



Herpesviruses, polyomaviruses, parvoviruses, papillomaviruses, and anelloviruses in vestibular schwannoma

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Abstract

Etiology of vestibular schwannoma (VS) is unknown. Viruses can infect and reside in neural tissues for decades, and new viruses with unknown tumorigenic potential have been discovered. The presence of herpesvirus, polyomavirus, parvovirus, and anellovirus DNA was analyzed by quantitative PCR in 46 formalin-fixed paraffin-embedded VS samples. Five samples were analyzed by targeted next-generation sequencing. Viral DNA was detected altogether in 24/46 (52%) tumor samples, mostly representing anelloviruses (46%). Our findings show frequent persistence of anelloviruses, considered normal virome, in VS. None of the other viruses showed an extensive presence, thereby suggesting insignificant role in VS.

Keywords Vestibular schwannoma · Viral etiology · Herpesvirus · Polyomavirus · Parvovirus · Anellovirus

Introduction

Vestibular schwannoma (VS) arises from the eighth cranial nerve (CNVIII) and can occur either sporadically or as a result of neurofibromatosis 2 (NF2) syndrome. The etiology of sporadic VS is unknown. Typical risk factors for malignancies, such as tobacco smoking and alcohol consumption, do not seem to play a significant role in this entity. The only identified risk factors are medical irradiation during early life and exposure to radiation hazards (Preston et al. 2002; Ron et al.

1988; Schneider et al. 2008). Studies have shown possible links of sporadic VS to certain genes (De Vries et al. 2015); however, the trigger for a tumor-forming cascade is unknown. A possible microbial etiology has been studied less.

Viruses and other microbes are estimated to cause 15% of all cancers worldwide (Plummer et al. 2016). This subject is even more topical than ever before as the rapid evolution in virus research has brought attention to newly discovered viruses. The role of viral infections as an etiological factor in intracranial tumors has previously been proposed. The prevalence of cytomegalovirus (CMV) in glioblastoma was as much as 84% among 1653 tumors (Rahman et al. 2018). Merkel cell polyomavirus (MCPyV) has been found in several head and neck tumors, including neurofibromas (1/3, 33%) and schwannomas (2/12, 17%) (Tanio et al. 2015).

Given this background, and the potential exposure of the CNVIII to middle ear viruses and other microbes, it is intriguing to speculate on a potential viral etiology of VS. A few studies have been published regarding this topic. Bhimrao et al. (2015) investigated the prevalence of certain herpesviruses in tissue microarray samples of sporadic VS and neurofibromatosis-related schwannomas, but none were detected. Håvik et al. (2018) applied ViroChip microarray ($n=15$ patients), RT-PCR for human endogenous retrovirus K (HERV-K) and herpes simplex virus-1 and -2 (HSV-1, -2) RNA ($n=46$), and whole genome sequencing ($n=2$). HERV-K was detected, but other findings remained modest. The ViroChip used in the study was published 20 years ago.

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The DNA-virus families in focus in the present study (*Herpesviridae*, *Parvoviridae*, *Polyomaviridae*, *Papillomaviridae*, and *Anelloviridae*) comprehend oncogenic and cancer-associated viruses, as well as viruses with oncolytic abilities (Beral et al. 1990; Chang et al. 1994; Feng et al. 2008; Gissmann et al. 1984; Kreuter et al. 2018; Nüesch et al. 2012; Syrjänen et al. 1983; Väisänen et al. 2019). Within the total of nine viruses of the human herpesvirus family, human herpesvirus 8 (HHV-8) causes Kaposi's sarcoma (Beral et al. 1990; Chang et al. 1994), and Epstein-Barr virus (EBV) is strongly associated with, e.g., Burkitt's lymphoma and nasopharyngeal carcinoma (Henle et al. 1970). HSV-1 and -2 and varicella zoster virus (VZV), on the other hand, are neurotropic and widespread, but without clear association with malignancies.

Human polyomaviruses comprise a group of 15 viruses, the majority discovered during the last decade, and most with a high seroprevalence among humans. MCPyV causes Merkel cell carcinoma in humans; however, the association of polyomaviruses to other tumors is still unknown (Csoboz et al. 2020; De-Thé et al. 1978; Feng et al. 2008). Among parvoviruses, many viruses have been discovered in the past two decades: human bocaviruses 1–4 (HBoV 1–4), parvovirus 4 (Parv4), and the protoparvoviruses bufaviruses (BuV), cutaviruses (CuV), and tusaviruses (TuV; (Söderlund-Venermo 2019). HBoV1 causes pediatric respiratory tract infections, whereas CuV has been associated with cutaneous T cell lymphoma (Kreuter et al. 2018; Phan et al. 2016; Qiu et al. 2017; Väisänen et al. 2019). However, the clinical roles of the other recently discovered viruses need further investigation.

Anelloviruses encompass a broad scale of viruses, including the ubiquitous torque teno viruses (TTV). No causal association to any disease has been made; thereby, they are considered a part of the normal human virome.

The unknown etiology of VS, with abundant virus load in the anatomical proximity constituting a potential trigger, formed the motivation for the present study. Quantitative PCR (qPCR) is the gold standard for virus detection due to its high sensitivity and specificity, whereas next-generation sequencing (NGS) enables wider multiplex-screening and analysis of the virus sequence. We performed a retrospective cross-sectional study to evaluate the prevalence of viral DNAs in VS by qPCRs and NGS, targeting many recently discovered virus entities not studied before.

Materials and methods

Ethics

A research ethics committee approval (HUS/31/07.03.2019) and an institutional research permission (HUS/332/2019) were obtained at the Helsinki University Hospital.

Patient characteristics and clinical specimens

The patients were treated at the Departments of Neurosurgery and Otorhinolaryngology – Head and Neck Surgery, Helsinki University Hospital, Helsinki, Finland, between 2008 and 2018. Altogether 46 FFPE tumor samples from 46 patients were collected from the Helsinki Biobank (permission no. §73/15.05.2019, HUS/118/2019). The clinical information was recorded from hospital charts.

There were 30 females (65%) and 16 males (35%). The mean age at operation was 52.8 years (median 53.1, range 32.3–80.9). Forty-four patients had sporadic tumors. One patient had bilateral VS suggestive of neurofibromatosis 2, and one patient had multiple meningiomas, although the genetic testing for neurofibromatosis was negative. Only six (13%) patients were known smokers, while the smoking status was unknown for 26 (57%) patients. The alcohol use was not recorded for 85% of the patients. Three (7%) patients used immunomodulatory drugs, and two (4%) patients had diabetes. VS had an extracanalicular extension in 44 (96%) cases. Radiation therapy (RT) was given altogether to six (13%) patients, but preoperatively only to one (2%) patient.

The samples were collected from FFPE tissue blocks as 2-mm punch biopsies in a PCR-sterile manner from the site of the tumor tissue — one sample for the qPCR and another from a nearby location for NGS. All samples were collected in 1.5 ml microcentrifuge tubes and stored until DNA extraction.

DNA extraction

DNA was extracted using QIAamp DNA mini kit (Qiagen, Heiden, Germany) according to the manufacturer's protocol, with slight modifications: paraffin removal was done twice and 40 µl of proteinase K was used. The DNA preps were eluted in 100 µl AE buffer and stored at –20 °C.

Evaluation of DNA quality and human cell quantity was done by subjecting the samples to the reference gene *RNase-P* qPCR, as described earlier (Toppinen et al. 2015).

Virus DNA detection by qPCR

The viruses analyzed with qPCR are listed in Supplemental Table 1. For the nine human viruses of *Herpesviridae*, a three-tube multiplex qPCR assay (HSV-1, HSV-2, and VZV; HHV-6A, HHV-6B, and HHV-7; EBV, cytomegalovirus (CMV), and HHV-8) was applied to target each virus (Pyöriä et al. 2020). For three virus members of *Polyomaviridae*, BK polyomavirus (BKPyV), JCPyV, and MCPyV, the LT genes were detected by singleplex qPCR assays

(Dumoulin and Hirsch 2011; Goh et al. 2009). As for the members of *Parvoviridae*, parvovirus B19 (B19V) was detected and quantified with a pan-B19 qPCR, targeting the *NS1* genes of all three genotypes (Toppinen et al. 2015), whereas HBoV1–4 were detected by a multiplex qPCR, targeting the NS1 regions of all four bocaviruses (Kantola et al. 2010). CuV, BuV, and TuV were detected and quantified by a multiplex qPCR (Väisänen et al. 2019) targeting the NS1 region of BuV and VP2 regions of TuV and CuV. For TTV detection, the conserved untranslated region (UTR) was targeted by a multiplex qPCR, which detects most TTV types (Toppinen et al. 2020; Väisänen et al. 2022). All real-time qPCR assays were performed with AriaMx Realtime PCR System (Agilent Technologies, Santa Clara, CA).

Molecular biology grade water was included in all PCR reactions as non-template control. Ten-fold diluted plasmids (10^1 – 10^6), containing each viral target amplicon, served as PCR standards and as positive controls. To prevent contamination, the master mix components, tumor samples, and plasmids were each handled in separate rooms. Laminar hoods were used when preparing and handling the samples and plasmids.

Virus DNA detection by NGS

The viruses analyzed with targeted NGS are listed in Supplemental Table 1. The DNA was mechanically fragmented with a Covaris E220 with a target length of 200 nt. Subsequently, the libraries were prepared with the KAPA HyperPlus kit (Roche) using unique Dual Index Adapters (Roche). Targeted enrichment of the viral DNAs was performed using a custom panel of biotinylated RNA-probes covering the full length of the viral genomes (Arbor Biosciences) as described (Toppinen et al. 2020). Each sample was individually enriched via two rounds of hybridization, following the manufacturer recommendations for low input DNA (MyBaits v5 kit; Arbor Biosciences). The probes were 100 bp in length and designed with $2\times$ tiling. Kapa Universal Blockers (Roche) were used to block unspecific binding to the adapters during hybridization.

During library preparation and viral enrichment, the libraries were amplified $3\times$ 13–25 cycles. The clean-up steps were performed with $0.8\times$ KAPA HyperPure Beads (Roche). The enriched libraries were quantified with the KAPA Library Quantification Kit (Roche) using Stratagene 3005P qPCR System (Agilent) and pooled for sequencing on NovaSeq 6000 (S1, PE151 kit; Illumina).

NGS analysis

The data analysis was done with TRACESPipeLite, a streamlined version of TRACESPipe (Pratas et al. 2020).

TRACESPipeLite is fully automatic and provides for each reconstructed viral genome, the consensus sequences, breadth and depth coverage, and the associated profiles, among other information and quality controls. The paired-end reads were trimmed and collapsed with AdapterRemoval, cutting ambiguous bases at the 5'/3' termini with quality scores below or equal to two. Reads shorter than 20 bases were discarded. FALCON-meta (Pratas et al. 2018) was used to find the highest similar reference from the NCBI viral database. The reads were aligned with BWA (Li and Durbin 2010) using a seed length of 1000 and a maximum diff of 0.01. Read duplicates were removed with SAMtools (Li et al. 2009) and the consensus sequences reconstructed with BCFtools (Li 2011). The coverage profiles were created with BEDtools (Quinlan 2014). When in low breadth coverage ($<15\%$), the individual reads were manually inspected and confirmed by BLAST. The pipeline is freely available, under MIT license, at <https://github.com/viromelab/TRACESPipeLite>, along with the code (included in the TRACESPipeLite repository).

Results

Presence of virus DNA by qPCR

The viruses analyzed with qPCR are listed in Supplemental Table 1. Altogether, 26 viral DNA findings were made by qPCR in 24/46 (52%) tumors (Table 1). One tumor had three viral DNA findings, and two tumors had two viral findings each. The viral loads ranged between 9 and 121,000 copies per 1 million cells (cpm). TTV DNA was detected in altogether 21/46 (46%) tumors, with viral loads of 1.38 – 8.91×10^4 , mean 1.08×10^4 cpm. In 15/46 (33%) tumors, TTV PCR was positive in duplicate analysis. MCPyV was detected in 3/46 (7%) tumors. One tumor was positive for EBV, one tumor for B19V, and one tumor for HHV-7 DNA. Duplicate analysis performed for all these six tumor samples tested negative, probably due to low virus loads (mean 44.8, median 39.3, range 8.75–89.3 cpm). Sixteen out of 21 viruses from our screen were not detected in any sample. The two patients with probable NF2 tested both positive for TTV but negative for all other viruses. The human *RNaseP* served as evaluation of DNA quality and human cell quantity and its qPCR results varied between 10^2 and 10^5 copies/ μ l among all samples with a mean of 4.4×10^3 .

Presence of virus DNA in NGS

The viruses analyzed with targeted NGS are listed in Supplemental Table 1. Tumor samples from five patients, previously analyzed with qPCR, were chosen for targeted enrichment and sequencing, four samples because of their viral DNA positivity (MCPyV, EBV, HHV-7) and one sample

Table 1 Different virus types detected with qPCR in 46 vestibular schwannoma samples

		Viral DNA findings	Virus load (virus DNA/1000 000 cells)
<i>Herpesviridae</i>	Human herpesvirus 7	1	2.54×10^1
	Epstein–Barr Virus	1	8.75×10^0
<i>Polyomaviridae</i>	Merkel cell polyomavirus	3	Mean 6.87×10^1
<i>Parvoviridae</i>	Parvovirus B19	1	2.86×10^1
<i>Anelloviridae</i>	Torque teno virus	21	1.08×10^4

without any viral DNA by qPCR as a negative control. Also, one peripheral neural tissue sample was used as a control. Human endogenous retrovirus (HERV) served as an internal control, and all the six samples were positive for it. One tumor gave a single read for B19V DNA (one manually confirmed read). Apart from HERV and B19V, all the samples were DNA negative for all the other 40 tested viruses.

Discussion

This study is an up-to-date qPCR and NGS screening to detect both well-known and recently discovered viruses (herpesviruses, polyomaviruses, parvoviruses, and papillomaviruses) in 46 VS. QPCR for 20 viruses of three virus families (herpesviruses, polyomaviruses, and parvoviruses; Supplemental Table 1) resulted in six positive virus DNA findings (MCPyV, EBV, B19V, HHV-7). MCPyV was detected in three (3/46, 6.5%) tumors, with approximately similar results of two in 12 schwannomas (17%) by Tanio et al (2015). A possible “hit-and-run” mechanism has been proposed for MCPyV (Prezioso et al. 2021), but in our study, we were unable to investigate such potential because of the lack of parallel lymph node samples. EBV, B19V, and HHV-7 were each detected once.

QPCR was supplemented with targeted NGS to both verify the findings, and to broaden the spectrum of screened viruses. Forty-one viruses of seven virus families (Supplemental Table 1) in five tumors, previously analyzed by qPCR, were investigated with targeted enrichment and sequencing. Only B19V DNA was detected in one tumor sample.

In addition, wide-spread anelloviruses, namely, TTVs, were screened by qPCR. Their DNA was found in 21/46 (46%) tumors. This was expected, since TTVs are considered normal flora (Arze et al. 2021). Given that TTV is actively replicating in healthy individuals and that its clinical significance has not yet been determined, the presence in VS is insufficient proof of association, not to mention causality.

QPCR offers a solid, sensitive base for virus screening. NGS with targeted enrichment is also well justified in virus research. We used a panel that includes 90% of the DNA viral families and majority of viruses with a clinical significance in humans (Supplemental Table 1). Whole genome sequencing has undeniably an even broader potential in the extent of screening, but its sensitivity for virus detection is low, and therefore, the approach with targeted viruses was preferred in this study. NGS overcomes the limitations of FFPE samples (low amount of DNA, DNA fragmentation, DNA deamination) that can potentially affect the performance of PCR.

QPCR and NGS results were largely consistent with each other, in that only limited amounts of virus DNAs were found. This is in accordance with previous studies (Bhimrao et al. 2015; Havik et al. 2018). The more recently identified viruses searched for in our study (bocaviruses, bufavirus, cutavirus, tusavirus, polyomaviruses) were not studied in the previous reports. HERV was detected in all our sequenced samples and serves as an internal control rather than a potential etiological factor.

In this study, we were unable to repeat our original qPCR results with duplicate qPCR analysis or detect the same viruses with NGS. This might be due to low virus load in combination with a second freeze–thaw round needed for NGS with possible degraded DNA. In addition, although FFPE samples have previously been shown to be a useful source for virus screening (Jauhiainen et al. 2021), it is possible that with this tumor type, larger samples and fresh tumor material would be beneficial as they may increase the amount of intact DNA and improve its quality (Mielonen et al. 2022).

When searching for possible viral etiology for VS, one has to bear in mind that the mere presence of virus DNA in a tissue sample does not indicate a causative role in disease development, and neither does its absence. A possible “hit-and-run” phenomenon may be involved as the trigger (Ferreira et al. 2021), but this would warrant more advanced techniques, not yet available, to be investigated.

Conclusion

Based on the current and previous studies, it seems unlikely that herpesviruses, polyomaviruses, parvoviruses, and papillomaviruses play a significant etiological role in VS. Thus, the etiology of VS remains to be studied further.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s13365-023-01112-8>.

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Data Availability For possible data requests, the first Author should be contacted.

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