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NOTE

Detection of KI polyomavirus and WU polyomavirus DNA by real-time polymerase chain reaction in nasopharyngeal swabs and in normal lung and lung adenocarcinoma tissues

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ABSTRACT

Polyomaviruses KI (KIPyV) and WU (WUPyV) were detected from 7 (3.0%) and 38 (16.4%) of 232 children with respiratory tract infections by real-time PCR. The rates of infection by KIPyV and WUPyV alone were 3 of 7 (42.9%) and 20 of 38 (52.6%), respectively. In the other samples, various viruses (human respiratory syncytial virus, human metapneumovirus, human rhinovirus, parainfluenza virus 1 and human bocavirus) were detected simultaneously. One case was positive for KIPyV, WUPyV and hMPV. There was no obvious difference in clinical symptoms between KIPyV-positive and WUPyV-positive patients with or without coinfection. KIPyV was detected in one of 30 specimens of lung tissue (3.3%). Neither of the viruses was detected in 30 samples of lung adenocarcinoma tissue.

Key words KI polyomavirus, real-time PCR, WU polyomavirus.

Polyomaviruses are small non-enveloped double-stranded DNA viruses of around 5000 base pairs that can infect mammals and birds (1). Until 2007, only two polyomaviruses, BKV (2) and JCV (3), were known to cause human disease. In 2007, two novel viruses, KIPyV and WUPyV, were cloned from respiratory tract samples in Sweden and the USA, respectively (4, 5). Studies in Australia, Korea, Canada, the UK, France, Germany, Italy, the Netherlands, Thailand, China and the Philippines have since reported detection of KIPyV and WUPyV in patients with RTIs (4–22) (Tables 1, 2). In 2008, a fifth novel polyomavirus was identified from Merkel cell carcinoma tumors and other tissues and named MCPyV (23). Although infections with polyomaviruses can produce tumors in animal models, there is no evidence of their role as cancer-producing operants in humans. MCPyV is strongly suspected to be oncogenic because its DNA has been found to be integrated within the genome of Merkel cell carcinoma (23). MCPyV has also been detected in patients with small cell lung cancer (24, 25) and in squamous cell carcinoma and basal cell carcinoma of the skin (26, 27). However, little is known about the oncogenicity of KIPyV and WUPyV (28, 29). In this study, we examined the prevalence of KIPyV and WUPyV in pediatric patients with RTIs to obtain epidemiological data for Japan. In addition, we investigated the prevalence of KIPyV and WUPyV in Japanese normal lung and lung adenocarcinoma tissues to evaluate the

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List of Abbreviations: BKV, BK virus; CT, threshold cycle; HBoV, human bocavirus; HCoV, human coronavirus; HEV, human enterovirus; hMPV, human metapneumovirus; hRSV, human respiratory syncytial virus; HRV, human rhinovirus; JCV, JC virus; KIPyV, KI polyomavirus KI; MCPyV, Merkel cell polyomavirus; NPS, nasopharyngeal swab; PIV, parainfluenza virus; RTI, respiratory tract infection; WUPyV, WU polyomavirus.

Table 1. Summary of KIPyV detection in respiratory specimens from patients with RTIs

Country	Number tested	Rate (%)	Method	Year reported	Authors	Ref
USA	2599	2.8	real-time PCR	2010	Hormozdi D.J.	21
UK	371	2.7	nested PCR	2008	Kiasari B.A. *et al*.	11
Sweden	637	0.94	nested PCR	2007	Allander T. *et al*.	4
France	537	0.6	nested PCR	2008	Foulongne V. *et al*.	9
Italy	222	0.45	PCR	2008	Babakir-Mina M. *et al*.	19
Italy	486	0.2	nested PCR	2010	Debiaggi M. *et al*.	22
The Netherlands	230	2.6	real-time PCR	2008	van der Zalm M.M. *et al*.	15
Australia	2866	2.6	nested PCR	2008	Bialasiewicz S. *et al*.	6
Korea	486	1.0	nested PCR	2007	Han T.H. *et al*.	20
China	406	2.7	nested PCR	2008	Yuan X.H. *et al*.	14
Thailand	302	1.99	nested PCR	2008	Payungporn S. *et al*.	7
Philippines	411	0.5	nested PCR	2010	Furuse Y. *et al*.	18
Japan	232	3.0	real-time PCR	2011	Teramoto S. *et al*.	this study

association between KIPyV and WUPyV infection and lung cancer.

After obtaining informed consent from their parents, 232 NPSs were collected from 219 children (115 boys and 104 girls) aged 1–90 months (average age, 19.0 months) with RTIs at four hospitals (see Acknowledgements) in Hokkaido, Japan during the period from June 2005 to May 2007. DNA was extracted from 200 μL of NPSs by Chomczynski's protocol (30). The elution volume of the extractions was 90 μL. Thirty fresh sample pairs of lung adenocarcinoma and adjacent non-cancerous normal lung tissue were obtained from surgical material at the University of Toyama Hospital in 2002, with the informed consent of the patients. These patients included 20 men and 10 women aged from 45 to 77 years, with an average age of 61.7 years. None of the patients from whom samples were obtained for this study had immunodeficiency diseases. The tissue samples were frozen in liquid nitrogen and stored at -80°C until used for DNA extraction. DNA from 100 mg aliquots of frozen tissue samples was extracted using a standard method with proteinase K digestion and phenol–chloroform (31). The concentration of DNA extracted from lung tissues and tumors was adjusted to 50 ng/μL. Detection and quantification of KIPyV and WUPyV were performed by real-time PCR. The primers and probe for KIPyV were 5′-ACC TGA TAC CGG CGG AAC T-3′ (forward), 5′-CGC AGG AAG CTG GCT CAC-3′ (reverse) and 5′-[FAM]-CCA CAC AAT AGC TTT CAC TCT TGG CGT GA -[TAMRA]-3′ (a TaqMan probe) (32). The primers and probe used for WUPyV were 5′-GGC ACG GCG CCA ACT-3′ (forward), 5′-CCT GTT GTA GGC CTT ACT TAC CTG TA-3′ (reverse) and 5′-[FAM]-TGC CAT ACC AAC ACA GCT GCT GAG C-[TAMRA]-3′ (a TaqMan probe) (32). The 50 μL amplification reaction mixture contained 50 ng (for lung tissues and tumors) or 5 μL (for NPSs) of sample DNA, 25 μL of TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA), 900 nmol/L of each primer

Table 2. Summary of WUPyV detection in respiratory specimens from patients with RTIs

Country	Number tested	Rate (%)	Method	Year reported	Authors	Ref
USA	410	1.2	PCR	2007	Gaynor A.M. *et al*.	5
Canada	79	2.5	PCR	2007	Abed Y. *et al*.	12
UK	371	1.08	PCR	2008	Kiasari B.A. *et al*.	11
Germany	1277	4.9	PCR	2008	Neske F. *et al*.	8
France	537	2.4	PCR	2008	Foulongne V. *et al*.	9
Italy	486	1.4	nested PCR	2010	Debiaggi M. *et al*.	22
The Netherlands	230	9.1	real-time PCR	2008	van der Zalm M.M. *et al*.	15
Australia	2866	4.5	PCR	2008	Bialasiewicz S. *et al*.	6
Korea	486	7.0	PCR	2007	Han T.H. *et al*.	20
China	406	4.2	PCR	2008	Yuan X.H. *et al*.	14
Thailand	302	6.29	PCR	2008	Payungporn S. *et al*.	7
Philippines	411	1.5	PCR	2010	Furuse Y. *et al*.	18
Japan	232	16.4	real-time PCR	2011	Teramoto S. *et al*.	this study

Table 3. Detection of KIPyV and WUPyV genomes in normal lung, lung cancer tissue and nasopharyngeal swab samples

Samples	KIPyV	WUPyV
Normal lung tissue	1/30 (3.3%)	0/30 (0.0%)
Adenocarcinoma	0/30 (0.0%)	0/30 (0.0%)
Nasopharyngeal swab	7/232 (3.0%)	38/232 (16.4%)

and 100 nmol/L of a probe. Amplification was performed using an ABI Prism 7000 Sequence Detection System (Applied Biosystems) with the following instrument settings: 50°C for 2 min, 95°C for 10 min and then 50 cycles of 95°C for 15 s and 60°C for 1 min. The plasmids pKIPyV-real and pWUPyV-real containing the PCR products of the qualitative PCR in the vector pT7Blue (Novagene, Madison, WI, USA) were used as positive controls and for standard curves. All real-time PCR reactions were performed in duplicate and the results analyzed using ABI Prism 7000 SDS software. Viral loads were calculated from the CT values of individual samples with respect to the standard curve. The minimum concentrations of KIPyV and WUPyV genomes that would allow reproducible quantification were 10 copies per reaction. These correspond to 2×10^2 copies/μg (lung tissues and tumors) and 2×10^3 copies/mL (NPSs). As an internal control, β-actin was also amplified using TaqMan β actin detection reagents according to the manufacturer's instructions (Applied Biosystems). Nested PCR for KIPyV and PCR for WUPyV were also performed for lung tissue samples and NPSs as described previously (4, 5). All specimens that were positive for KIPyV and WUPyV were also assayed for the presence of twelve other respiratory viruses: hRSV, hMPV, HRV, HBoV, PIV 1–3, influenza A and B viruses, HEV, HCoV, and adenoviruses. The PCR and RT-PCR protocols used for detecting these twelve viruses were the same as those previously described (33, 34).

The KIPyV genome was detected in 7 of the 232 NPSs (3.0%) (Table 3), consistent with previously reported detection rates (0.5 to 5%) (28). The median viral load of all KIPyV-positive NPSs was 1.8×10^4 copies/mL, and the maximum value 4.1×10^5copies/mL. The prevalence of KIPyV in NPSs determined by real-time PCR

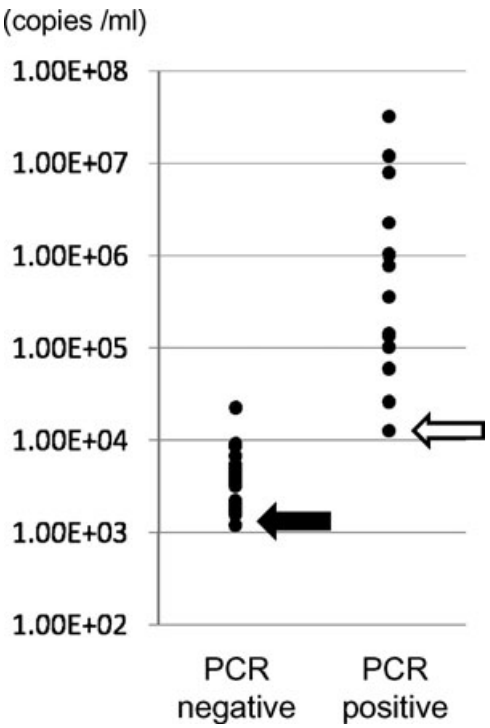

Fig. 1. WUPyV copy numbers of NPSs detected by real-time PCR. Each dot represents the copy number of WUPyV detected by real-time PCR. The first row shows the copy numbers of WUPyV that were negative by PCR and the second row shows the copy numbers of WUPyV that were positive by PCR. The black arrow shows the detection limit of real-time PCR (10 copies per reaction, which is equal to 2×10^3 copies per mL), and the white arrow shows the detection limit of PCR (100 copies per reaction, which is equal to 2×10^4 copies per mL).

was the same as that determined using nested PCR (4), and direct sequencing of PCR products of the seven samples showed that they were completely identical to the published sequence of KIPyV strain Stockholm 350 (Genbank accession number EF127907 bases 1536 to 1860) (4). The WUPyV genome was detected in 38 of the 232 NPSs (16.4%) by means of real-time PCR (Table 3). The median viral load of all WUPyV-positive NPSs was 5.1×10^3copies/mL, and the maximum value was 3.2×10^7 copies/mL. Three of the seven

Table 4. Coinfection with other viruses in nasopharyngeal samples

	Coinfection with another virus					Coinfection with two other viruses		
	hRSV	hMPV	HRV	HBoV	PIV1	hMPV+HBoV	hRSV+HBoV	HRV+HBoV
KIPyV	0	2	0	0	0	1	0	0
WUPyV	7	4	1	2	1	0	1	1
KI/WUPyV	0	1	0	0	0	0	0	0
total	7	7	1	2	1	1	1	1

Table 5. Clinical symptoms in KIPyV-positive and WUPyV-positive patients with or without coinfection

	KIPyV		WUPyV	
	Single-infection	Coinfection	Single-infection	Coinfection
Number of patients	3	4*	20	18*
Symptom				
Cough	3/3	4/4	19/20	18/18
Rhinorrhea	2/3	3/4	18/20	14/18
Hypoxia	0/3	0/4	1/20	1/18
Wheezing	2/3	3/4	12/20	9/18
Fever (>37.5°C)	3/3	4/4	18/20	17/18
Maximum (°C)	40.5	39.5	40.4	40.2
Average (°C)	38.9	38.9	39.2	39.0
Mean duration of fever(days)	3.0	5.8	3.6	3.8
Hospitalization required	3/3	4/4	17/20	17/18
Mean duration of hospitalization (days)	6.0	7.3	5.4	4.9

*One case was positive for KIPyV, WUPyV and hMPV.

KIPyV-positive samples were detected simultaneously with other viruses (two with hMPV and one with hMPV and HBoV). Among the 38 WUPyV-positive samples, 17 were detected simultaneously with other viruses (7 with hRSV, 4 with hMPV, 1 with HRV, 2 with HBoV, 1 with PIV1, 1 with hRSV and HBoV, and 1 with HRV and HBoV). One case was positive for KIPyV, WUPyV and hMPV (Table 4). Seven KIPyV-positive samples were collected during the period from March to September (one sample in March, one in April, one in June, two in July, one in August and one in September) (Supplemental figure). Thirty-eight WUPyV-positive samples were collected in all months except February (two samples in January, one in March, four in April, five in May, five in June, four in July, one in August, one in September, two in October, eight in November and five in December) (Supplemental figure).

The prevalence of WUPyV in NPSs in this study was obviously higher than previously reported prevalences (0.4 to 9%) (28), though by switching the detection method from real-time PCR to PCR (5), the rate of WUPyV-positive NPSs fell from 16.4% (38 of 232) to 5.6% (13 of 232). The detection limit of PCR (5) (100 copies per reaction, which is equal to 2×10^4 copies per mL) and that of real-time PCR (10 copies per reaction, which corresponds to 2×10^3 copies per mL) explains the prevalence gap for WUPyV in NPSs in this study. The copy numbers of 13 NPSs that were real-time PCR-positive but PCR-negative were all below 2×10^4 copies per mL except for one (Fig. 1). Direct sequencing of the PCR products of the 13 PCR-positive samples showed that 10 of the 13 sequences were completely identical to the published sequence of WUPyV strain B0 (Genbank accession number EF444549 bases 1331 to 1580), and 3 of the 13 sequences had a single-base-pair substitution (G1369C, A1396C and C1432A) in the VP3 gene (5).

Clinical and laboratory features of the KIPyV- and WUPyV-positive patients are shown in Table 5 and Supplemental table. The ages of patients with KIPyV-positive samples ranged from 3 months to 2 years 11 months, and the ages of patients with WUPyV-positive samples ranged from 1 month to 4 years 11 months. All seven of the KIPyV-positive patients (100%) and 34 of the 38 WUPyV-positive patients (89.5%) were admitted to hospital for 3 to 11 days. The clinical diagnoses of the KIPyV-positive patients were wheezy bronchitis (three patients), bronchitis (three) and pneumonia (one). The clinical diagnoses of the WUPyV-positive patients were bronchitis (15 patients), wheezy bronchitis (14), pneumonia (5), asthma (1), laryngotracheitis (1), acute pharyngolaryngitis (1), and acute pharyngitis (1). There was no obvious difference in clinical symptoms among KIPyV- and WUPyV-positive patients with or without coinfection.

Using real-time PCR, the KIPyV genome was detected in 1 of the 30 specimens of normal lung tissue (3.3%), whereas the WUPyV genome was not detected in any of the 30 specimens of normal lung tissues (Table 3). The viral load in KIPyV-positive normal lung tissue was 3.58×10^2 copies/μg. The sequence of KIPyV detected in normal lung tissue could not be determined because the KIPyV genome was not successfully amplified by nested PCR. On the other hand, neither the KIPyV genome nor the WUPyV genome was detected in 30 Japanese lung adenocarcinoma tissue samples (Table 3). The mean β actin values of the normal and adenocarcinoma lung tissues were 3.6×10^5 DNA molecules (range, 7.6×10^4 to 6.9×10^5) and 2.8×10^5 DNA molecules (range, 9.8×10^4 to 6.6×10^5) per μg, respectively.

In a study in Italy, the KIPyV genome was detected in 1 of 20 specimens of normal lung tissue (5.0%) (29), in agreement with our data. The oncogenic potential of KIPyV and WUPyV in human lung adenocarcinoma could not be determined in the present study. Although the KIPyV genome was detected in 9 of 20 specimens of lung cancer tissue (45.0%) in the study in Italy (29), because these authors did not provide the pathological classification of the lung cancer specimens, the relationship between KIPyV and lung adenocarcinoma remains unclear. Further studies on a larger number of specimens of malignant lung tissue of different types should be performed to evaluate the oncogenic properties of KIPyV and WUPyV.

To our knowledge, this is the first report of detection of KIPyV and WUPyV in Japanese children with RTIs. Results of nested PCR and PCR for KIPyV and WUPyV in NPSs from Japanese children with RTIs are consistent with the results of studies in other countries, and the sequence similarities to previous findings indicate worldwide distribution of the same virus lineage. The presence of KIPyV in normal lung tissue suggests that KIPyV may establish infection in lung tissue; alternatively it may be an innocent bystander. Our negative findings for KIPyV and WUPyV in lung adenocarcinoma tissue indicate that there may be no relationship between these viruses and lung adenocarcinoma transformation; alternatively the overall prevalence of the viruses might have been too low to be distinguished in our small number of samples. A larger sample size is needed to determine whether they are present in lung adenocarcinoma and their association with lung adenocarcinoma progression.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

Figure S1 Clinical characteristics of KIPyV-poitive and WUPyV-positive patients.

Table S1 Seasonal distribution of KIPyV- and WUPyV-positive cases and numbers of samples collected.

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