Title: Survey of KI, WU, MW and STL polyomavirus in cancerous and non-cancerous lung tissues

Authors: Eszter Csoma[1*], László Bidiga[2], Gábor Méhes[2], Melinda Katona[1], Lajos Gergely[1]

Authors' affiliation:

[1] University of Debrecen, Faculty of Medicine, Department of Medical Microbiology, Hungary

[2] University of Debrecen, Department of Pathology, Hungary

Running head: Study of human polyomaviruses in cancerous and non-cancerous lung samples

[*]**corresponding author:**

Eszter Csoma

Department of Medical Microbiology, Faculty of Medicine, University of Debrecen

Nagyerdei krt. 98., 4032 Debrecen, Hungary

E-mail: csoma.eszter@med.unideb.hu

Tel.: +36 52 255425

ABSTRACT

Background/Aims: The pathogenesis of human polyomavirus KI, WU, MW and STL has not been elucidated yet. Respiratory transmission is suggested, but the site of the replication, tissue and cell tropism is not clarified. KIPyV and WUPyV DNA and/or antigen were detected in normal lung tissues previously by others. In fact, KIPyV DNA sequence was found in lung cancer samples. Up to date, there is no publication about the DNA prevalence of MWPyV and STLPyV neither in normal nor in cancerous lung tissues. The aim of the present study was to examine DNA prevalence of these polyomaviruses in cancerous and non-cancerous lung tissue samples, in order to study the possible site for viral replication and/or persistence, and the potential association of these viruses with lung carcinogenesis, as well.

Methods: 100 cancerous and 47 non-cancerous, formalin-fixed paraffin-embedded lung tissue samples were studied for KIPyV, WUPyV, MWPyV and STLPyV by real-time PCR.

Results and conclusion: Neither of the viruses was found in samples from small cell, non-small cell (adenocarcinoma, squamous cell carcinoma and large cell neuroendocrine lung cancer), mixed type and non-differentiated lung carcinoma, and non-cancerous lung tissues (from patients with pneumonia, emphysema and fibrosis).

INTRODUCTION

Members of the family *Polyomaviridae* are in growing numbers during the last decade, but only the "old" human polyomaviruses (PyV) BK and JC, the novel Merkel cell polyomavirus (MCPyV) and Trichodysplasia spinulosa–associated polyomavirus are linked to diseases. Pathogenesis of the other human polyomaviruses has not been clarified [1].

Human polyomavirus KI and WU (KIPyV and WUPyV) were described as new viral genomes from respiratory samples in 2007 [2, 3]. Later, both viruses were found mainly in respiratory samples: the viruses were detected (positivity rates are shown in brackets) in respiratory secretions (0.5-6.5 % for KIPyV and 0.35-16.4 % for WUPyV) and in tonsillar tissue samples (2 % for KIPyV and 2.2-12 % for WUPyV) [4-10]; and both viruses was demonstrated in non-cancerous lung tissues (3.3 and 9.5 % for KIPyV and case studies to detect antigens of WUPyV and KIPyV) [11-15], while WUPyV was found in 7.8 and 27.7 % of adenoids studied [6, 7], as well. MW polyomavirus (MWPyV) genome was discovered from stool sample in 2012 [16], but beside stool samples (2.3 %), it was detected also in a condyloma specimen [17], nasal swab and nasopharyngeal aspirate samples (1.5-9.2%) [18, 19], tonsils (2 and 6 %) and adenoid tissue (1 %) [8, 20]. STL polyomavirus (STLPyV) complete genome was also found in 0.25-1.1 % of faecal samples studied [21], but it was

detected in 2% of tonsillar tissue samples analyzed, as well [8].

Based on seroepidemiological studies these viruses are ubiquitous in the human population, childhood primary infections are suggested, and seropositivities increase with age. Seropositivity rates are 55-91% for KIPyV, 69-98% for WUPyV, 42-99 % [22-25] for MWPyV [25-27] and 68-70% for STLPyV [28]. Since all the studied viruses are ubiquitous and have been detected in respiratory and faecal samples, respiratory and/or faecal-oral transmission is suggested.

Despite the oncogenic potential of all human pathogenic polyomaviruses linked to large and small tumour antigens (LTAg and sTAg), only MCPyV is thought to be an ethiologic agent in tumourigenesis, namely Merkel cell carcinoma [29, 30]. The association between seropositivity for KIPyV and for WUPyV and lung cancer was studied previously, antibodies against VP1 (viral protein 1) and sTAg were detected and analysed. Colombara et al. found that previous infections with these polyomaviruses were not associated with lung cancer [31, 32]. DNA prevalence of KIPyV and WUPyV was studied in different cancer types [9, 11-13, 33-39], but only KIPyV was detected in lung carcinomas [11] and in benign skin tumour [35]. To date, DNA prevalence of MWPyV was examined and detected in tonsillar cancer [9], but it was not found in mucosal melanoma [33] . STLPyV was studied only in tonsillar cancer, but the virus was not detected [9].

The aim of the present study was to examine the DNA prevalence of KIPyV, WUPyV, MWPyV and STLPyV in cancerous and non-cancerous lung tissue samples, in order to study the possible site for viral replication and/or persistence, and the potential association of these viruses with lung carcinogenesis, as well.

MATERIALS AND METHODS

Patients and samples

The study was approved by Regional and Institutional Ethics Committee, University of Debrecen (IX-R-052/00016-29/2012).

One hundred and forty seven formalin-fixed paraffin-embedded lung tissue samples from 143 patients diagnosed routinely between 2012 and 2016 in Department of Pathology, University of Debrecen were analysed. Data of patients and numbers of cancerous and non-cancerous samples are detailed in Table 1. Nucleic acid isolation was performed from a 10 μM tissue section using High Pure FFPET DNA Isolation Kit (Roche, Switzerland) according to the manufacturer's instruction. Deparaffinization was carried out with xylene according to the protocol of the kit.

Quality and quantity of nucleic acid were checked by a NanoDrop 2000c Spectrophotometer (Thermo Scientific, USA).

Detection of KIPyV, WUPyV, MWPyV and STLPyV DNA

Human polyomaviruses were detected by quantitative, real-time PCR (qPCR) in an Applied Biosystem 7500 real-time PCR instrument and analysed by 7500 Software v2.0.6 (Applied Biosystems, USA). To control nucleic acid isolation, PCR with human β-globin primers PCO3 and PCO4 was carried out as detailed previously [39]. Primers used are summarized in Table 2.

Protocol for KIPyV and WUPyV qPCR and positive controls were the same as described previously [39]. Plasmid containing KI polyomavirus isolate Stockholm 60 and AP-p002 plasmid with the half genome of WU polyomavirus were kindly provided by Tobias Allander and David Wang.

MWPyV qPCR was carried out with 5 μL template nucleic acid using primers ES105 and ES106, probe ES107 and protocol as described by Siebrasse et al. [16]. A partial sequence of Malawi polyomavirus (GenBank: JQ898291.1, from 3966 nt to 4927 nt) was synthesized and cloned into pOK vector (GeneArt Gene Synthesis), than served as a positive control in qPCR.

STLPyV DNA was detected in 5μL nucleic acid by qPCR published by Bialasiewicz et al. using 6.25-6.25 pmol STL-LT-F, STL-LT-R primers and 5 pmol STL-LT-Prb TaqMan probe (VIC-MGB) in a final volume of 25 μL [40]. STLPyV partial sequence (GenBank: JX463183.1, from 3922 nt to 4776 nt) was sythesized (GeneArt Gene Synthesis), cloned into pJET1.2/blunt vector (Thermo Fisher Scientific, USA), than used as a positive control in qPCR.

RESULTS AND DISCUSSION

Nucleic acid isolation was successful from 47 non-cancerous lung tissue samples and 100 cancerous lung tissue samples proved by human β-globin PCR: amplifiable, good quantity DNA presented in each sample. Mean and median concentration of nucleic acid was 164 and 152 ng/μL; the range of A_{260}/A_{280} ratio was 1.68-2.05 (median 1.86).

Tissue samples from small cell, non-small cell (adenocarcinoma, squamous cell carcinoma and large cell, neuroendocrine lung cancer), mixed type and non-differentiated lung carcinoma, and samples from non-cancerous lung tissues (from patients with pneumonia, emphysema and fibrosis) were negative for KIPyV, WUPyV, MWPyV and STLPyV DNA.

Despite the prevalence studies and growing number of data published, the pathogenesis of KIPyV, WUPyV, MWPyV and STLPyV has not been clarified. KIPyV and WUPyV were examined and found (positivity rates are shown in brackets) most frequently in respiratory secretions (0.35-16.4 % for KIPyV and 0.5-6.5% for WUPyV), but also in stool (0.5-11.6 % for KIPyV and 0.5-8.1 % for WUPyV), cerebrospinal fluid (1.6 % for WUPyV), blood (1-3.2 % for KIPyV DNA and 0.8-4.6 % for WUPyV) and urine (2 % for KIPyV and 12 % for WUPyV), suggesting that these viruses spread within the body and may establish persistent, even latent infection [4, 41]. Different (other than blood) tissue samples – normal and malignant, fresh or archived (frozen or formalin-fixed paraffin-embedded) – were also examined. Results published are summarized to date in Table 3. As mentioned previously, based on the prevalence data, direct human-to-human, indirect (via food, water or surfaces), respiratory and/or faecal-oral transmission are suggested [42, 43]. Respiratory transmission and portal entry is also strengthened by some prevalence data of studies with tissue samples from the respiratory tract, namely adenoids, tonsils and lung. In adenoid samples only WUPyV DNA was found, but KIPyV was not [6, 7]. WUPyV was detected in tonsillar tissues in each study [5-10] , but KIPyV was found in two tonsils only by Peng et al [8]. Beside these, both viruses were detected in brain autopsy samples [44], in lymphoid tissues [14, 45], and examined, but not found in various, non-cancerous tissue samples detailed in Table 3. [13, 46]. MWPyV was also detected in respiratory samples [18, 19], in one adenoid tissue [20] , and in tonsils, but only in two [8, 20] out of the four studies [8-10, 20] Interestingly, tonsillar squamous cell carcinoma samples were positive for MWPyV DNA, but were negative for KIPyV, WUPyV and STLPyV [9]. STLPyV was detected in tonsillar samples in one study [8], while others did not find the viral sequence in tonsillar tissue samples [9, 10].

Normal and cancerous lung tissue samples have been studied previously by two research teams for the presence of KIPyV DNA, nucleic acid isolation was carried out from fresh samples [11, 12]. Babakir-Mina et al. found high rate of KIPyV VP1 DNA positivity in cancerous lung tissue samples (9/20; 45%), and they detected viral VP1 sequence in one normal lung tissue sample surrounding the tumour, and in a lung biopsy from a transplant child (2/21; 9.5 %), as well. However, only two cancerous lung samples were positive using other PCR targeting the early region of the virus [11]. Teramoto et al. examined KIPyV and WUPyV in 30 lung adenocarcinoma tissues and 30 adjacent, normal lung sample pairs, but only KIPyV was detected in one normal lung tissue [12]. Toptan et al. developed a pan-human polyomavirus immunohistochemistry test and analyzed 1250 samples from various, also lung tumours and normal tissues (detailed in Table 3.), and antigen positivity was

revealed in a lung tissue sample from a patient with chronic lymphocytic leukaemia, then the presence of WUPyV DNA was also proved [13]. Siebrasse et al. studied KIPyV and WUPyV in lung tissue samples from two patients not only by PCR, but also by antibodies, revealing antigen positivity [14, 15]. KIPyV positive cells were identified as CD68+, suggesting that alveolar macrophages might be infected by the virus. Beside lung, other tissue samples were also examined, and KIPyV antigen positivity was detected in a spleen sample [14]. Immunohistochemistry was performed on lung, trachea, liver, kidney, and gastrointestinal tract tissue sections from a bone marrow transplant patient by anti-VP1 WUPyV antibody. Viral antigen positive cells were found in trachea and lung sections, some cells were CD68+ and showed macrophage morphology [15].

Other cancerous tissue samples – detailed in Table 3. – have been examined for KIPyV, WUPyV and MWPyV yet, but beside the above mentioned, KIPyV was detected solely in Spitz naevus, a benign tumour [35].

Based on immunohistochemistry and PCR studies, it is suggested that KIPyV and WUPyV may infect lung (even productively), but it requires more data to prove. To our knowledge, DNA prevalence of MWPyV and STLPyV have not been studied in lung tissue samples before this study. The PCR methods used in this study were developed and tested previously [16, 40, 47], it has high importance to compare data. Although we examined higher number of cancerous (n=100) and non-cancerous lung (n=47) tissue samples in the present study than was studied in any previous one, all were negative for KIPyV, WUPyV, MWPyV and STLPyV DNA by PCR. Despite the possible oncogenic potential hypothetically linked to LTAg and STAg, and that DNA of KIPyV and MWPyV was detected in cancerous tissue samples, further epidemiological studies with high number of cancerous and non-cancerous tissues, and in vitro studies, as well are essential to evaluate whether these novel polyomaviruses are ethiological agent in tumourigenesis or not.

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344 **Table 1. Data of patients and tissue samples**

	Tissue samples		number of patients / number of samples	female	male	age (year) of patients: min-max (median)
lung carcinoma	small cell carcinoma		4/4	2	2	53.4-67 (65.4)
	non-small cell carcinoma	squamous cell carcinoma	17/17	3	14	57.7-75.9 (66.4)
		adenocarcinoma	69/71	37	32	38.3-73.3 (61.3)
		large cell neuroendocrine carcinoma	2/2	1	1	28.1-56.5
	mixed type carcinoma		4/4	2	2	45-71.6 (59.6)
	non-differentiated type lung carcinoma		2	1	1	35.6-61.9
	total		98/100	46	52	28.1-77.3 (61.8)
control	lung tissue sample from patients with pneumonia, fibrosis or emphysema		45/47	21	24	13.1-78.8 (59.6)
total			143/147	67	76	13.1-78.8 (61.5)

Table 2. Details of PCR used

PCR	Primer name	Sequence 5'→ 3'	Target gene	Amplicon size (bp)	Sensitivity of PCR GEq/PCR	Reference
KIPyV and WUPyV, duplex, real-time PCR	KIPyV	CTATCCCTGAATACCAGTTGGAAAC	VP2-3	74	5	[47]
	KIPyV	GTATGACGCGACAAGGTTGAAG				
	KIPyV VP2-3	FAM- TTCCGGGCATCCCAGACTGGC-MGBNFQ				
	WUPyV	AACCAGGAAGGTCACCAAGAAG	VP1	76	5	
	WUPyV	TCTACCCCTCCTTTTCTGACTTGT				
	WUPyV VP1 probe	NED-CAACCCACAAGAGTGCAAAGCCTTCC-MGBNFQ				
MWPyV real-time PCR	ES105	TGAGAAGGCCCCGGTTCT	LT	73	10	[16]
	ES106	GAGGATGGGATGAAGATTTAAGTTG				
	ES107	FAM-CCTCATCACTGGGAGC-MGBNFQ				
STLPyV real-time PCR	STL-LT-F	TGCAGAGGTCCCTTCATCATC	LT	132	10	[40]
	STL-LT-R	TTTTCTTTTTAGGGCGGACAATAT				
	STL-LT-Prb	VIC-CCACCATTGCTCCCAAGCAGGAGTAC –				
control PCR for human DNA	PCO3	ACACAACTGTGTTCACTAGC	human beta-globin gene	110	no data	[39]
	PCO4	CAACTTCATCCACGTTCACC				

VP1: viral protein 1 gene; VP2: viral protein 2 gene; VP3: viral protein 3 gene; LT: large T antigen gene

KIPyV: KI polyomavirus; WUPyV: WU polyomavirus; MWPyV: MW polyomavirus; STLPyV: STL polyomavirus

GEq/PCR: genome equivalent / PCR

Table 3. Prevalence of human polyomavirus 3, 4, 10 and 11 in tumour and normal tissue samples based on literature

			number of positive samples/number of samples tested (%)			
	reference	**tissue**	**KIPyV (KIPyV)**	**WUPyV (WUPyV)**	**MWPyV (MWPyV)**	**STLPyV (STLPyV)**
tumour tissues	[33]	mucosal melanoma	0/55	0/55	0/55	nd
	[34]		0/38	0/38	nd	nd
	[35]	**Spitz naevus**	**4/25 (16%)**	0/25	nd	nd
		keratoacanthoma	0/22	0/22	nd	nd
	[36]	CNS tumours (ependyoma, astrocytoma, medulloblastoma, other gliomas, other neoplasms)	0/25	0/25	nd	nd
		neuroblastoma	0/31	0/31	nd	nd
	[37]	neuroendocrine tumours (brain, thymus, digestive system, lung, skin, thyroid gland)	0/50	0/50	nd	nd
	[38]	neuroendocrine tumours (lung, gastrointestinal tract, female reproductive system, soft tissue, head and neck region, bladder)	0/74	0/74	nd	nd
	[39]	renal neoplasia (adenoma, angiomyolipoma, oncocytoma,	0/187	0/187	nd	nd

		leiomyosarcoma, carcinoma renocellulare renis) and bladder uroepithelial carcinoma)				
	[11]	**lung cancer**	**9/20 (45%)**	nd	nd	nd
	[12]	lung adenocarcinoma	0/30	0/30	nd	nd
	[13]	lung adenocarcinoma (n=136), lung squamous cell carcinoma (n=100), oral cavity, stomach, colon, bladder, kidney, skin, breast, brain, mesothelium tomours, Non-Hodgkin's lymphoma tumour	0*/1157	0*/1157	0*/1157	0*/1157
	[9]	**tonsillar squamous cell carcinoma**	0/38	0/38	**7/38 (18.4 %)**	0/38
normal tissues	**[44]**	**brain autopsy**	**8/54 (14.8%)**	**6/54 (11.1%)**	nd	nd
	[12]	**lung**	**1/30 (3.3%)**	0/30	nd	nd
	[11]		**2/21 (9.5%)**	nd	nd	nd
	[14]		**2*/2 (100%)**	nd	nd	nd
	[15]		nd	**1*/1**	nd	nd
	[13]		**1*/1**	0/1	0/1	0/1
	[15]	**trachea**	nd	**1*/1**	nd	nd
	[8]	**tonsil**	**2/99 (2%)**	**11/99 (11.1%)**	**2/99 (2%)**	**2/99 (2%)**

	[20]		nd	nd	**6/100 (6%)**	nd
	[9]		0/40	**3/40 (7.5%)**	0/40	0/40
	[10]		0/78	**3/78 (3.8%)**	0/78	0/78
	[7]		0/51	**2/51 (3.9%)**	nd	nd
	[6]		0/50	**6/50 (12%)**	nd	nd
	[5]		0/229	**5/229 (2.2%)**	nd	nd
	[20]	**adenoid**	nd	nd	**1/100 (1%)**	nd
	[7]		0/51	**4/51 (7.8%)**	nd	nd
	[6]		0/83	**23/83 (27.7%)**	nd	nd
	[45]	**lymphoid tissue (lymph node and spleen)**	**4/97 (4.1%)**	**3/97 (3.1%)**	nd	nd
	[14]	**lymphoid tissue (lymph node and spleen)**	**1*/4**	nd	nd	nd
	[13]	stomach, colon, bladder, kidney, skin, brain, mesothelium, Non-Hodgkin's lymphoma	0*/93	0*/93	0*/93	0* /93
	[46]	placenta, heart and liver from foetus	0/535	0/535	nd	nd

nd: no data, not examined

KIPyV: KI polyomavirus

WUPyV: WU polyomavirus

MWPyV: MW polyomavirus

STLPyV: STL polyomavirus

* antigen was detected by immunohistochemistry assay