ORIGINAL ARTICLE

Occurrence of newly discovered human polyomaviruses in skin of liver transplant recipients and their relation with squamous cell carcinoma *in situ* and actinic keratosis – a single-center cohort study

Yilin Wang[1], Anne Keinonen[2], Sari Koskenmies[2], Sari Pitkänen[2], Nanna Fyhrquist[3,4], Mohammadreza Sadeghi[1,5], Heikki Mäkisalo[6], Maria Söderlund-Venermo[1] & Klaus Hedman[1,7]

1 Department of Virology, University of Helsinki, Helsinki, Finland
2 Skin Cancer Unit, Department of Dermatology, Helsinki University Hospital, Helsinki, Finland
3 Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden
4 Department of Bacteriology and Immunology, University of Helsinki, Helsinki, Finland
5 Department of Virology, University of Turku, Turku, Finland
6 Organ Transplantation and Liver Surgery Unit, University of Helsinki, Helsinki, Finland
7 Helsinki University Hospital, Helsinki, Finland

Correspondence
Yilin Wang MSc, Department of Virology, University of Helsinki, Haartmaninkatu 3, FI-00290 Helsinki, Finland.
Tel.: +358 417051619;
fax: +358 2941 26491;
e-mail: yilin.wang@helsinki.fi

SUMMARY

To date 14 human polyomaviruses (HPyVs) have been identified. The newly found HPyVs have not been examined with regard to post-transplant skin carcinogenesis. To determine the occurrences in skin and possible pathological associations of the HPyVs, we studied their geno-prevalences in squamous cell carcinoma (SCC) in situ or actinic keratosis and benign skin in liver transplant recipients (LiTRs); and of healthy skin in immunocompetent adults. We used highly sensitive and specific HPyV PCRs of two types. Overall, Merkel cell polyomavirus (MCPyV), human polyomavirus 6 (HPyV6), human polyomavirus 7 (HPyV7), trichodysplasia spinulosa polyomavirus (TSPyV), and Lyon IARC polyomavirus (LIPyV) were found in 58/221 (26.2%) skin biopsies. MCPyV DNA was detected in 5/14 (35.7%) premalignant vs. 32/127 (25.2%) benign skin of LiTRs, and in 12/80 (15%) healthy skin of immunocompetent adults, with no statistically significant difference in viral DNA prevalence or load. TSPyV DNA was found in a single skin lesion. LIPyV, HPyV6 and HPyV7 DNAs occurred exclusively in benign skin. Overall, the viral findings in premalignant versus benign skin were alike. The occurrences of HPyVs in skin of LiTRs and immunocompetent individuals speak against a role for any of the 14 HPyVs in SCC development.

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Key words
actinic keratosis, cancer, human polyomavirus, immunosuppression, post-transplant, squamous cell carcinoma

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Introduction

Human polyomaviruses (HPyVs) are ubiquitous DNA viruses, with high seroprevalences in the general population [1]. The first two HPyVs, BK virus (BKPyV) and JC virus (JCPyV) were discovered in 1971; BKPyV was found in urine of a kidney transplant recipient and JCPyV in brain tissue of a patient with progressive multifocal leukoencephalopathy [2,3]. Over the past 11 years, 12 new HPyVs have been identified: KI and

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WU polyomavirus (KIPyV, WUPyV) in the respiratory tract of children [4,5]; Merkel cell polyomavirus (MCPyV) in the tumor of Merkel cell carcinoma patients [6]; trichodysplasia spinulosa polyomavirus (TSPyV) in hair and skin biopsies of a transplant recipient with trichodysplasia spinulosa [7]; human polyomavirus 6 (HPyV6) and human polyomavirus 7 (HPyV7) in normal skin of healthy volunteers [8]; HPyV9 in sera of kidney transplant recipients [9]; HPyV10 in fecal sample from children with acute gastroenteritis [10]; HPyV11 in stool of a healthy child [11]; HPyV12 in liver and gastrointestinal tissue of patients with malignant diseases [12]; HPyV13 in epithelial cells of a pancreatic transplant patient [13]; and the latest virus Lyon IARC polyomavirus in skin swab and oral gargles of cancer-free individuals (LIPyV, 2017) [14].

While several HPyVs have been found in skin of asymptomatic individuals (e.g., MCPyV, HPyV6, HPyV7, TSPyV, HPyV9) [8,15,16], some can cause severe disease especially in the immunocompromised: MCPyV is the causative agent of the corresponding carcinoma [6]; as is the TSPyV of the TS disease of skin [17]; HPyVs 6 and 7 appear to be associated with pruritic dermal dyskeratinizations [18,19]. HPyV9 has not been associated with any disease.

Cutaneous squamous cell carcinoma (SCC) is an epithelial skin cancer occurring in organ transplant recipients 65–250 times more frequently than in the general population [20,21]. Its pathogenesis is multifactorial, including advanced age, ultraviolet exposure, and viruses such as human papillomavirus [22]. Actinic keratosis (AK) and SCC *in situ* (SCCis), the precursors of invasive SCC, constitute a risk of malignant transformation especially in immunosuppression [23]. Thus, identification of factors of pathogenic potential in these premalignant lesions is important for understanding the etiology of SCC.

The associations of newly found HPyVs with clinical diseases in immunosuppressed individuals have not been fully explored. Especially the new ones discovered after TSPyV lack studies with regard to post-transplant (post-tx) skin carcinogenesis. As the incidences of SCC pre-stages are increased after solid organ transplantation [23], we wanted to determine the genoprevalences of HPyVs in biopsies of premalignant lesional versus non-lesional skin in liver transplant recipients (LiTRs) followed long post-tx; as well as of healthy skin in immunocompetent individuals. To this end, we used bead-based PCRs and quantitative real-time PCRs (qPCRs) for detection of the viral DNAs in fresh-frozen skin biopsies.

Materials and methods

Study population

Liver transplant recipients

Altogether 126 adult LiTRs were included (Fig. 1). All the LiTRs had been recruited for follow-up skin examination at Helsinki University Hospital between October 2012 and December 2016. The examinations were conducted and documented by dermatologists of the Dermatology Unit. Any premalignant lesions were diagnosed histologically. AK is defined as keratinocytic atypia involving the upper layers of epidermis; and SCCis is defined as full-thickness epidermal dysplasia. Of the LiTRs, 12 [median age at diagnosis 68 years, median post-tx (first, if repeated) time 11 years] had SCCis or AK; the remaining 114 (median age at diagnosis 62 years, median post-tx time 10 years), did not.

Altogether, 14 punch biopsies (of 5 mm) were collected from lesional sites and 127 from non-lesional sites (Fig. 1) and were stored at −70 °C. At least one biopsy of benign skin was taken from each LiTR. Additionally, sera were available from 118 LiTRs.

Immunocompetent adults

Immunocompetent asymptomatic adults (n = 80, median age 43 years) participated in epicutaneous testing with irritants. Skin biopsies (4-mm punch) were taken from the backs of all individuals and were stored in *RNA-later* at −80 °C.

Written informed consents were obtained from all subjects, and the tissues and sera were collected and handled in accordance with the ethical rules of the Ethics Committee of Helsinki and Uusimaa Hospital District.

Nucleic acid extraction

All the skin specimens were sliced with disposable scalpels and digested with proteinase K overnight. DNA was isolated with Qiagen DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Standard precautions to avoid contamination were taken. The isolated DNA from each skin was eluted in 60 μl of AE buffer (Qiagen).

The DNA was also isolated from serum of 37 LiTRs with HPyV DNA detectable in biopsies (serum from one patient was not available). The DNA from each serum (200 μl) was extracted with Qiagen DNA blood

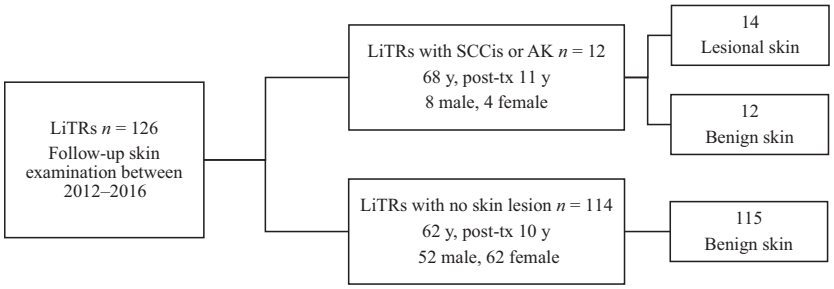

Figure 1 Lesional and benign skin biopsies collected from liver transplant recipients. AK, actinic keratosis; LiTRs, liver transplantation recipients; post-tx, post-transplant; SCCis, squamous cell carcinoma.

mini kit (Qiagen), and the yield was eluted in 100 μl of AE buffer (Qiagen). All DNA extracts were stored at −20 °C.

HPyV detection with Luminex-based PCRs

Viral DNA of each sample was measured using a bead-based multiplex PCR for the first 13 HPyVs [24], and a separate bead-based singleplex PCR for the LIPyV, as described [14] except that the multiplex PCR annealing temperature was 57.5 °C (Appendix S1). This was shown to result in a higher analytical sensitivity compared to the prior approach [24] (Appendix S2: Tables S2–S4). Each run included as positive controls the plasmids of all 14 HPyVs.

Quantification and confirmation of multiplex PCR findings

The samples with positive bead-based PCR findings (MCPyV, HPyV6, HPyV7, TSPyV) were re-examined with the corresponding qPCRs [7,25,26] (Table S1). Samples were considered positive when both the multiplex PCR and the corresponding confirmatory qPCRs were positive. The viral DNA loads were given per million cells, determined with the human house-keeping gene *RNaseP* [27]. The PCR product of the LIPyV-positive sample was purified with Diffinity RapidTip (Sigma-Aldrich, St. Louis, MO, USA) and Sanger-sequenced. The resulting sequences were compared with the reference DNA sequences of the NCBI Entrez Nucleotide database (accession number KY404016 [14]), using NCBI Blast program.

Statistical analysis

Fisher's exact test, Mann–Whitney *U* test, chi-squared test, unpaired nonparametric Kruskal–Wallis test and Dunn's multiple comparison test were performed for conducting comparisons using GRAPHPAD PRISM version 7.00 (GraphPad Software, La Jolla, CA, USA). A *P*-value <0.05 was considered significant.

Results

Liver transplant recipients

Among the 141 skin biopsies from the 126 LiTRs, 47 samples were positive in bead-based PCR, and 45 also in the corresponding qPCRs or sequencing. The other two samples remained negative in qPCRs targeting both MCPyV LT and VP1 regions. Three non-lesion skins were MCPyV-positive in bead-based PCR and VP1 qPCR (with low copy numbers; $<1.7 \times 10^2$ per 10^6 cells), but not in Large T qPCR. *RNaseP* (human house-keeping gene) qPCR showed 10^3–10^5 copies per reaction for all 141 samples, indicating successful DNA isolation and absence of notable PCR inhibition. The overall prevalences of HPyVs in skin biopsies are shown in Table 1.

The dermal occurrences of MCPyV in lesions versus non-lesions were similar, both in detection rates 5/14 (35.7%) vs. 32/127 (25.2%) and DNA loads (lesion mean, 1.2×10^2 per 10^6 cells vs. healthy skin mean, 1.4×10^2 per 10^6 cells), with no significant difference (rates $P = 0.52$, Fisher's exact test; loads $P = 0.18$, Mann–Whitney *U* test). The MCPyV-DNA-positive individuals with lesion(s) versus non-lesion(s) matched in ages (median 69 years vs. 61 years) and post-tx years (median 10 years vs. 10 years). The characteristics of the HPyV-DNA-positive LiTRs with premalignant lesions are given in Table 2. A single patient (P-070) presented with MCPyV in lesion but not in healthy skin (Table 2).

Trichodysplasia spinulosa polyomavirus DNA was detected (at merely 1.1 copies per 10^6 cells) in one skin lesion in a LiTR (P-016) who had another SCCis in his chest. The latter specimen as well as this patient's healthy skin were negative for all HPyVs.

Table 1. HPyVs genoprevalence in lesion and non-lesion skin in liver transplant recipients and healthy adults.

	HPyV DNA in skin biopsies		
	Liver transplant recipients		
Virus	Lesional $n = 14$	Benign $n = 127$	Immunocompetent adults Healthy $n = 80$
MCPyV	5 (35.7%)	32 (25.2%)	12 (15%)
HPyV6	–	5 (3.9%)	2 (2.5%)
HPyV7	–	1 (0.8%)	–
TSPyV	1 (7.1%)	–	–
LIPyV	–	1 (0.8%)	–

HPyV, human polyomavirus; HPyV6, human polyomavirus 6; HPyV7, human polyomavirus 7; LIPyV, Lyon IARC polyomavirus; MCPyV, Merkel cell polyomavirus; TSPyV, Trichodysplasia spinusa polyomavivus.

Human polyomavirus 6 and HPyV7 DNAs were present in only non-lesion skin, in 5/127 (3.9%) and 1/127 (0.8%), respectively (Table 1). The viral loads of the two were similar (HPyV6 2.1×10^3 per 10^6 cells vs. HPyV7 7.7×10^3 per 10^6 cells). These LiTRs were alike in age (HPyV6 median 55 years, range 45–76 years vs. HPyV7 75 years) and post-tx years (median 8 years vs. 9 years).

Lyon IARC polyomavirus was repeatedly positive in a single biopsy of a LiTR with no premalignant lesions (52 years female, 14 years post-tx). The bead-based 155-bp PCR product was confirmed by sequencing, showing 100% identity to the reference LIPyV genome (KY404016).

Human polyomaviruses co-infection was seen in two individuals with no premalignant lesion (addressed also above): (i) HPyV6 and MCPyV in a 45 years male (5 years post-tx); (ii) LIPyV and MCPyV in a 52 years female (14 years post-tx).

Overall, HPyV DNA was found in biopsies of 38 LiTRs including five patients with pre-stage SCC. Except for two patients with HPyV DNA present exclusively in pre-stage SCC, in all other 10 pre-stage SCC patients the viral DNA findings in premalignant and healthy tissues were alike.

Of note, the DNA of any HPyV was undetectable in serum of any biopsy-positive LiTR (one serum was unavailable).

Immunocompetent adults

In total, 14 of 80 skin biopsies were HPyV DNA positive by both multiplex PCR and the corresponding qPCRs. Of the 80 skin biopsies, 12/80 (15%; median age 48 years) contained MCPyV DNA, and 2/80 (2.5%; median age 54 years) contained HPyV6 DNA (Table 1). A 52-year female had MCPyV-HPyV6 co-infection. All the copy numbers were low (MCPyV mean, 65 copies per 10^6 cells vs. HPyV6 mean, 2.1×10^4 copies per 10^6 cells).

Statistically, the prevalence and loads of HPyV6 DNA in skin biopsies from LiTRs versus healthy adults were equal (prevalence $P = 1$, Fisher's exact test; load $P = 1$, Mann–Whitney U test). The same held for MCPyV DNA in premalignant versus benign skin of either LiTRs or healthy adults (prevalence $P = 0.1$, chi-squared test; load $P = 0.37$, unpaired nonparametric Kruskal–Wallis test and Dunn's multiple comparison test).

Discussion and conclusion

This is the first comprehensive study on the DNA prevalences of the 14 HPyVs presently known, in any clinical material. We examined the HPyV DNAs in premalignant lesional versus non-lesional skin biopsies of post-tx patients and immunocompetent individuals. To ensure genome preservation we used fresh-frozen rather than formalin-fixed paraffin-embedded tissues. The bead-based PCRs performed at an annealing temperature of 57.5 °C were highly sensitive with a limit of detection of five copies/reaction for each of the HPyVs in both singleplex and multiplex platforms, and also highly specific with no cross amplification between viruses at 5×10^4 copies/reaction (Appendix S2: Tables S2–S4).

Altogether, five HPyVs were encountered. All these HPyVs in premalignant/benign skin occurred in low copy numbers, unlike the same viruses in their associated skin diseases [7,18,19,28]. This is concordant with previous studies [28–30] on MCPyV, HPyV6, HPyV7, TSPyV, and HPyV9 in SCC precursors and SCC, pointing to virus latency or shedding, rather than activation.

Table 2. Clinical characteristics of SCCis/AK patients with HPyV findings.

Patient	Age at diagnosis	Years post-tx	Sex	Immunosuppression	Localization	Skin	HPyVs	Copies/10^6 cells
P-007	62	4	M	Mycophenolic acid	Left arm	Non-lesion	MCPyV	4.9×10^1
					Neck	SCCis	MCPyV	1.6×10^2
P-015	66	14	M	Tacrolimus	Left collarbone	Non-lesion	MCPyV	1.3×10^1
					Left temple	SCCis	MCPyV	6.3×10^1
P-045	83	9	M	Cyclosporine	Collarbone	Non-lesion	MCPyV	1.8×10^2
					Right temple	AK	MCPyV	3.9×10^2
P-070	71	11	M	Cortisosteroid, cyclosporine, mycophenolic acid	Collarbone	Non-lesion	Neg	Neg
					Back	SCCis	MCPyV	4.9×10^2
					Arm	SCCis	MCPyV	7.4×10^1
P-016	66	7	M	Cyclosporine, azathioprine	Collarbone	Non-lesion	Neg	Neg
					Forehead	SCCis	TSPyV	1.1
					Chest	SCCis	Neg	Neg

AK, actinic keratosis; post-tx, post-transplant; SCCis, squamous cell carcinoma in situ.

Of the additional five new HPyVs studied here, only LIPyV was encountered in skin.

The occurrence of MCPyV in the premalignant versus benign skin of either LiTRs or healthy controls was statistically similar, speaking against a role for this carcinogenic virus in SCC development. Previously, HPyV6 and HPyV7 have been found at low prevalence in some SCC patients [28,31]. In our much larger series, HPyV6, HPyV7 were present exclusively in non-diseased skin. That HPyV6 occurred slightly more frequently than HPyV7, is in line with the corresponding prevalences in skin swabs [15], and also with the HPyV6 and HPyV7 seroprevalences in the general population [1]. As in a previous study [29], our low prevalences of HPyV6 and HPyV7 do not point to SCC pathology among LiTRs.

Lyon IARC polyomavirus was discovered in skin of cancer-free individuals [14]. To our knowledge, LIPyV DNA has been searched neither in any cancer nor among the immunocompromised. In our cohort, LIPyV DNA was found in skin tissue of a single LiTR but not in her serum. Based on the low DNA prevalence here as in the original report [14], as well as the low LIPyV seroprevalence [32,33], it is tempting to think over whether this agent belongs to the human virome.

This study covered all the currently known HPyVs, and was focused on their occurrence in SCC premalignant lesions. While speaking against a role for any HPyV in SCC carcinogenesis, further studies with larger materials are warranted, particularly with high-risk SCC because of its aggressiveness. In light of the large number of cancer types and their multifactorial pathogenesis, other risk factors and their combinations with HPyV types could provide additional information on the driving forces of cancer development.

In conclusion, we determined the prevalences of HPyV DNA in skin biopsies from post-tx patients and constitutionally healthy individuals. Our data do not support a role for any of the 14 HPyVs in SCC pathogenesis.

Authorship

YW: carried out the experiments, acquired and analyzed the data, and drafted the manuscript. SK, AK, and SP: contributed to conception, study design, provision of study materials and clinical data. MS: participated in study design and experimentation. HM: directed the liver transplantations and performed some of them. MS-V and KH: designed and coordinated the project, and participated in manuscript writing. All authors

read, revised, and approved the final version of the manuscript.

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Conflicts of interest

The authors have declared no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1. Methods.

Appendix S2. Results.

Table S1. Primer and probe sequences of the reference qPCRs used in this study.

Table S2. Limits of detection of 13 human polyomaviruses in singleplex and multiplex format at an annealing temperature of 57.5 °C.

Table S3. Specificities of 13 type-specific probes employed in multiplex human polyomaviruses genotyping at an annealing temperature of 57.5 °C.

Table S4. Specificities of the multiplex platform with HEK293 cells.

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