lesions from kidney transplant recipients (KTRs) alongside the viral DNA. A total of 111 samples, corresponding to 79 skin lesions abscised from 17 KTRs, have been analyzed. The initial PCR analysis demonstrated that β-HPV-DNA was highly present in our tumor series (85%). Using a combination of antibodies raised against the E4 and L1 proteins of the β-genotypes, we were able to visualize productive infection in 4 out of 19 actinic keratoses, and in the pathological borders of 1 out of 14 squamous cell carcinomas and 1 out of 31 basal cell carcinomas. Increased expression of the cellular proliferation marker MCM7, that extended into the upper epithelial layers, was a common feature of all the E4-positive areas, indicating that cells were driven into the cell cycle in areas of productive viral infections. Although the present study does not directly demonstrate a causal role of these viruses, the detection of E4 and L1 positivity in actinic keratosis and the adjacent pathological epithelium of skin cancer, clearly shows that β-HPV are actively replicating in the intraepidermal precursor lesions of KTRs and can therefore cooperate with other carcinogenic agents, such as UVB, favoring skin cancer promotion.

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Introduction

Solid organ transplantation is a treatment offered to an increasing number of patients with end-stage organ diseases. Although life-saving, organ transplantation is associated with an overall 3-5 fold increased risk of malignancies.[1–4] Most of these cancers are caused by reactivated viruses whose oncogenic potential is suppressed by immunological reactions in healthy individuals, like Epstein-Barr virus-associated B-cell lymphomas, Kaposi's sarcoma, caused by the reactivation of human Herpesvirus type 8, and Merkel cell carcinomas (MCC) of the skin, associated with Merkel cell polyomavirus (MCPyV or MCV).[5–9]

Of the cancers presenting in organ transplant recipients (OTRs) that have no established infectious etiology, skin cancer is the most frequent form (95%), including non-melanoma skin cancers (NMSC); squamous cell and basal cell carcinomas in particular (SCC and BCC, respectively).[10–13] The incidence of NMSC, the most common cancer in fair-skinned populations, is at least 50-fold higher in OTRs.[14–16] Large numbers of skin tumors (often more than ten) tend to develop over time in these at-risk subjects, thus presenting an enormous challenge for the patients and those responsible for their care.[17,18]

Nearly all adults are persistently infected in the skin with many viruses belonging to the Human Papillomavirus (HPV) family.[19,20] Although they appear not to cause overt clinical symptoms in a great majority of infected individuals, they can cause cancer, either in skin or in genital sites, particularly in subjects with impaired immune function.[21–25] To date, more than 150 HPV types have been completely sequenced and classified into five genera (and a number a species within each genus) based on DNA sequence analysis.[26,27] Of these, *Alphapapillomaviruses* (α-HPVs) are associated with genital cancer (e.g. HPV16 and 18) and some genotypes with common skin warts.[28] Skin-tropic *Betapapillomaviruses* (β-HPVs) (e.g. HPV5 and 8) are evolutionarily distinct from genus *Alpha* and appear to cause widespread unapparent or asymptomatic infections in the general population, but they have been associated with skin cancer in the immunocompromised host.[29]

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The relationship between β-HPV infection and NMSC has been clearly defined in patients suffering from the rare inherited disease epidermodysplasia verruciformis (EV), an autosomal recessive disease characterized by a predisposition to infection by specific types of β-HPV.[30–32] In these patients, HPV5 and 8 replicate very efficiently and reveal their full transforming potential, inducing multiple NMSC.[33,34]

Emerging evidence also supports the role of β-HPV in skin cancer development in immunosuppressed individuals. Sero-epidemiological studies have associated NMSC in OTRs with the presence of anti-β-HPV antibodies, and PCR-based studies have identified β-HPV DNA in over 80% of skin tumors from these patients.[22,35–43] Despite these findings, a causal role of these viruses has been difficult to verify because of their ubiquitous prevalence in the general population and their absence in some cancers.[25,44] The major weakness of the available studies is that the proposed association is mostly based on the presence of viral DNA in tumor tissues or positive antibody responses. Very few studies have addressed whether the β-HPV detected in these cases are actually localized within the malignant cells or whether they are transcriptionally active, the confirmation of which would greatly strengthen the evidence for a carcinogenic role of these pathogens.

In a recent study, we examined the β-HPV life cycle in skin tumors obtained from EV patients and found that, similar to what has been seen with other PV types, the onset of vegetative viral genome amplification coincides closely with the cytoplasmic expression of the viral protein E4 during productive infection.[33] This pattern was observed in all areas of the tumors where there was still some morphological differentiation of the epithelium. In addition, we have also demonstrated using fluorescent *in situ* hybridization (FISH) that the abundance of β-HPV seen in some lesions was a direct result of genome amplification within the carcinoma tissue.[34] Overall, our results indicated that E4 staining could be exploited as a marker of viral expression during β-HPV-associated skin cancer progression as reported in the HPV8 transgenic mouse model and in cervical disease for the alpha genotypes.[33,45-47]

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In order to extend the data obtained so far in EV patients to other groups of patients at high risk of developing NMSC, here we studied a series of skin lesions from kidney transplant recipients (KTRs) attending our University Hospital by systematically analyzing for the presence of β-HPV infection both at the DNA (PCR) and protein level. Using a combination of antibodies raised against the E4 and L1 proteins of the β-genotypes, we were able to visualize the completion of viral life cycle in some precancerous lesions such as actinic keratosis or at the periphery of more advanced disease. These data demonstrate that β-HPV transcription is occurring at site of skin transformation in the OTR setting and points to its possible involvement in the process of skin carcinogenesis.

Material and Methods

Sample collection.

Tissue sections were obtained from 111 formalin-fixed and paraffin-embedded (FFPE) blocks, previously collected from 79 skin lesions and stored in the University Hospital medical material archives. For some lesions, only a single block was available, denominated 'whole lesion' (WL); while for others, two blocks existed, corresponding to the core (C) and edges (E) of the lesion. All lesions were excised from 17 KTRs, while one amongst them was also heart-transplant twice, (16 male and 1 female) receiving transplants between 1998 and 2009. Data on skin lesion development and characteristics were retrieved from pathology archives and clinical records. The mean length of patient follow-up following kidney transplantation was 15 years and 4 months ± 7 years and 8 months. A single FFPE block from a wart-like lesion of a patient with epidermodysplasia verruciformis was also analyzed in the present study as positive control.

Written informed consent was obtained by all subjects according to the Declaration of Helsinki and approval was obtained from local ethic committee.

β-HPV DNA detection and genotyping with the PM-PCR RHA method

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Two consecutive 10 μm-thick sections were retrieved from FFPE samples and used to extract DNA by means of the QIAamp Tissue Kit (Qiagen srl, Milan, Italy) according to the manufacturer's instructions. To prevent cross-contamination, the microtome was thoroughly cleaned following the sectioning of each block and a new blade installed for the next. β-HPV detection and genotyping was carried out using the PM-PCR reverse hybridization assay (RHA) method (Skin β-HPV prototype research assay; Diassay BV, Rijswijk, The Netherlands), as previously described.[48] The method was designed to identify the 25 established β-HPV types (i.e., HPV genotypes 5, 8, 9, 12, 14, 15, 17, 19, 20, 21, 22, 23, 24, 25, 36, 37, 38, 47, 49, 75, 76, 80, 92, 93, and 96). The PM-PCR reverse hybridization assay, which generated a biotinylated amplimer of 117 bp from the E1 region, was carried out following all the precautions indicated by the manufacturer to avoid recurrence of cross-contamination. Briefly, PM-PCR was performed in a final reaction volume of 50μL, containing 10μL of the isolated DNA, 2.5mM $MgCl_2$, 1x GeneAmp PCR buffer II, 0.2mM deoxynucleoside triphosphates, 1.5 U Amplitaq Gold DNA polymerase and 10μL of the PM primer mix. The PCR was performed by a 9 min preheating step at 94°C, followed by 35 cycles of amplification comprising 30 s at 94°C, 45 s at 52°C, and 45 s at 72°C. PCR was ended by a final elongation step of 5 minutes at 72°C. As a positive PCR control, a β-HPV plasmid clone was included. Identification of the amplified HPV types was performed by reverse hybridization analysis of the amplimers on genotyping strips provided by the manufacturer. In each strip a probe line contains a mix of "universal" HPV probes: in some cases these probes are positive without a probe for specific β-HPV being positive, indicating the presence of an unspecified β-HPV. These cases are indicated as 'unspecified β-papillomavirus genotype'.

Antibodies and generation of anti-L1 antibodies

To generate antibodies that can broadly identify the L1 proteins of the β-genotypes, sequence alignments were first carried out, and the highly conserved region between amino acid 200 and 300 was chosen. This region of the HPV5 full length protein (Ref. Seq.: NP_041372.1) was amplified

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by PCR from an HPV5 genomic clone recently isolated from an EV patient. The primer sequences used were: L1 forward: 5´-CGCGGATCCCGTCTGTATCCAAAACCCTTG-3´; L1 reverse: 5´-CCGCTCGAGTTATCCTATAGTCTTTTGAGCTTG-3´. The forward primers included a BamHI site (underlined) upstream of the ATG initiation codon, while the reverse primers contained a XhoI sequence (underlined). Amplimers were cloned into the pGEX-4T-2 vector (GE Healthcare Europe GmbH, Milan, Italy), and then expressed in the *Escherichia coli* host BL21. GST-L1 fusion protein was dialyzed overnight at 4°C in 50mM Tris-HCl pH 8.5, 0.5% β-mercaptoethanol and 3M urea, and then re-dialyzed for 12h at 4°C in 50mM Tris-HCl pH 8.5 and protease inhibitors (Sigma). Anti-sera were raised by injecting rabbits with the dialyzed GST-L1 fusion protein. Animals were bled 1 week after the fourth immunization and serum immunoglobulins obtained by precipitation with 45% saturated ammonium sulfate. The precipitate was then resuspended in phosphate-buffered saline (PBS) and purified on a protein A affinity column (GE Healthcare) according to the supplier's specifications. Preimmune serum was collected prior to the initiation of the immunization protocol and was used as a control in the immunostaining experiments.

The production of polyclonal antibodies to HPV5E4, which crossreact with many genotypes from species 1, including HPV8, 14, 20, 24, 25, 36, has been described previously.[33] Antibodies to minichromosome maintenance protein 7 (MCM7) were obtained from Neomarkers Fremont (MS-862-p1).

Immunofluorescent detection

Consecutive 5-µm thick sections obtained from FFPE tissues were processed for immunofluorescent detection of antigens coupled to DNA-FISH as previously described.[49] For the other markers analyzed (E4, MCM7, L1), antigen unmasking was performed by heating the slides in a conventional decloaking chamber, where they were placed for 10 min at 121°C in 10 mM citrate buffer at pH 6.0 (Vector Laboratories, Burlingame, CA). The immunofluorescent protein-protein double detection (HPV5 E4-MCM7) was carried out by incubating the slides overnight at 4 °C with the primary antibody anti-MCM7 (1:200) diluted in 5% normal goat serum (NGS) followed

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by the amplification with TSA (Tyramide Signal Amplification; Perkin Elmer, Waltham MA); then, slides were incubated with the primary antibody anti-HPV5 E4 (1:1000) for 3 hours and subsequently with the appropriate fluorescent secondary antibody. The immunofluorescence for L1 detection was carried out by incubating the slides overnight at 4 °C with the primary antibody anti-L1 (1:1000) diluted in 5%NGS-PBS followed by incubation with the appropriate fluorescent secondary antibody. Images were acquired using a digital scanner (Pannoramic MIDI, 3D Histech Kft., Budapest, Hungary). For the assessment of histological features, the slides analyzed by HPV5 E4-MCM7 were disassembled and stained with hematoxylin and eosin (H&E).

Results

Patients and skin lesion characteristics

The baseline characteristics of the study population and the histological features of the cutaneous lesions, alongside the relative number of lesions, the year in which they occurred following transplantation, and their location at sun-exposed or unexposed sites are listed in Table 1. The median time of skin lesion occurrence was 7±5 years after transplantation. Patients are numbered in ascending order based on the number of tumors developed. Within 10 years from transplantation, 5 patients developed more than 5 lesions (patients 13 to 17), mostly in sun-exposed sites, confirming sun exposure to be a major contributor to epithelial transformation. The skin lesions included: SCC (n=14), and BCC (n=31), followed by precancerous lesions such as actinic keratosis (n=19) (AK) and Bowen's disease (n=1) (BD), keratoacanthoma (n=7) (KA), and benign lesions such as seborrheic keratosis (n=7) (SK). The proportion of lesions affecting sun-exposed skin areas (head, hands and forearms) was 59 out of 79 lesions (75%).

β-HPV type determination

The results of β-HPV testing of the DNA extracted from the 79 lesions are shown in Table 2. In 28 cases, multiple biopsy blocks were available corresponding to either the core or edges of the lesions. Out of the 111 FFPE blocks analyzed, 94 were HPV positive (85%), and 86 samples

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(77%) could be genotyped as containing at least one of the 25 β-HPV genotypes available on the RHA (Table 2).

The spectrum of β genotypes identified for each lesion from the 17 study patients is reported in the Supplementary Table 1. In cases of multiple biopsies, the genotypes found in the core and edges of the tumors are combined. The most frequently observed genotypes are HPV5 and 8 (both belonging to species 1), which were found in 51 (65%) and 20 (25%) of the 79 lesions, respectively. HPV5 was detected as a single infection in 18 out of the 79 lesions (23%) and HPV8 was detected as a single infection in 2 lesions (3%). Multiple infections with 2 genotypes were found in 13 lesions (16%), while more than 2 genotypes were found in 28 lesions (35%). In the five patients exhibiting more than 5 tumors, HPV5 alone or in multiple infections was found in 38 (66%) of the 58 lesions present on these 5 patients. Considering just the genotypes belonging to species 1, including HPV5, 8, 12, 14, 19, 24, 25, 36 and 93, 61 tumors (77%) turned out to be positive for at least one genotype. For the lesions split into more FFPE blocks (n=36), at least one β1 genotype was consistently found in 15 (42%) of them (data not shown).

Visualization of viral protein expression in skin lesions

Previous studies from our group have demonstrated that detection of the abundant viral E4 protein is helpful for the visualization of active β-HPV infection in skin tumors from EV patients.[33,34] Figure 1 shows representative images of tissue sections from a benign wart-like lesion from an EV patient displaying the unequivocal histological features associated with the infection, including acanthosis and the disorganization of the granular layers defined by an abrupt variation in keratoyaline granules. The E4-positive cells are enlarged and display prominent blue-grey pallor with occasional perinuclear halos. HPV8 DNA-FISH analysis confirmed that these cells are supporting viral genome amplification as visualized by many positive nuclei for overlapping cytoplasmic E4 staining. Completion of the viral life cycle was also defined by L1 staining performed using in-house polyclonal antibodies raised against a common region of the β-genus

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major capsid protein. As expected, L1-positive nuclei were detected in the more superficial layers of the E4-positive area.[50] The cellular proliferation marker MCM7 was also strongly increased in the lesion compared with the adjacent normal epithelium; being most apparent in the basal and suprabasal layers indicating that cells were stimulated to enter the cell cycle.[49,52]

To investigate whether similar images of productive infection could be visualized in OTR skin lesions, tissue specimens from the available FFPE blocks were co-stained using immunofluorescence (IF) analysis with anti-E4 and anti-L1 antibodies to detect viral antigen expression, and with MCM7, a marker of cellular proliferation.

Out of the 111 FFPE blocks analyzed, E4-positive cells were found in 6 FFPE blocks (for brevity, referred to as IF-positive), originating from patients 5, 8, 16, and 17, and corresponding to 4 AK lesions, and the adjacent pathological area of 1 SCC and 1 BCC. Patients 16 and 17 were transplanted in 1993 and 1999, respectively, and developed 14 skin lesions each in their post-transplant period. The other two patients received transplants in 2002 and 2005, respectively. Table 3 summarizes all the lesions developed by the IF-positive patients and reports the details of the genotyping results for each FFPE block available, distinguishing the different parts of the lesions (edges, core or whole lesion). As expected from the spectrum of the polyclonal anti-E4 antibody used, all the IF-positive sections harbored genotypes belonging to species 1.

Having established the EV staining pattern, we carefully went through all the E4-positive areas identified in our tumor series, looking for their major histological characteristics and staining patterns, and the most representative images are presented. Figure 2 shows one of the two E4-positive areas detected in the adjacent pathological epithelium from a BCC of the neck. The epithelium displays acanthosis and both the E4 and L1 staining patterns show great resemblance to those obtained from the EV patient shown in Figure 1. Figure 3 summarizes the staining obtained from patient 8, focusing on the positive area found in the bowenoid AK, located next to the SCC. This area shows clear signs of β-HPV-related cytopathic effects, some cells display pleomorphism and nuclear atypia, and again both early and late viral markers of β-HPV infection are present.

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Three hypertrophic AK, from the same patient (patient 16), are reported in Figure 4 (panels A-C). Panel A shows a clear-cut area of positivity found in an epithelial crevice, where disruption of the granular layer was clearly evident alongside parakeratosis. In this case, it was also possible to perform FISH with the single genotype found, specifically HPV25; where FISH-positive nuclei neatly overlapped with some E4-positive cells in the cytoplasm, while L1 positivity was confined to some parakeratotic nuclei. The positive area reported in panel B was found in the pathological edge of an AK and shows the typical staining pattern and cytopathic effects of productive β-HPV infection. The AK of panel C shows a positive area corresponding to a highly parakeratotic epithelium that abruptly changed to normal orthokeratosis on both sides. As expected from the high grade of parakeratosis, many L1-positive nuclei were found above the E4-positive cells. Consistent with the staining seen in the EV lesion shown in Figure 1, MCM7 expression was increased in all the E4-positive areas, being present in the basal layer and above (see especially Figure 4B). This staining decreased following the onset of E4 expression, although a region of overlap was well apparent where E4-MCM7 double-positive cells were found.

A clear area of positivity was also found in the adjacent pathological epithelium of a SCC, as reported in Figure 5. Here, the cytopathic effect is remarkable and unequivocally similar to what is generally found in intraepidermal precursor lesions from EV patients. Many E4-positive cells are present, as well as superficial L1-positive nuclei. Unfortunately, although performed and present, we are not able to show FISH-positivity with HPV24 because this slide turned out to be very fragile and got severely damaged during processing.

In addition, the H&E staining of all the tissue sections which were negative for viral markers were carefully screened for the signs of any cytophatic effects, while none observed.

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Discussion

To our knowledge, this is the first study in which the association between skin lesions and β-HPV infection has been assessed at both the DNA level by PCR and at the protein level by immunofluorescence analysis. The main goal of this study was to determine whether detection of β-HPV gene products, as defined in skin tumors from EV patients, could also be observed in lesions from KTRs. We chose to compare the KTR setting with EV because they share the following two main features: i) a status of chronic immunosuppression, which may favor persistence or reactivation of latent skin-tropic viruses including β-HPV; and ii) the high risk of developing multiple skin cancers.[9,10,12] These commonalities are also valid for patients affected by primary immunodeficiencies in general.[51] In addition, in this study the EV model served as an important tool for optimizing the staining procedure with anti-E4 and L1 antibodies, and was used as a positive control when examining KTR tissue sections. A polyclonal antibody recognizing the E4 protein from species 1 was used alongside a newly generated polyclonal antibody raised against a highly conserved region of the L1 protein from the β genus. In addition, to increase the chances of success, the biopsy blocks containing the normal surrounding areas of the tumors were also processed for the viral markers for a total of 111 FFPE corresponding to 79 skin lesions abscised from 17 KTRs.

The initial PCR analysis demonstrated that β-HPV-DNA was highly present in our specimens, with 94 blocks (85%) resulting positive. Multiple infections were observed in 41 lesions (52%), and at least one genotype belonging to the β1 species was found in 79 of the 111 blocks analyzed by PCR (71%).

When we analyzed the same specimens by immunofluorescence, 6 blocks showed areas of positivity corresponding to 4 AK, and the adjacent pathological epithelium of 1 BCC, and 1 SCC. The E4-positive areas were found in the context of the disorganized epithelium in the AK lesions, while in the more advanced tumors, such as SCC and BCC, they were always localized to the adjacent pathological epithelium. In all the specimens, the viral E4 protein displayed the expected

cytoplasmic localization in the middle-superficial layers of the epithelium accompanied by an increase of the cellular proliferation marker MCM7 in the basal and suprabasal layers. The disappearance of E4-positivity in the adjacent normal epithelium constantly overlapped with a reduction in MCM7 expression, which was restricted to the basal layer. These findings support the hypothesis that β-HPV replication drives the cells above the basal layer to enter the cell-cycle in order to facilitate the amplification of its genome. Consistent with this observation, FISH-positive nuclei for the viral genome were found in some of the E4-positive cells. As further proof that the viral life cycle was being completed, all the E4-positive areas showed expression of the major coat protein (L1), which also occurred in a subset of E4-positive cells in the upper layers. This is fully consistent with what has been seen with other PV types, where the onset of vegetative viral genome amplification coincides closely with the expression of cytoplasmic E4 and nuclear L1 in some very superficial cells during productive infection.[49,50,52] In one highly differentiated AK, the number of L1 positive nuclei was very high and most of them were in the thick layers of parakeratosis.

A common trait of cancers with a well-documented viral etiology is their high incidence in immunosuppressed individuals.[5-9] Since skin cancer is the most prevalent tumor in the OTR setting, one would expect that viruses may contribute to this well documented increased susceptibility and incidence. The discovery and detection of β-HPV-DNA and its active infection in EV patients provided an important new insight into the viral oncogenesis of skin cancer which might be extendable to other subsets of patients including other primary immunodeficiencies (PIDs) and particularly long-lasting iatrogenically immunosuppressed patients, such as OTRs. Despite many efforts, direct evidence of active β-HPV infection in tumors from non-EV patients were still missing and the major criticism against a direct involvement of these viruses in skin cancer in the general population is that they do not seem to be maintained in high-grade tumors, such as SCC. To date, the evidence suggesting a causal role of β-HPV in skin cancer has been tainted by: the finding that viral DNA is also found in normal skin, that discovery the viral genome copy number is usually

much less than one viral genome per tumor cell, and the fact that viral transcripts have not been identified by high-throughput mRNA sequencing.[19,53-56] Alongside the fact that ubiquitous β-HPV DNA carriage does not necessarily indicate active infection, these issues have created and so far maintained a state of uncertainty about the causative role of β-HPVs in non-EV skin cancer. Although the present study does not directly demonstrate a causal role of these viruses, the detection of E4 and L1 positivity in i) AK, which are widely regarded to be SCC precursors or in situ carcinoma, and ii) the adjacent pathological epithelium of 1 SCC and 1 BCC, clearly shows that β-HPV are actively replicating in the lesional skin of OTRs and can therefore cooperate with other carcinogenic agents, such as UVB, favoring skin cancer promotion and progression.

Our data are fully consistent with the model proposed by Weissenborn et al. in 2005,[54] who demonstrated that the highest β-HPV loads are present in AK, rather than in SCC, suggesting that their persistence may not be necessary for the maintenance of the malignant phenotype. Instead, they may act as a co-factor that enhances the carcinogenic potential of UV damage, as shown by Wallace et al.,[57] who also demonstrated that β-HPV E6 expression can enhance the carcinogenic potential of UV exposure by promoting p300 degradation.

If β-HPV infections play a role in skin cancer, then they must do so transiently and very likely upon reactivation or persistence in the immunocompromised host. Our findings, showing active β-HPV infection in precancerous lesions and in the vicinity of malignant tumors, are fully compatible with a role of these viruses in the early steps of skin carcinogenesis in at least the immunosuppressed setting. These data are consistent with the "hit and run" mechanisms of carcinogenesis, with cutaneous HPV being possibly important for tumor initiation and progression but not necessary for tumor maintenance.[58,59] Consistent with a causal role in the early stage of the disease, E4-positivity was clearly visualized in an AK that was localized adjacent to a SCC in the neck region. In addition, increased MCM7 expression that extended into the upper epithelial layers was a common feature of all the E4-positive areas, indicating that cells were driven into the cell

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cycle in areas of productive viral infections.[23,49,50] The observed stimulation of basal cell proliferation may contribute, in association with other transforming agents, such as UVB irradiation, to the transformation process without necessarily being maintained in the more advanced disease.

Assuming that viral replication is more active at very early stages of carcinogenesis and perhaps when lesions are not yet even clinically evident, thus it is reasonable to propose that when lesions are removed, especially in more advance stages, only some residual areas are detectable that exhibit viral replication. It is also worth mentioning that the areas of positivity were usually found in lesions which were originally split in more than one FFPE blocks. This procedure is currently applied when the surgical specimen is quite big and the different areas of the lesion are macroscopically evident as it is occurring when the erythematosus area surrounding the tumor is also surgically removed. This can also be defined as field cancerization. These assumptions can explain the low percentage of positivity found in this kind of study and the need of more thorough investigation of the perilesional skin where the likely-hood of finding active β–HPV infection should be higher.

In summary, our work shows that careful detection of β-HPV gene products at the single cell level allows us to visualize their sites of replication, especially in intraepidermal precursor lesions and the marginal zones of more advanced disease. However, these findings require further research to improve our knowledge of β-HPV natural infection and reactivation which will help to understand how it is influenced by the epithelial site, UVB exposure, and the immune system.

Disclosure/Conflict of interest

The authors declare no conflict of interest.

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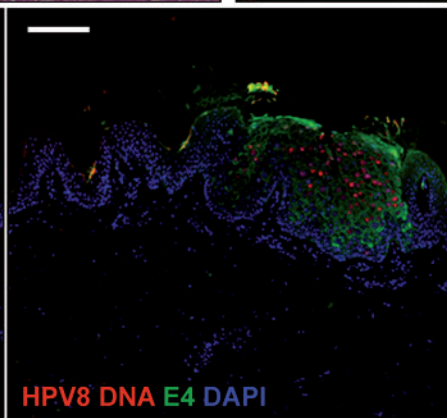
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Figure legends

Figure 1. Distribution of the viral and cellular markers E4, L1, HPV8 DNA, and MCM7 in a wart-like lesion from an EV patient (elbow). The top images show the scan of the H&E histological staining (left) and IF staining (MCM7 in red; E4 in green) (right) of the same tissue section. The white dotted line indicates the basal layer. In the lower panel, the above mentioned MCM7/E4 staining is reproduced (left picture) and serial sections were double stained for the presence of viral genome amplification by HPV8 DNA-FISH (red) and for E4 expression (green) (middle picture); and stained with antibodies to L1 (red) (right picture). The region shown corresponds to the red square highlighted in the H&E image. The white arrows indicate nuclear L1 staining. All sections were counterstained with DAPI (blue) to visualize cell nuclei. Scale bars: 100 μm.

Figure 2. Distribution of the viral and cellular markers E4, L1, and MCM7 in a basal cell carcinoma (BCC) from a KTR (patient 5, neck). The top picture shows the scan of the tissue section using H&E staining (scale bar: 1000 μm). The blue circle shows the BCC and the red square indicates the area of interest in the adjacent epithelium. The region shown in the lower panels (scale bars: 100 μm) corresponds to the red square highlighted in the overall H&E image, magnified in the left hand lower panel. In the middle picture, the same section was double stained using antibodies to E4 (green) and MCM7 (red), and a serial section was stained with antibodies to L1 (red) (right picture). The white arrows indicate nuclear L1 staining. All sections were counterstained with DAPI (blue) to visualize cell nuclei.
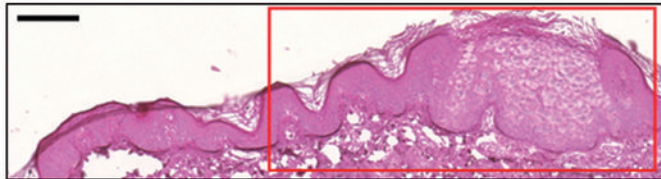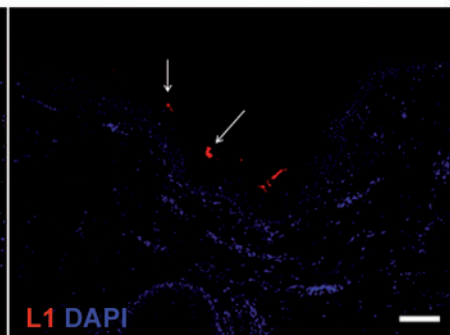
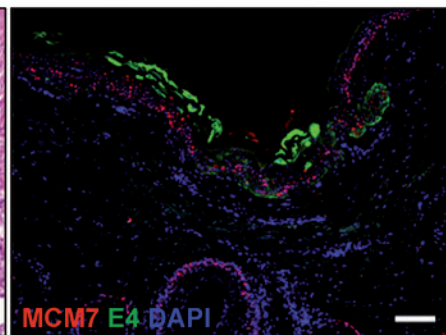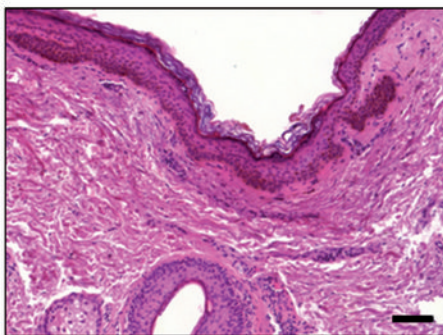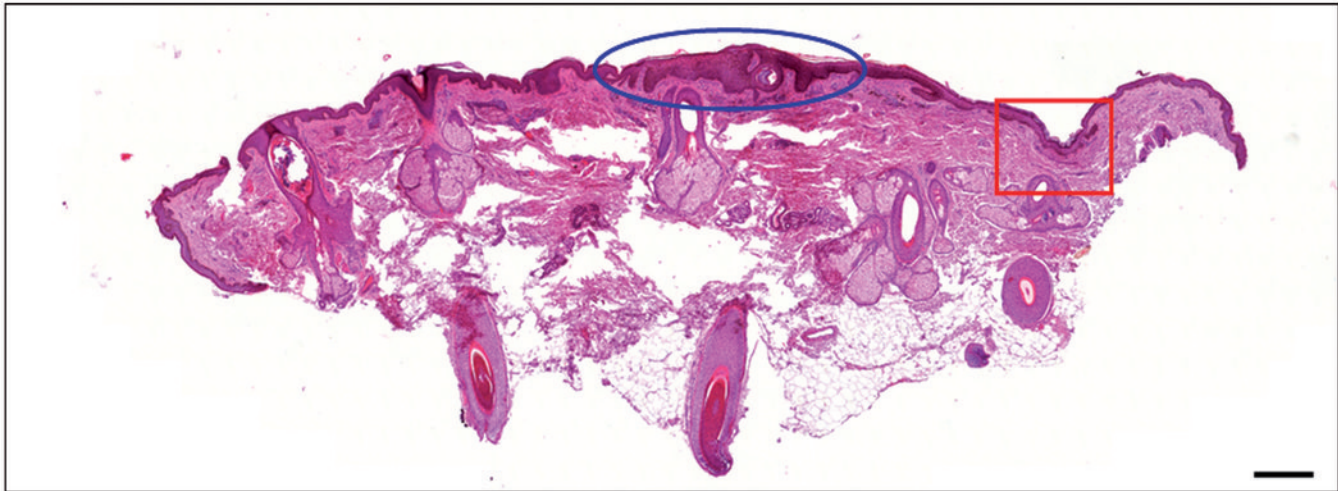**Figure 3. Distribution of the viral and cellular markers E4, L1, and MCM7 in a bowenoid actinic keratosis (AK) from a KTR (patient 8, neck).** The top pictures (scale bars: 1000 μm) show the scan of the tissue sections using H&E staining; the right hand panel shows an AK with the

23

area of interest boxed in red and the left panel shows a nearby squamous cell carcinoma (SCC). The region shown in the lower panels corresponds to the red square highlighted in the H&E image, magnified in the left hand lower picture. The same section was double stained using antibodies to E4 (green) and MCM7 (red) (middle picture) and a serial section was stained with antibodies to L1 (red) (right picture). The white arrows indicate nuclear L1 staining. All sections were counterstained with DAPI (blue) to visualize cell nuclei. Scale bars: 50 µm.

Figure 4. Distribution of the viral and cellular markers E4, L1, HPV25 DNA, and MCM7 in three cases of hypertrophic actinic keratosis (AK) from a KTR (patient 16, A. hand; B. face; C. hand). A: The top picture (scale bar: 1000 µm) shows the scan of the tissue section of the first AK using H&E staining. The region shown in the lower panels corresponds to the red square highlighted in the over H&E image, reproduced in the lower left hand picture. The same section was double stained using antibodies to E4 (green) and MCM (red) (second picture); serial sections were double stained for the presence of viral genome amplification by HPV25 DNA-FISH (red) and for E4 expression (green) (third picture); and also stained with antibodies to L1 (red) (fourth picture). The white arrows indicate nuclear L1 staining. All sections were counterstained with DAPI (blue) to visualize cell nuclei. Scale bars: 50 µm) **B:** The top pictures (scale bars: 1000 µm) show the scan of the tissue sections (using H&E staining) of the second AK (left picture) and its edge (right picture). The right handed middle panel shows the histology (H&E staining) of the area from the edge where viral markers were expressed (red square) and an overall picture of the IF staining (E4 in green; MCM7 in red) of the same tissue section (scale bars: 200 µm). The white dotted line indicates the basal layer. In the lower panel, the above MCM7/E4 staining is reproduced (left picture) and serial sections were stained with antibodies to L1 (red) (right picture). The white arrows indicate nuclear L1 staining. Scale bars: 100 µm. All sections were counterstained with DAPI (blue) to visualize cell nuclei. **C:** The top picture shows the scan of the tissue section of the

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third AK using H&E staining (scale bar: 1000 µm). The black rectangle shows a magnification (scale bar: 400 µm) of the positive parakeratotic epithelium that abruptly changed to normal orthokeratosis on both sides. The region shown in the lower panels corresponds to the red square highlighted in the H&E image, reproduced in the lower left hand picture. The same section was double stained using antibodies to E4 (green) and MCM7 (red) (middle picture) and a serial section was stained with antibodies to L1 (red) (right picture). Scale bars: 50 µm). All sections were counterstained with DAPI (blue) to visualize cell nuclei.
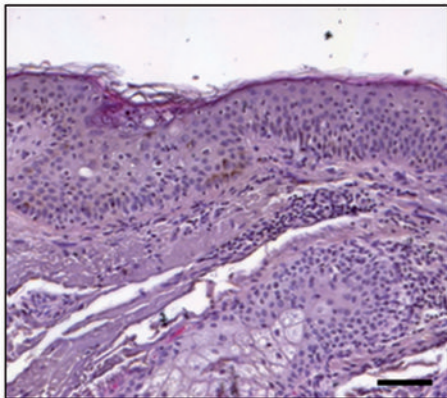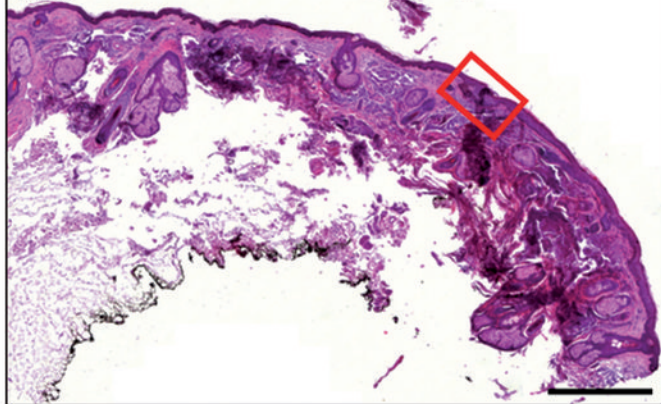
Fig 5. Distribution of the viral and cellular markers E4, L1, and MCM7 in the adjacent epithelium of a SCC from a KTR (patient 17, ear). The top pictures shows the scan of the tissue sections (H&E staining) of the SCC (left picture; scale bar: 500 µm) and its edge (right picture; scale bar: 1000 µm). In the lower panel, the same sections were stained using antibodies to E4 (green) (middle picture) and serial sections were stained with antibodies to L1 (red) (right picture). The region shown corresponds to the red square highlighted in the H&E image. Scale bars: 50 µm). All sections were counterstained with DAPI (blue) to visualize cell nuclei. The inset in the lower left picture shows the cytopathic effects typical of a productive β-HPV infection (scale bar: 20 µm).
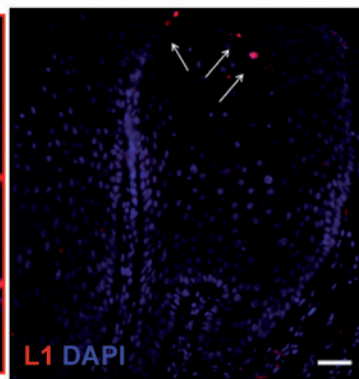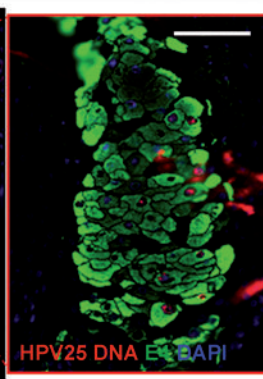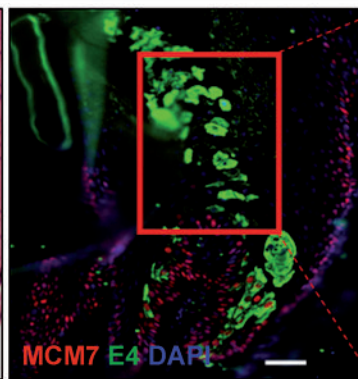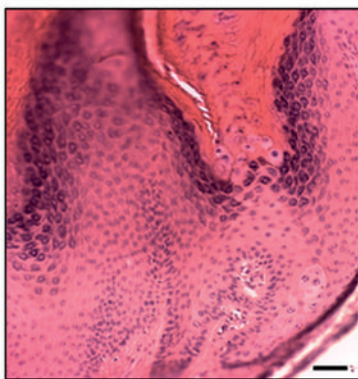
MCM7 E4

MCM7 E4 DAPI

HPV8 DNA E4 DAPI

L1 DAPI
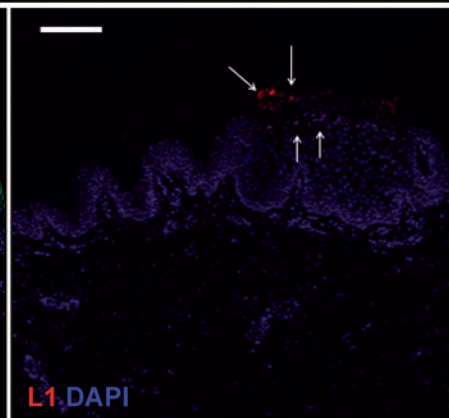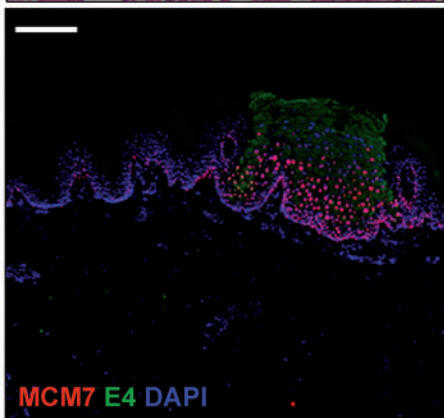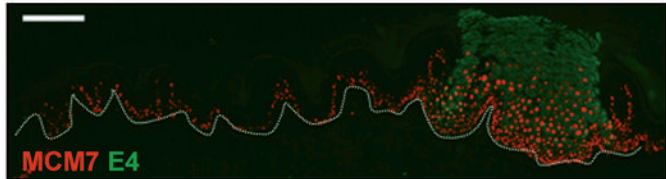
Borgogna et al., Figure 1

MCM7 E4 DAPI

L1 DAPI

Borgogna et al., Figure 2

SCC AK

MCM7 E4 DAPI

L1 DAPI

Borgogna et al., Figure 3

MCM7 E4 DAPI

HPV25 DNA E4 DAPI

L1 DAPI

Borgogna et al., Figure 4A

AK

Edge

MCM7 E4

MCM7 E4 DAPI

L1 DAPI
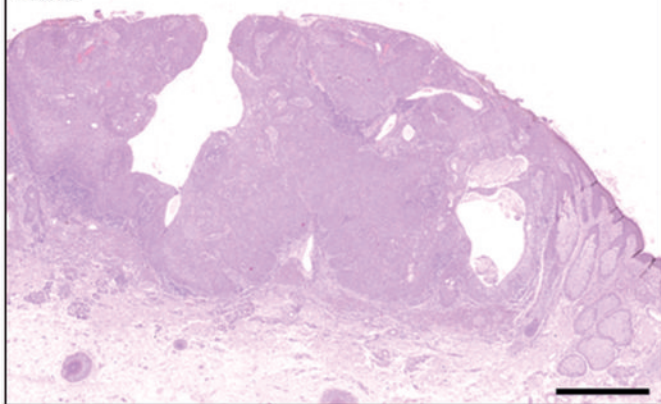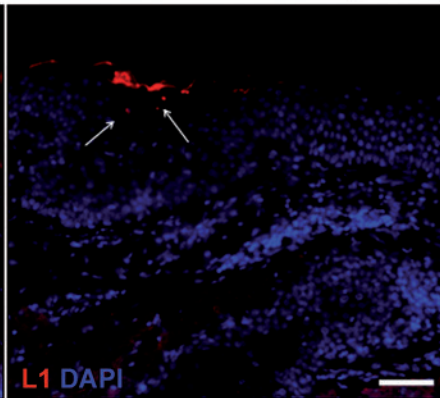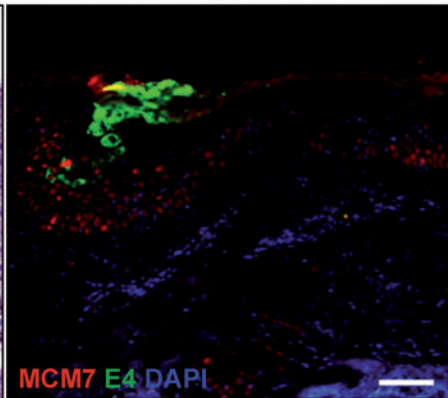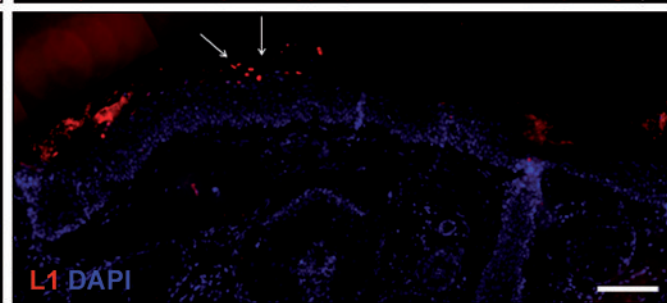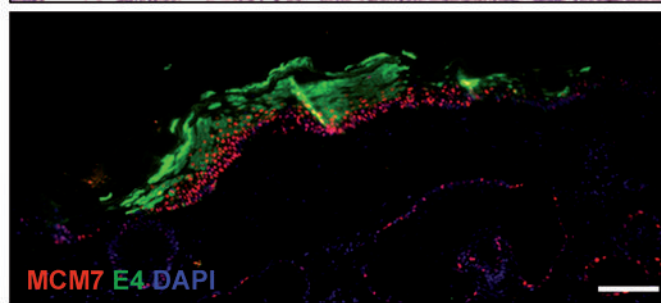
Borgogna et al., Figure 4B

MCM7 E4 DAPI

L1 DAPI

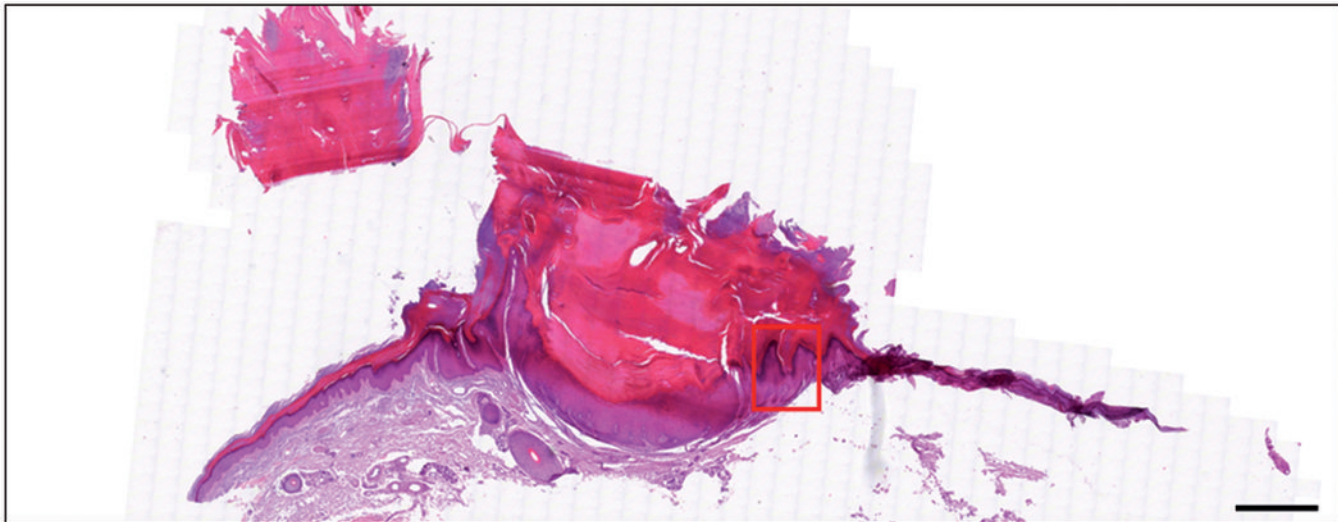**Borgogna et al., Figure 4C**
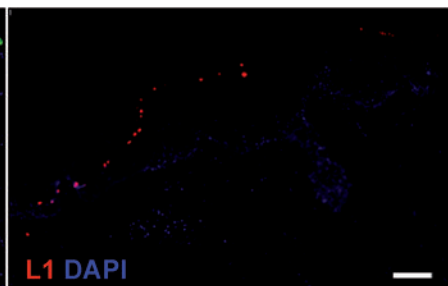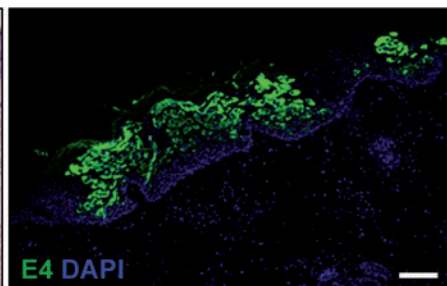
Borgogna et al., Figure 5

Table 1 Baseline characteristics of the study cohort of kidney transplant recipients (KTRs)

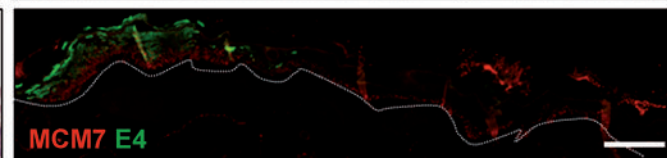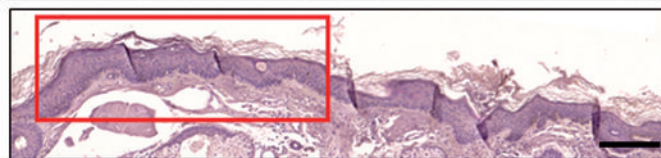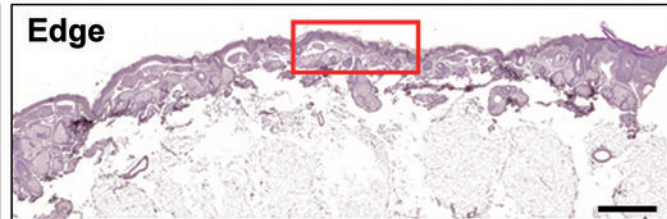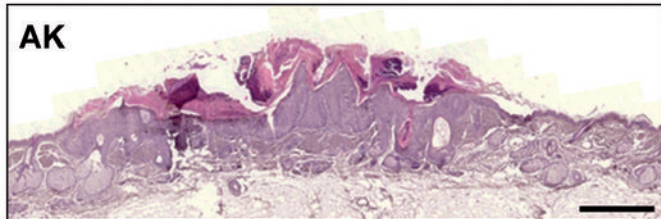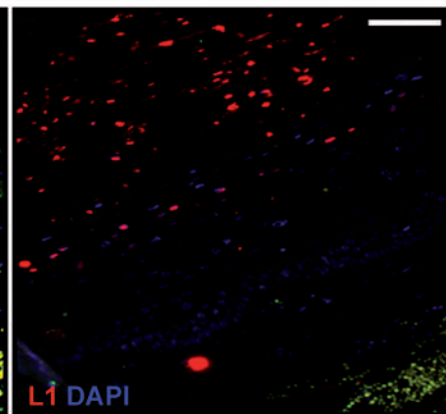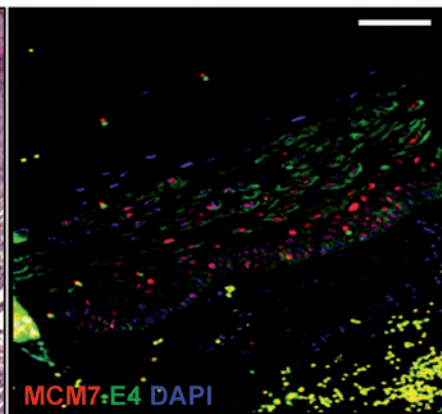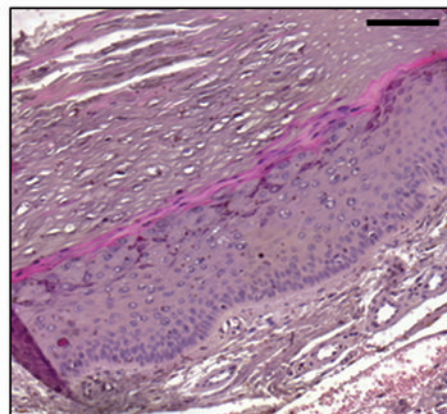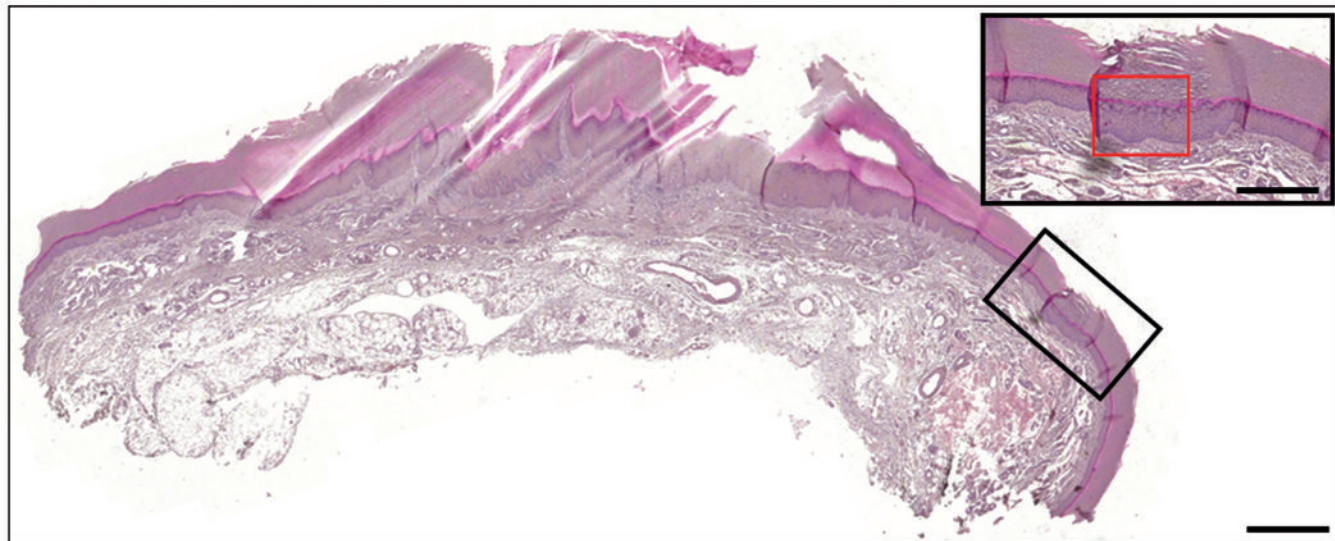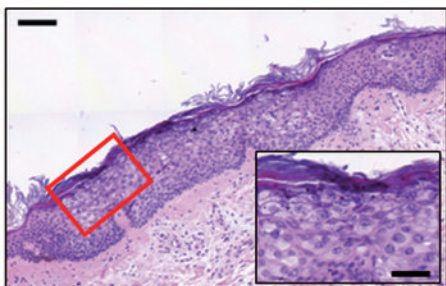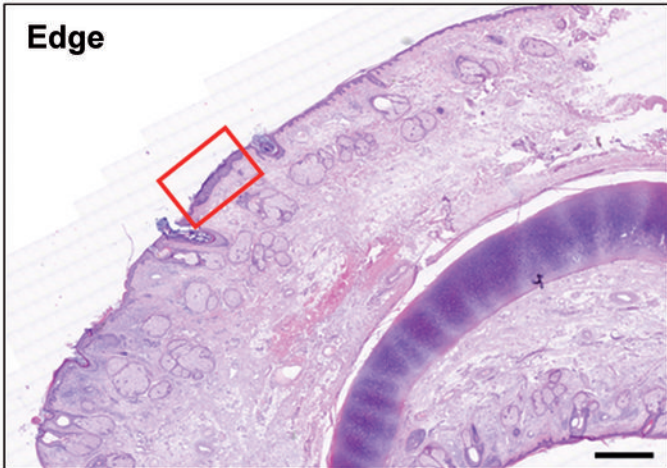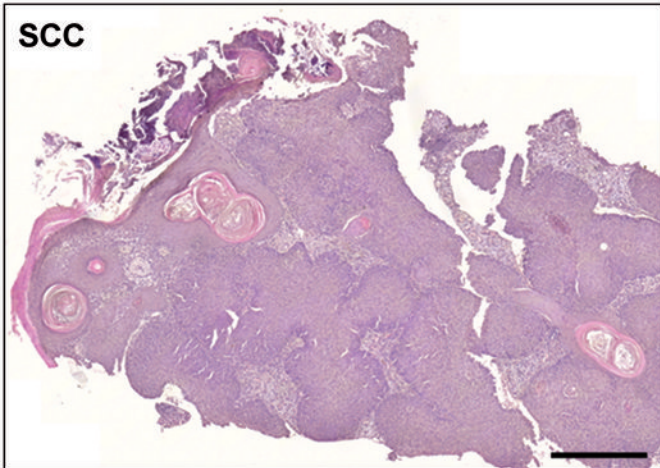| Patients	Birth date	Tx date	Years after transplantation																										Follow Up (years)	Total n. of Lesions
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	21	22	25	27	28	29	31	32		
1 M	1961	2005 K				BCC*																								8	1
2 F	1946	1995 K												SCC																18	1
3 M	1952	1996 K					BCC																							17	1
4 M	1939	1994 K											BCC*																	19	1
5 M	1952	2002 K					BCC*																							11	1
6 M	1940	1988 K																		SK*										25	1
7 M	1943	2009 K		AK*																										4	1
8 M	1938	2005 K						AK* SCC*																						8	2
9 M	1940	2004 K	AK*					AK* SCC*																						9	2
10 M	1941	1988 H 1996 H 2006 K																		AK* SCC*										25	2
11 M	1932 2008†	1992 K				SCC*										BD* BCC*		BCC												16	4
12 M	1969	1992 K										2BCC* BCC*					SK													21	4
13 M	1934	1981 K												SK BCC										SK BCC		KA	KA*	SCC*	2KA* KA		32	8
14 M	1940	2000 K						AK* SCC*	3BCC* 2BCC*			BCC	SK* BCC	BCC																13	10
15 M	1946	2005 K	BCC	BCC		BCC*		SK 2BCC 2SCC*	AK* 2BCC* BCC																					8	12
16 M	1948 2005†	1993 K			AK*			2AK* 2AK* 3SCC*		AK* KA*	2AK*		AK* KA*																	12	14
17 M	1940	1999 K	BCC*	SCC*			1SK* SCC* 2SCC*	SK* 3AK*				2BCC* BCC*	BCC*	BCC*																14	14

SK: Seborrheic keratosis; KA: Keratoacanthoma; BD: Bowen's disease; BCC: Basal cell carcinoma; AK: Actinic keratosis ; SCC: Squamous cell carcinoma

*: Lesions in sun-light exposed body sites

Table 2 Beta HPV DNA distribution in the 111 formalin fixed paraffin embedded (FFPE) blocks obtained from the 79 lesions of the kidney transplant recipients (KTRs) cohort

Lesions	Total n = 79			SK n = 7			KA n = 7			BD n = 1	BCC n = 31			AK n = 19			SCC n = 14		
	FFPE blocks n = 111			FFPE blocks n = 8			FFPE blocks n = 13			FFPE blocks n = 1	FFPE blocks n = 44			FFPE blocks n = 25			FFPE blocks n = 20		
Beta HPV	Whole Lesion n = 51	Core n = 28	Edge n = 32	Whole Lesion	Core	Edge	Whole Lesion	Core	Edge	Whole Lesion	Whole Lesion	Core	Edge	Whole Lesion	Core	Edge	Whole Lesion	Core	Edge
Positive	43	25	26	5	1	1	1	5	6	1	17	8	8	11	5	6	8	6	5
Genotype not defined*	4/43	3/25	1/26	1/5							1/17	1/8	1/8		1/5		2/8	1/6	
Negative	8	3	6	1					1		4	2	5	2	1		1		

SK: Seborrheic keratosis; KA: Keratoacanthoma; BD: Bowen's disease; BCC: Basal cell carcinoma; AK: Actinic keratosis; SCC: Squamous cell carcinoma.

*: unspecified β-HPV genotype (see M&M)

Table 3 β-HPV genotyping analysis of each FFPE block obtained from the four patients with lesions positive for the β-HPV E4 and L1 proteins (IF-positives)

Patients	Birth Date	Tx date	Lag	1st	Lag	2nd	Lag	3rd	Lag	4th	Lag	5th	Lag	6th	Lag	7th	Lag	8th	Lag	9th	Lag
5 M	1952	2002 K	5	**BCC** **WL** **8† (E4/L1)**																		
8 M	1938	2005 K	6	SCC WL 5, 23, 36	6	**AK** **WL** **8, 12, 36 (E4/L1)**															
16 M	(1948 2005†)	1993 K	3	AK WL 5, 19, 24	6	SCC E: 5 | C: 5	6	AK WL 5	6	SCC WL 5	6	AK WL 15, 75	6	**AK** **WL** **25 (E4/L1)**	6	SCC WL 5, 15	6	**AK** **E: 36, 75 (E4/L1)** | **C: 25**	8	**AK** E: 5, 9 | **C: 8, 14, 19 38, 75 (E4/L1)**	8
17 M	1940	1999 K	1	BCC E: Pos | C: Pos	2	SCC E: 5 | C: 5	5	**SCC** E1: 5, 9 | **E2: 24 (E4/L1)** | C: 38	5	SK WL 5, 9, 24	5	SCC WL 5, 8, 80	5	SCC WL Pos	6	AK WL Neg	6	SK WL 8	6	AK WL 9, 24	6

Tx: transplant; Lag: years since transplantation; SK: Seborrheic keratosis; KA: Keratoacanthoma; BD: Bowen's disease; BCC: Basal cell carcinoma; AK: Actinic keratosis ; SCC: Squamous cell carcinoma;

WL: Whole Lesion; E: Edge; C: Core; E1: Edge 1; E2: Edge 2

†: β-HPV genotype; Pos: unspecified β-HPV genotype (see M&M)

Table 3 (Continued)

Patients	Birth Date	Tx date	10th		Lag	11th		Lag	12th			Lag	13th	Lag	14th		
5 M	1952	2002 K															
8 M	1938	2005 K															
16 M	(1948 2005†)	1993 K	AK		9	KA		9	AK			11	KA	11	AK		
			E	C		E	C		E	C			WL		WL		
			80	5, 24		5, 38, 80	5		5, 38, 80	5			5		5		
17 M	1940	1999 K	AK		10	BCC		10	BCC			11	BCC	12	BCC		
			E	C		WL			E1	E2	C		WL		E1	E2	C
			Neg	Pos		5, 38			80	5, 8, 9, 38	38		5, 38		Neg	5, 38, 80	5, 8

Tx: transplant; Lag: years since transplantation; SK: Seborrheic keratosis; KA: Keratoacanthoma; BD: Bowen's disease; BCC: Basal cell carcinoma; AK: Actinic keratosis ; SCC: Squamous cell carcinoma;

WL: Whole Lesion; E: Edge; C: Core; E1: Edge 1; E2: Edge 2

†: β-HPV genotype; Pos: unspecified β-HPV genotype (see M&M)