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Oncogenic Viruses in Skull Base Chordomas

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BACKGROUND: Chordomas are rare tumors assumed to derive from notochordal remnants. We believe that a molecular switch is responsible for their malignant behavior. The involvement of oncogenic viruses has not been studied, however. Thus, in the present study, we investigated the presence of oncogenic viruses in chordomas.

METHODS: DNA and RNA from snap-frozen chordoma (n = 18) and chondrosarcoma (n = 15) specimens were isolated. Real-time PCR or RT-PCR was performed to assess the presence of multiple oncogenic viruses, including herpesviridae (herpes simplex virus [HSV]-1, HSV-2, Epstein-Barr virus [EBV], cytomegalovirus, human herpesvirus [HHV]-6, HHV-7, and Kaposi’s sarcoma-associated herpesvirus), polyomaviridae (parvovirus B19 [PVB19], BK virus, JC virus, Simian virus 40, Merkel cell polyomavirus, human polyomavirus [HPyV]-6, and HPyV-7), papillomaviridae, and respiratory viruses. Immunohistochemistry (IHC) and in situ hybridization (ISH) were used to validate the positive results.

RESULTS: PVB19 DNA was detected in 4 of 18 chordomas (22%) and in 1 of 15 chondrosarcomas (7%). IHC recognizing the VP2 capsid protein of PVB19 showed a positive cytoplasmic staining in 44% of the cases (14 of 32). HHV7 DNA was present in 6 of the 18 chordomas (33%).

CONCLUSIONS: Viral involvement in the etiology of chordomas is likely, with PVB19 the most distinguishing.

INTRODUCTION: Chordoma is a rare neoplasm that is believed to originate from remnants of notochordal cells, located almost exclusively in the axial skeleton. Long-term management of these tumors is limited, given that a high percentage of patients will experience regrowth of the tumor after multimodal therapy. Due to this predicament, research over the last decade has focused on the etiology of chordoma tumors in the hope of revealing biomarkers that can enhance our understanding of tumor growth and treatment management. A widely accepted view is that notochordal cells develop into chordomas subsequent to a benign notochordal tumor stage. The pathophysiological mechanism underlying this switch from indolent cells to malignant local invasive behavior is unknown. However, while consolidated knowledge is accumulating on the role of cellular and molecular mechanisms responsible for this malignant phenotype, no Genomic DNA of EBV was found in 22% of the samples; however, no positive results were found on ISH. None of the chordoma cases showed any presence of DNA from the remaining viruses.

Key words
- Cancer
- Chondrosarcoma
- Chordoma
- Oncogenic virus
- PVB19
- Virus

Abbreviations and Acronyms

CMV: Cytomegalovirus
EBV: Epstein-Barr virus
HHV: Human herpesvirus
HPV: Human papillomavirus
HPyV: Human polyomavirus
HSV: Herpes simplex virus
ISH: In situ hybridization
KSHV: Kaposi’s sarcoma-associated herpesvirus
MCPyV: Merkel cell polyomavirus

PVB19: Parvovirus B19
TMA: Tissue microarray

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Viral infections are an important etiological factor in the development of many tumors and are therefore a common target for investigation. A small group of oncoviruses (oncoviruses), including hepatitis viruses, human papillomaviruses (HPVs), human herpesviruses (HHVs), parvoviruses, and human polyomaviruses (HPyVs), are known to be responsible for a large proportion of virus-related cancers. Such infectious agents are estimated to be responsible for 12%–15% of human tumors worldwide and to account for approximately 20% of cancer-related deaths. Although the exact mechanisms by which oncoviruses induce malignant biological behavior has yet to be unraveled, one proposal assumes causation by dysregulation of apoptosis due to genetic and epigenetic mechanisms. A viral etiology for chordomas may be plausible, given that merely a fraction of the patients with notochordal remnants develop a malignant chordoma phenotype. In addition, Stephens et al. described the existence of chromosomal instability in chordomas and proposed a single crisis as responsible for this phenomenon, otherwise known as chromothripsis. Because viruses have been implicated in the induction of chromosomal instability, a possible association has been investigated.

In the present study, we examined the incidence of the most prevalent oncoviruses in skull base chordomas, their control tissues, and chordrosarcomas. The viruses under investigation include the hepatitis B virus, hepatitis C virus, HPVs, Merkel cell polyomavirus (MCPyV), and HHVs, including herpes simplex virus (HSV)-1/2 (HHV-1/2) Epstein-Barr virus (EBV; HHV-4), cytomegalovirus (CMV; HHV-5), HHV-7, and Kaposi’s sarcoma-associated herpesvirus (KSHV; HHV-8). In addition, we also examined the involvement of HPyV-6 and -7, JC virus, BK virus, parvovirus B19 (PVB19) and Simian virus 40.

**MATERIALS AND METHODS**

**Tumor Tissues and DNA/RNA Isolation**

Snap-frozen tissue specimens from 18 skull base chordomas and 15 chordrosarcomas were collected from surgeries performed at the Neurosurgery Department, Maastricht University Medical Center. Normal tissue, consisting of either bone or fat tissue removed for surgical exposure, was also collected from these surgeries. A tissue microarray (TMA) was generated from 32 formalin-fixed, paraffin-embedded blocks from 24 patients with chordomas, obtained from the Maastricht Pathology Tissue Collection. The storage and use of tissue and patient data were conducted in accordance with the Code for Proper Secondary Use of Human Tissue in The Netherlands (http://www.fmwv.nl). The use of tissue was approved by the local Ethical Committee (METC 16-4-267). DNA and RNA isolation was performed using TRIzol reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA), according to the manufacturer’s protocol. To prevent contamination, the isolation of nucleic acids was performed in a specially designed, isolated environment for DNA and RNA extraction. For real-time PCR, all samples were spiked with murine CMV DNA or RNA, which served as an amplification control following extraction.

**PCR Analysis for Herpesviruses, BK and JC Viruses, and Respiratory Viruses**

Real-time PCR for all herpesviruses was performed using primers and probes as described previously, with the exception of primers targeting HHV-7, for which the primers and probe consisted of the following nucleotide sequences: forward primer, 5’- AACGTGATGCTAAACACGCA-3’; reverse primer, 5’-TAGTTCCACAGCCTGCAATCCG-3’; probe, FAM-5’-TGTATGCTGATGCGTGGAAACAGG-3’. For respiratory virus detection, a separate reverse-transcription step was performed using TaqMan reverse-transcriptase reagents, random hexamers (Applied Biosystems, Foster City, California, USA), and incubation for 10 minutes at 25°C, for 30 minutes at 48°C, and finally for 5 minutes at 95°C. Consequently, for all targets, the PCR mix consisted of 20 μL of isolated DNA (or produced cDNA), primers and probes, and 1× Absolute QPCR Mix (ABgene, Epsom, UK).

The PCR protocol consisted of 15 minutes at 95°C, followed by 42 cycles of 15 seconds at 95°C and 1 minute at 60°C. All qualitative real-time PCR reactions were performed using an ABI Prism 7900 HT PCR machine (Applied Biosystems, Foster City, California, USA), and Ct values were determined using a threshold value of 0.05 and automatic baselining. The quality of the assays was ensured by positive and negative controls, as well as by a test on amplification inhibition in each sample by an external amplification control. DNA or RNA controls served as positive controls. Artificial DNA controls were constructed by cloning the PCR product into pGEM-3Z vectors, culturing of *Escherichia coli* containing the construct and subsequent plasmid isolation. The isolated plasmids were used as artificial DNA controls. For the generation of artificial RNA controls, RNA was initially constructed using pGEM-z2 vectors containing T7 RNA polymerase promoters flanking the multiple cloning region (Promega, Leiden, The Netherlands) into which the respective ampiclons were cloned. Subsequently, RNA constructs containing the ampiclons were generated and used as artificial RNA controls using T7 RNA polymerase.

**PCR Analysis for HPVs**

Analysis for HPVs was performed as described previously using GP5+/6+-mediated PCR. In short, a step at 94°C for 4 minutes, 40 cycles of 94°C for 1 minute, 40°C for 2 minutes, and 72°C for 1.3 minutes, and then a final step at 72°C for 4 minutes were completed on the PTC-200 PCR apparatus (Bio-Rad, APP/PCR-001). Acrylamide gels were analyzed using a microplate reader (Bio-Rad). For the assessment, both high-risk and low-risk HPV-positive controls were applied.

**PCR Analysis for Polyomaviruses**

Detection of Simian virus 40 was performed using previously published primers with the following PCR conditions: 10 minutes at 94°C, 40 cycles of 30 seconds at 94°C, 30 seconds at 55°C, 45 seconds at 72°C, and 7 minutes at 72°C. The positive control was kindly provided by Dr. Verschoor from the Biomedical Primate Research Centre, Rijswijk, The Netherlands. RT-PCR for MCPyV (VP1 and M antigens), HPyV-6, and HPyV-7 were performed as described by Schwalter et al. and Schrama et al.
Immunohistochemistry and PCR Analysis for PVB19

Real-time PCR for PVB19 was performed with primers and a TaqMan probe as described by Dennert et al. The PCR mix consisted of 20 µL of isolated DNA, a final concentration of 600 nM of each primer, and 200 nM of the probe and 1× Absolute QPCR Mix. All real-time PCR reactions were performed in an ABI Prism 7900 PCR machine and quantified using a standard curve. The PCR assay used had a linear quantitative range from $10^7$ copies to $1 \times 10^5$ with a detection probability >95%. Below this range, semiquantitative detection was performed by extrapolation of the standard curve. The quality of the assays was ensured by positive and negative controls, as well as by a test on amplification inhibition in each sample with an additional external amplification control. For quantification of viral loads, standard curves were included for each run.

Immunohistochemistry (IHC) analyses were performed using the EnVision FLEX Mini Kit (Agilent Technologies, Santa Clara, California, USA) using an indirect method.32,33 Slides were cut to 4-µ thickness, adhered to Superfrost Plus Micro Slides (VWR, Radnor, Pennsylvania, USA), deparaffinized, and subjected to antigen retrieval using Tris/EDTA Target Retrieval solution buffer, pH 9 (Agilent Technologies) for 20 minutes at 97°C. Endogenous peroxidase activity was quenched with 3% H2O2 for 30 minutes. After overnight incubation at 4°C with primary anti-PVB19 antibody (1:100 MAB293), VP2 capsid protein; MilliporeSigma, Darmstadt, Germany), the secondary antibody horseradish peroxidase—conjugated goat anti-mouse/rabbit (EnVision FLEX, High pH, K801021-2, Agilent-Dako) was incubated for 2 hours at room temperature. Peroxidase activity was visualized by incubation of the slides in 3,3′-diaminobenzidine tetrahydrochloride plus chromogen/substrate buffer (EnVision). Counterstaining was performed by incubation in hematoxylin and eosin using the Mayer’s staining protocol. A positive control sample was acquired from a cardiac biopsy specimen from a patient with chronic dilated cardiomyopathy with a high PVB19 viral load in the heart.

In Situ Hybridization EBV

Colorimetric in situ hybridization (ISH) was performed manually on 4-µm sections of formalin-fixed, paraffin-embedded biopsy tissue on Superfrost Plus slides using the RNAscope 2.0 Brown Assay Kit (Advanced Cell Diagnostics, Hayward, California, USA). In brief, sections were treated with heat and protease, followed by hybridization with the probe cocktail, which includes 17 pairs of probes within a target region (25–968) of the virus. The development of hybridization was visualized with 3,3′-diaminobenzidine (Advanced Cell Diagnostics). Slides were counterstained with 50% hematoxylin for 2 minutes and then mounted with xylene-based SHUR/Mount (Triangle Biomedical Sciences, Durham, North Carolina, USA).

RESULTS

PVB19 DNA was identified in 4 of 18 (22%) chordoma specimens. In contrast, only 1 of 15 (7%) chondrosarcoma specimens showed a positive signal after 39 PCR cycles. Viral load in the chordoma samples ranged from 104 copies/mL to 2106 copies/mL. The single positive chondrosarcoma sample had 52 copies/mL. The samples were excised from a group of adult patients with a median age of 51 years (range, 35–75 years), with an exact 2:1 male to female ratio (12 males, 6 females).

In 4 patients, normal control tissue was also available. For these patients, we assessed whether PCR would detect the presence of PVB19 only in the tumor tissue, or in both the tumor tissue and normal tissue. We found viral involvement in 2 out of the 4 patients (50%). In both of these patients, viral DNA was found in both normal tissue and tumor tissue.

Of the 32 chordoma tissue cores in the TMA stained for chordoma, positive cytoplasmic staining for parvovirus B19 VP2 capsid protein was seen in 14 (44%) (Figure 1). The 4 samples that were positive on PCR were from cases in which no formalin-fixed, paraffin-embedded material was available at the time of the TMAcompilation, and thus a correlation could not be evaluated. None of the chordomas tested positive for other paroviruses (BK virus, JC virus, Simian virus 40, MCpYV, HHV-6, and HHV-7) by PCR.

Chordomas were also tested for the presence of herpesviruses. Four of the 18 samples (22%) were positive for EBV, with copy numbers ranging from 59 to 290/mL. Normal control tissue from the patients with chordomas was used to help determine the specificity of the signal. Our analysis revealed EBV RNA only in the control tissues, and not in the corresponding chordoma tissues of the same patient. ISH analysis for the location of EBV RNA in the tumor samples revealed no RNA (Figure 2).

For HHV-7, in 6 out of 18 patients (33%), PCR indicated amplified DNA, with copy numbers ranging from 112 to 4985 copies/mL. Similar to EBV, compared with normal tissue from the same patients, HHV-7 was positive in 2 of 4 chordoma samples.

Tests of other herpesviruses, including CMV, HHV-1/2, HHV-6, and HHV-8, as well as the aforementioned respiratory viruses, did not show the presence of viral DNA in the chordoma samples.

Tables 1 and 2 provide an overview of the positive results.

When considering all the expression data presented, it is to be noted that at least 1 of the 3 viruses—PVB19, EBV, or HHV-7—was present in one-half of all the chordoma specimens analyzed. In 2 specimens, all 3 viruses were expressed.

DISCUSSION

Chordomas are believed to originate from notochordal remnants, which are present in 2% of adults. The scarce nature of this tumor and chromosomal distortion indicate a possible malignant switch in phenotype in these remnants, resulting in chordomagenesis. One of the potential mechanisms by which tumor formation can occur is through the involvement of oncogenic viruses. Approximately 15% of all human tumors are caused by viruses, involving intricate processes that remain incompletely understood.

Here we describe, for the first time, the relationship of well-known oncogenic viruses and their occurrence in chordoma tissue samples. One of the most recently discovered oncogenic viruses, PVB19 was discovered in 1975 in healthy blood donors and was later related to the existence of erythema infectiosum as the causative agent. Later the virus was associated with other pathologies, including papillary thyroid carcinoma and germ cell tumors. In our cohort, the PVB19 genome was found in 22% of the chordoma specimens, compared with only 7% of the chondrosarcoma samples. In 4 of these patients, viral DNA was also detected in one-half of the normal surrounding tissues, indicating...
a larger area of previous infection. The prevalence of PVB19 infection, determined by the presence of IgG and IgM, is high in the general adult population, 70%–80%, and highest in the elderly population. However, the presence of DNA in normal tissues and blood is very rare, with reported rates ranging from 0 to 10%. Thus, an incidence of 22% for chordomas in contrast to chondrosarcomas and the general public is significantly higher. In addition, IHC staining showed a high percentage...
of positive samples, demonstrating that focal positivity of PVB19 in chordomas is not an uncommon finding. In chordomas, early PVB19 infection might explain the chromothripsis observed and thus could be responsible in part for the switch in notochordal remnants leading to chordomagenesis. Another potential mechanism could be an effect of PVB19 on the phenotype of the established tumor, either contributing to its progression rather than causing the carcinogenesis or causing impairment of the cancer cells, making them resistant to chemotherapeutic drugs. Finally, viral infection might be an opportunistic infection due to iatrogenic immunosuppression. However, considering that this phenomenon has not been observed in patients undergoing organ transplantation, such a concept is unconvincing. Thus, a more comprehensive investigation of the potential role of PVB19 in a larger cohort of chordoma samples with regard to its role in carcinogenesis, chemoresistance, and prognosis is needed.

Herpesviruses are implicated in the pathophysiology of many cancers. However, in contrast to HSV-1 and HSV-2, little is known about the contribution of HHV-7 to the etiology of cancer. HHV-7 was discovered in 1990, and an active infection has since been related to exanthema subitum in children. In addition to the skin, HHV-7 has an apparent predilection for the central nervous system, considered to be related to febrile seizures as well as viral DNA, being present in 14% of primary brain tumors. In line with this observation, our present cohort, 33% of the resected chordomas contained the HHV-7 genome. Given the location of skull base chordomas in the clival region, close to the sphenoid sinus, and the almost absent detection of HHV-7 in other internal organs, potential contamination of notochordal remnants by HHV-7 might be occurring. Considering the ubiquitous nature of HHV-7 in human saliva and mandibular glands, this likely explains its existence in bony structures surrounding the tumor.

In contrast to HHV-7, EBV has been extensively studied and has well-recognized associations with a multitude of tumors, including Burkitt’s lymphoma, gastric cancer, breast cancer, and nasopharyngeal cancer. Variable positive fractions of these tumors contain EBV DNA, ranging from 10%–16% in conventional gastric adenocarcinomas to 10%–51% in breast tumors. The difference in persistent EBV infection reported could be explained by the heterogenetic presence of the virus in tissue when examined by ISH analysis and the potential of EBV to persist lifelong in the memory B-cell compartment. With this in mind, the presence of EBV in chordomas merits further investigation, with the contrast between positive results in EBV PCR and negative results in EBV ISH possibly explained by heterogeneity in the

<table>
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<tr>
<th>Patient</th>
<th>Sample</th>
<th>PVB19 (c/mL)</th>
<th>HHV-7 (c/mL)</th>
<th>EBV (c/mL)</th>
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<td>Patient 1</td>
<td>Normal</td>
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<td>360</td>
<td>800</td>
</tr>
<tr>
<td>Patient 1</td>
<td>Tumor</td>
<td>91</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Patient 2</td>
<td>Normal</td>
<td>2621</td>
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<td>-</td>
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<td>Patient 2</td>
<td>Normal Tumor</td>
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<tr>
<td>Patient 4</td>
<td>Tumor</td>
<td>-</td>
<td>-</td>
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</tr>
</tbody>
</table>

Two sample moments (primary resection and resection of a recurrent chordoma) were available for patient 1.

PVB19, parvovirus B19; HHV-7, human herpesvirus 7; EBV, Epstein-Barr virus.
samples or the presence of EBV in neighboring non-neoplastic cells, termed bystander cells.

In conclusion, our present study indicates the prevalence of oncogenic viruses in chordomas, with variable presence of genomic DNA of BPV19, EBV, and HHV7 in our cohort. One-half of our patients demonstrated the presence of genomic DNA of at least 1 of these 3 viruses. These findings support the idea of the potential involvement of viruses in the etiology of chordomas.

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