Kosulin et al. Int J Cancer Clin Res 2015, 2:3
DOI: 10.23937/2378-3419/2/3/1019

International Journal of
Cancer and Clinical Research

ISSN: 2378-3419

Research Article: Open Access

Screening for the Polyomaviruses BKV, JCV and SV40 in Pediatric Malignancies

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Abstract

Different studies suggested an oncogenic potential of the polyomaviruses JC virus, BK virus and simian virus 40, particularly in brain tumors and neuroblastoma, which belong to the most frequent malignancies in children. However, currently available data are controversial, possibly due to the different regional prevalence of the viruses and the detection techniques used. To elucidate the presence of these polyomaviruses in the indicated tumor entities and in childhood cancer in general, we have investigated a broad spectrum of pediatric malignancies, with particular emphasis on neuroblastoma from different geographic regions. More than 500 diagnostic specimens derived from 16 different pediatric cancer entities including solid tumors, leukemias and lymphomas were screened by highly sensitive and specific real-time quantitative PCR assays targeting important viral domains such as the large T-antigen, small T-antigen and virus protein 1. To ensure adequate power of the analysis, a minimum of 30 specimens were analyzed in each tumor entity. The vast majority of tumors investigated revealed negative findings, with only anecdotal presence of JC virus large/small T-antigen in individual cases of acute myeloid leukemia (1/30) and oligodendroglioma (1/30). Moreover, within a total of 111 neuroblastoma samples from different European countries, only a single case from Spain tested positive for BKV sequences. Our observations therefore reveal no evidence for the common presence of JC virus, BK virus or simian virus 40 in tumor tissue of pediatric malignancies and, in contrast to some earlier reports, provide no suggestion for an association between persistent infection with these viruses and childhood cancer.

Keywords

Polyomavirus, Large T antigen, Pediatric cancer, Brain tumor, Neuroblastoma

Abbreviations

ALL: Acute Lymphocytic Leukemia, AML: Acute Myeloid Leukemia, BKV: BK virus, CML: Chronic Myeloid Leukemia, HL: Hodgkin´s lymphoma, HPyV: Human Polyomavirus, JCV: JV virus, MCPyV: Merkel Cell Polyomavirus, NCCR: Non-coding Control Region, NHL: Non-Hodgkin´s lymphoma, RQ-PCR: Real-time Quantitative Polymerase Chain reaction, SV40: Simian virus 40, TAg: T-antigen, VP1: Virus Protein1.

Introduction

Human polyomaviruses (HPyV) belong to the family of small, non-enveloped DNA viruses. For several years, this family included only the human JC virus (JCV), which causes progressive multifocal leukoencephalopathy [1], and the human BK virus (BKV), causing mainly urinary tract infections [2]. Recent technological improvements permitted the identification of eight novel HPyV members in the last six years [3]. Most primary infections with the ubiquitous HPyV occur during the early childhood, usually without leading to serious clinical symptoms [4]. After primary exposure, the viral DNA can persist in individual cells by establishing a latent form of infection, and persistence of BKV and JCV can be detected in 75-90% of healthy individuals [5]. The possible role of BKV, JCV and the simian virus 40 (SV40) in human tumor formation has been a matter of debate for several decades [6,7] because these viruses display strong oncogenic potential after inoculation into rodents, with particular tropism to neural tissue [8]. The presence of BKV was documented in urothelial carcinoma, and the virus was suggested to play a contributory role in the pathogenesis of neuroblastoma [6,9,10]. The brain apparently serves as a sanctuary for the persistence of different viruses including JCV, and several studies reported the presence of viral sequences in various brain tumors [7,11,12]. As

Citation: Kosulin K, Zimmerhackl A, Hainfellner JA, Haberler C, Amann G, et al. (2015) Screening for the Polyomaviruses BKV, JCV and SV40 in Pediatric Malignancies. Int J Cancer Clin Res 2:019. doi.org/10.23937/2378-3419/2/3/1019

Received: June 11, 2015: **Accepted:** July 29, 2015: **Published:** July 31, 2015

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opposed to viral persistence as a putative source of oncogenic hits, the frequent detection of SV40 in brain tumors and other malignancies like Wilms´tumor, Ewing sarcoma, osteosarcoma or lymphoma was generally linked to the use of virus-contaminated polio vaccines several decades ago [13-16]. In contrast to studies describing the presence of BKV, JCV or SV40 in various tumors, other reports did not reveal any evidence for these viruses in brain tumors, other solid tumors or hematologic malignancies [17-22]. The conflicting data on the presence or absence of polyomavirus DNA in human malignancy might be, at least in part, attributable to regional differences in the occurrence of polyomaviruses or the technical approaches to virus detection providing different levels of sensitivity and specificity. One of the recently identified HPyV, the Merkel cell polyomavirus (MCPyV), has been under intensive investigation as a potential human tumor virus since its detection in patients with Merkel cell carcinoma, a rare type of skin cancer [23,24]. However, there are pronounced differences in DNA sequence to other HPyV, and the original cell type for MCPyV replication has not been elucidated so far.

With regard to the oncogenic potential of polyomaviruses, the main focus of attention centered on the early regulatory protein, the large T-antigen (large TAg). This protein is mainly located in the nucleus and displays binding sites for cellular proteins involved in growth regulation, such as the tumor suppressor proteins retinoblastoma protein, p53 and p300 [25,26]. Several studies have focused on the large TAg of SV40, which is closely related to that of the human polyomaviruses BKV and JCV, and reported an oncogenic capacity in rodents as well as the ability to transform human cells in culture [27-29]. A group studying BKV in neuroblastoma investigated the interaction of large TAg with p53 suggesting an inhibition of the antiproliferative effect of wild type p53 in this tumor [9]. The large TAg genes comprise nearly half of the entire viral genome and display an overlapping area at the 5´end coding for the small T-antigen (small TAg), which is known to contribute to the oncogenic capacity of HPyV in rodents [30]. The gene responsible for the oncogenic properties of MCPyV was also shown to be large TAg. Interestingly, in Merkel cell carcinoma only a truncated version of this gene has been detected, which is not compatible with viral replication [31].

The inconclusive data on the possible involvement of the polyomaviruses BKV, JCV and SV40 in the pathogenesis of human tumors, particularly of pediatric malignancies, prompted us to carry out the present study. We have investigated a large spectrum of pediatric malignant disorders including different solid tumors, leukemias, and lymphomas by highly sensitive real-time quantitative PCR (RQ-PCR) assays targeting viral genes demonstrated to be crucial for the oncogenic properties of BKV, JCV and SV40, with the aim to shed more light on the hitherto enigmatic potential role of these viruses in human neoplastic disorders.

Materials and Methods

Patients

Archived diagnostic specimens from pediatric patients with various types of solid tumors and leukemias including 337 fresh-frozen and 240 paraffin-embedded samples were collected for viral DNA screening. The fresh-frozen specimens tested were derived from patients treated at our clinical center, the St. Anna Children´s Hospital, Vienna, Austria, and included all leukemia (n=120) and lymphoma (n=71) samples analyzed as well as Wilms´ tumor (n=30), Ewing sarcoma (n=30), neuroblastoma (n=39), embryonal rhabdomyosarcoma (n=26) and osteosarcoma (n=21) samples, with informed consent. The leukemias investigated included diagnostic peripheral blood or bone marrow samples of the following entities: B-cell acute lymphocytic leukemia (B-ALL; n=30), T-cell acute lymphocytic leukemia (T-ALL; n=30), acute myeloid leukemia (AML; n=30) and chronic myeloid leukemia (CML; n=30). Lymph nodes from the time of diagnosis of Hodgkin´s lymphoma (HL; n=33) and Non-Hodgkin´s lymphoma (NHL; n=38, including both B-cell and T-cell NHL) were tested.

Table 1: RQ-PCR analysis in tumor cells for BKV, JCV and SV40

Tumors	No. of samples	HPyV positive
Leukemias and lymphomas		
B-cell acute lymphoid leukemia	30	
T-cell acute lymphoid leukemia	30	
Acute myeloid leukemia	30	1 (JCV)
Chronic myeloid leukemia	30	
Non Hodgkin's lymphoma	38[1]	
Hodgkin's lymphoma	33	
Solid tumors		
Neuroblastoma	39	
Wilms' tumor	30	
Ewing´s sarcoma	30	
Rhabdomyosarcoma	35[2]	
Osteosarcoma	30	
Ependymoma	30	
Medulloblastoma	30	
Glioblastoma	30	
Oligodendroglioma	30	1 (JCV)
Astrocytoma	30	
Total	**505**	

[1] including 29 B- and 9 T-Non Hodgkin´s lymphoma specimens

[2] including 26 embryonal and 9 alveolar rhabdomyosarcoma specimens

Table 2: Testing for BKV DNA in neuroblastoma specimens from different European countries

Country	No. of samples	BKV positive
Austria	39	0
Belgium	10	0
France	17	0
Germany	10	0
Portugal	5	0
Romania	15	0
Spain	15	1 (positive for LT, VP1 and NCCR)
Total	**111**	

Additionally, paraffin-embedded diagnostic tumor tissue was kindly provided by the Dept. of Clinical Pathology, Medical University of Vienna, including alveolar rhabdomyosarcoma (n=9) and osteosarcoma (n=9). Three 10µm sections of the rhabdomyosarcoma and osteosarcoma specimens were cut from each block using fresh disposable microtome blades to prevent cross-contamination. Moreover, brain tumor specimens including ependymoma (n=30), glioblastoma (n=30), oligodendroglioma (n=30), astrocytoma (n=30), and medulloblastoma (n=30) were kindly provided by the Institute of Neurology, Medical University of Vienna (Table 1). The glioblastoma, oligodendroglioma and five of the ependymoma specimens were derived from patients beyond 18 years of age. Punch sections of about 1mm in diameter targeting regions with high density of tumor cells were excised from the paraffin-embedded brain tumor tissue. Furthermore, paraffin-embedded diagnostic neuroblastoma samples were obtained from other geographic regions including Belgium (Center for Medical Genetics, Ghent University Hospital; n=10), France (Centre Leon Berard, Unite d´Oncologie Moleculaire, Lyon; n=17), Germany (Center for Pediatrics, University of Freiburg; n=10), Portugal (National Institute de Saude, Lisbon; n=5), Romania (Hospital de Cioii Louis Turcanu, Pediatric Clinic III, Temesvar; n=15), and Spain (Department of Pathology, Medical School, University of Valencia; n=15) (Table 2).

DNA extraction

Peripheral blood and bone marrow specimens from leukemia patients were subjected to DNA extraction by the QIAmp Blood DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommendations. DNA extraction from lymphoma samples and paraffin-embedded tissue was done by the QIAmp tissue DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommendations. In our earlier studies, the indicated kits were shown to facilitate efficient DNA isolation

Table 3: Primers and probes for RQ-PCR analysis of BKV, JCV and SV40 target sequences

Target	bp	5´label	Oligonucleotide sequence (5´-3´)	3´label	µM
BKV LT forw			TGTAAGGAATTTCACCCTGACAAAG		400
BKV LT rev			TGAGCTACCTTTACATCCTGCTCC		400
BKV LT probe[1]	98	FAM	AAAGTATTCATTCTCTTCATTTTATCCTCGTCGCCC	TAMRA	200
BKV VP1 forw			TCCAGGGGCAGCTCCC		400
BKV VP1 rev			GATCCCCCATTTCTGGGTTTAG		400
BKV VP1 probe[2]	146	FAM	AGGAGGAGTAGAAGTTCTAGAAG	NFQ	200
BKV NCCR forw			CCAGCCAGTGGCAGTTAATAGTG		400
BKV NCCR rev			GGGACAAGGCCAAGATTCCT		400
BKV NCCR probe[1]	137	FAM	CATGTCTGTCTGGCTGCTTTCCACTCCTT	TAMRA	200
JCV LT forw			AATAGGGAGGAATCCATGGAGC		400
JCV LT rev			GTCCCCACCTTTATCAGGGTG		400
JCV LT probe[2]	129	FAM	TCTCATGACAGGAATGTT	NFQ	200
JCV VP1 forw			AAATGTTCCTCCAGTTCTTCATATAACAA		400
JCV VP1 rev			GTCACCTTTGCAAAGTGGCC		400
JCV VP1 probe[2]	88	FAM	ACACCAAATTCATCAAGCA	NFQ	200
SV40 LT forw			GGAATATTCCTCTGATGAGAAAGGC		400
SV40 LT rev			CTCCATCTTCCATTTTCTTGTACAGAG		400
SV40 LT probe[1]	123	FAM	ATCTCCTCCTTTATCAGGATGAAACTCCTTGCATTT	TAMRA	200
SV40 VP1 forw			ACCAGTGCAAGTGCCAAAGC		400
SV40 VP1 rev			ATCAGGATTGCCCATTTGAGG		400
SV40 VP1 probe[1]	121	FAM	AAGCACTCCACCTCAGTGAAGCTGTCTACTCC	TAMRA	200

[1]TaqMan probes are labelled with FAM: 6-carboxyfluorescein and TAMRA: 6-carboxytetramethylrhodamine

[2]minor groove binding probes are labelled with FAM: 6-carboxyfluorescein and NFQ: Non-Fluorescence Quencher,

LT: Large T Antigen, NCCR: Non-Coding Control Region, VP1: Virus Protein1.

from a variety of viruses including HPyV [32]. In other solid tumor specimens, DNA extraction was performed by conventional tissue lysis using proteinase K at 56°C overnight, followed by DNA precipitation with 96% ethanol. The DNA yield and purity were determined by photometric analysis using the Spectrophotometer U-2000 (HITACHI, Japan).

RQ-PCR analysis

Primers and TaqMan® probes (Eurogentec) or minor groove binding probes (Life technologies) for RQ-PCR analysis of JCV, BKV and SV40 DNA target sequences were designed using the Primer Express software Version 2.0.0 (Life technologies). The primer/probe systems targeting virus protein 1 (VP1) and an overlapping region coding for the large TAg and small TAg genes were designed and optimized for uniform amplification protocols with an annealing temperature of 60°C (Table 3). For BKV analysis, additional primers and probe were designed for the non-coding control region (NCCR) to cover a greater proportion of the viral genome (Table 3).

All reactions were set up in a total volume of 25µl containing the RQ-PCR Mastermix Plus (Eurogentec) and 6µl DNA template. The amplifications were carried out using the ABI Prism© 7700 or ABI Prism© 7900 Sequence Detector (Life technologies) with an initial denaturation at 95°C for 10 minutes, followed by 50 cycles consisting of denaturation at 95°C for 15 seconds and annealing plus extension at 60°C for 60 seconds. Strict precautions were undertaken to prevent exogenous contamination of the PCR assays. To reduce the risk of false-positive results due to contamination with PCR products, desoxythymidintriphosphat was partially replaced by desoxyuraciltriphosphat in the reaction mastermix, and a desoxyuraciltriphosphat glycosylase step was performed prior to each PCR reaction, as described previously [32]. Moreover, multiple negative controls lacking template DNA were included in each assay to monitor and exclude the possibility of contamination [32]. Plasmid standards of JCV (pJCV GS/B) [33] and SV40 (Simian virus 40 strain 777), were used for the establishment of standard curves and as positive controls in each PCR run as well as for spiking experiments in urine samples using defined viral copy numbers. The plasmids were kindly provided by K. Dörries, Institute of Virology und Immunobiology, University of Würzburg, Germany). BKV-positive samples from stem cell transplant recipients obtained from our diagnostic center (LabDia Labordiagnostik/Children´s Cancer Research Institute, Vienna, Austria) including urine (n=12), peripheral blood (n=5), and cerebrospinal fluid (n=4) containing precisely quantified virus copy numbers ranging from 10^3 to 10^{10}/ml have been exploited as positive controls and reference for the establishment of detection limits of the PCR detection systems described herein.

Specificity and sensitivity of RQ-PCR assays

Potential cross-reactivity of the primers and probes with related polyomaviruses or human DNA sequences has been excluded by testing against different polyomavirus strains and human genomic DNA. For all viruses tested, the detection limit of the indicated assays (Table 3) was 10-100 target copies per reaction, as determined by serially diluted standards and spiking experiments.

Quality control, virus copy number quantification, and dynamic range

Adequate quality and quantity of DNA isolated from tumor specimens was assessed by parallel RQ-PCR amplification of the human single-copy housekeeping gene beta2-microglobulin [32]. Results exceeding $5x10^3$ control gene copies per reaction were obtained in all specimens from the entire range of tumor entities, medulloblastoma being the only exception, where lower control gene copy numbers were observed in 18/30 specimens investigated. For standardization of the quantitative virus detection assays, serial dilutions of plasmid standards quantified by spectrophotometric and fluorometric measurements were used as external references for the calculation of virus particle numbers. Standard curves with these serial dilutions were established to permit linear virus quantification across at least seven logs covering a range from 10^2 to 10^7 virus particles. The calculation of virus copy numbers per cell was based on the quantification of beta2-microglobulin gene copies in each specimen tested, and was generally expressed as virus copy number per 10^6 cells.

Statistics

The one-sided 95% confidence intervals of the proportion of virus-positive samples were calculated for each tumor entity. In the presence of negative test results for viral sequences in the tumor samples investigated, the analysis of ≥30 different specimens from each tumor entity provided a probability of 90% for the lack of correlation between the presence of virus and the respective tumor type.

Results

Testing for BKV, JCV and SV40 DNA sequences in diagnostic tumor samples

More than 500 specimens from 16 tumor entities outlined in Table 1 were screened for the presence of large TAg /small TAg and VP1 gene sequences from the polyoma viruses BKV, JCV and SV40. The selection of target genes was based on their documented importance for the oncogenic properties of these viruses in experimental animal models. Among the specimens tested, only two revealed positive findings, including one AML and one oligodendroglioma specimen, which displayed large TAg/small TAg signals of the JC virus (Table 1). Quantitative analysis of the viral signals indicated the presence of 10^3-10^4 virus copies per 10^6 tumor cells. None of the tumor specimens within this series revealed evidence for the presence of BKV or SV40 DNA. The rare occurrence of JCV sequences appears to reflect an accidental phenomenon which might be attributable to occasional persistence of the virus in the respective tissue following primary infection. Hence, despite the employment of highly sensitive and specific detection methods, our systematic screening covering a large spectrum of primarily pediatric malignancies does not support published evidence for the presence of these polyoma viruses in tumor tissue, including particularly brain tumors [7,11]. It is necessary to consider, however, that all patients included in this series were diagnosed and treated in Austria, thus leaving the question unanswered whether geographic differences in the occurrence of these viruses might play a role in their association with malignant disease.

Screening for BKV DNA in neuroblastoma specimens from different European countries

A study performed by Flaegstad et al. in Swedish patients reported a 100% incidence of BKV in a series of 18 neuroblastoma samples [9]. In contrast to these findings, our analysis of 39 Austrian patients did not reveal any evidence for BKV DNA sequences in the diagnostic tumor specimens. To address the possibility of regional differences, we have sought to obtain neuroblastoma samples from different European countries, and were successful in receiving diagnostic specimens from six additional countries including Belgium, France, Germany Portugal, Romania and Spain (Table 2). A total of 111 neuroblastomas, including the samples from Austria, were screened by RQ-PCR for BKV genes coding for the large T-antigen, small T-antigen, VP1 and the NCCR. Virtually all neuroblastoma samples investigated tested negative for the indicated viral genes, the only exception being a single sample from Spain which revealed signals for BKV large T-antigen, VP1 and NCCR genes, suggesting the probable presence of the entire viral genome (Table 2). The assessment of virus copy numbers indicated less than 10^4 virus copies per 10^6 cells. The anecdotal presence of BKV in less than 1% of the neuroblastoma patients analyzed by a highly sensitive and specific technique does not support the prominent association of BKV with neuroblastoma reported earlier [9]. However, our study cannot exclude a regional association of BKV with neuroblastoma which might be prevalent in Scandinavia. In order to firmly establish whether the frequent detection of BKV sequences in neuroblastoma reported in the indicated study indeed represents an intriguing epidemiological phenomenon, and was not attributable to other factors including technical issues, investigation of independent patient cohorts from the region of interest would be warranted.

Discussion

The lack of evidence for the presence of JCV, BKV and SV40 in the study presented may be related to various factors beyond regional differences. The considerable sequence similarity between polyomaviruses, which is in the range of 70%, raises the issue of cross-reactivity between detection assays for these viruses, and possibly other viral sequences contained in the human genome. Efficient isolation of viral DNA from clinical specimens is a prerequisite for reliable downstream analyses, and the extraction methods employed in the present study were shown to be appropriate [32]. Aliquots of the tumor DNA samples investigated had been used previously in PCR-based screening for adenoviruses, and the presence of low virus copy numbers was demonstrated in several instances, thus indicating adequate purification of amplifiable viral DNA [12]. Screening techniques used decades ago may have displayed insufficient specificity facilitating false-positive findings. The high level of specificity and sensitivity provided by the RQ-PCR approaches employed in the current study in the presence of appropriate controls should largely preclude incorrect results. Moreover, the implementation of appropriate measures preventing carryover contamination in PCR-based analysis has certainly improved over the past years [12,34,35]. Nevertheless, confirmation of PCR-positive findings by independent techniques, such as fluorescence in situ hybridization (FISH) to intracellular viral sequences, is certainly desirable to render any observations of PCR-based screening approaches more reliable [12].

In contrast to viruses such as the human T-lymphotropic virus 1, the differences in the endemic occurrence of polyoma viruses may be less pronounced. Nevertheless, the reported prevalence of BKV in the United Kingdom is >80%, in China >40%, and in Japan only 5%, and such differences could, at least in part, explain the disparity between reports on virus detection in tumor tissue [7,13,36,37]. A geographic impact therefore cannot be entirely excluded. This notion is exemplified by the lack of evidence for the presence of SV40 in our study, because the contaminated vaccine held responsible for the occurrence of SV40-related diseases apparently was never used in Austria [14,15]. It may be of interest in this regard that some of the studies reporting a high prevalence of SV40 in brain tumors are from Italy where the population was exposed to the SV40-contaminated polio vaccine [14,38]. However, epidemiological data from Italy do not show a particularly high prevalence of brain tumors. These occur at a considerably higher frequency in other parts of Europe including Scandinavia and Southeastern European countries where the contaminated vaccine was not used [39]. Recent studies based on systematic screening of brain tumors reported occasional occurrence of HPyV, but did not provide any evidence for an association between presence of the virus and tumor formation [20,40]. These results highlight the importance of functional studies to link detection of viral sequences in tumor tissue to the pathogenesis of the malignancy investigated. In this regard, the screening for the presence of viral DNA can only be regarded as a first step towards understanding the processes involved in viral oncogenesis.

Conclusion

The absence of viral sequences in virtually all tumor specimens included in the present study despite the use of a highly sensitive and specific screening approach does not support an implication of the polyomaviruses BKV, JCV or SV40 in brain tumors, neuroblastoma or other pediatric malignancies.

Acknowledgements

This study was performed with financial support from the Jubiläumsfonds of the National Bank of Austria (grant no. 11168). The authors wish to express their gratitude to colleagues from the Center for Medical Genetics, Ghent University Hospital, Belgium; Centre Leon Berard, Unite d´Oncologie Moleculaire, Lyon, France; Center for Pediatrics, University of Freiburg, Germany; National Institute de Saude, Lisabon, Portugal; Hospital de Cioii Louis Turcanu, Pediatric Clinic III, Temeswar, Romania; Department of Pathology, Medical School, University of Valencia, Valencia, Spain, for kindly providing their archived neuroblastoma samples for the present study.

Ethical Statement

For the human specimens informed consent is available.

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