Otarine herpesvirus-1: a novel gammaherpesvirus associated with urogenital carcinoma in California sea lions (Zalophus californianus)

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Abstract

The incidence of neoplasia in California sea lions (CSLs) is considered to be unusually high. Electron microscopic examination of some of these urogenital tumours revealed the presence of virions with typical herpes-like structure. While current attempts to cultivate this virus have not been successful, molecular studies employing DNA extracted from tumour tissues allowed both the classification of the agent and its identification in tumours and archived tissue samples. Two genome fragments generated using degenerate primers in PCR demonstrated highest identities with other mammalian gammaherpesviruses. Phylogenetic analysis showed that this novel virus, tentatively designated Otarine herpesvirus-1 (OtHV-1), grouped with members of the gammaherpesvirus subfamily and was distinct from PHV-2, a previously described pinniped gammaherpesvirus. An OtHV-1 specific PCR was established and used to investigate the presence of this virus in CSL tissues. PCR of DNA isolated from animals with these tumours, demonstrated that this virus was present in 100% (16/16) of tumours. Furthermore, DNA extracted from archived brain and muscle tissues was also positive in 29% (4/14) and 50% (7/14) of cases examined. This preliminary study provides evidence to support the hypothesis that the presence of this novel gammaherpesvirus is a factor in the development of urogenital carcinoma in CSLs. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Herpesvirus; Sea lion; Tumour; PCR

The nucleotide sequence data reported in this paper have been submitted to GSDB and have been assigned the accession numbers AF236050 (OtHV-1 Dpol) and AF236051 (OtHV-1 terminase).

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1. Introduction

The occurrence of tumours in free-ranging California sea lions (CSLs: *Zalophus californianus*) has been reported to be unusually high. Previous studies have shown that 18% (66/370) of sub-adult and adult animals admitted to a Pacific marine mammal rehabilitation centre over a 15-year period had histopathologic evidence of neoplasia, the most common of which was a disseminated carcinoma (Gulland et al., 1996). This distinct neoplasm is associated with extensive metastases typically involving sub-lumbar lymph nodes (Gulland et al., 1996; Lipscomb et al., 2000). Electron microscopic examination of some of these tumours has revealed the presence of non-enveloped nuclear and enveloped cytoplasmic virions with typical herpes-like structure (Lipscomb et al., 2000 and Lowenstine, L.J., personal communication). Preliminary classification of this virus as a gammaherpesvirus was supported by immunohistochemistry using a cross-reactive Epstein–Barr virus (EBV) monoclonal antibody and nucleotide sequence data (Lipscomb et al., 2000). Gammaherpesviruses have been implicated as being critical factors in the etiology of a number of human tumours such as Kaposi’s sarcoma, Burkitt’s lymphophoma and nasopharyngeal carcinoma.

The aim of this study was to provide further evidence to support the hypothesis that the presence of a herpesvirus is associated with the development of urogenital carcinoma in CSL. Furthermore, since the phylogenetic relationship between the herpesvirus present in CSL tumours and other pinniped herpesviruses, particularly phocine herpesvirus-2 (PHV-2), is poorly defined, an additional objective of this study was to produce comparative sequence data for these viruses. PHV-2 is a gammaherpesvirus that has been isolated on a number of occasions from Atlantic harbour seals (*Phoca vitulina*); the clinical significance of infection has not yet been established (Lebich et al., 1994; Harder et al., 1996). Unfortunately, since there is no overlap in the sequences available for PHV-2 and the CSL herpesvirus, comparisons have not been possible to date. PHV-2 had also been reported to share antigenic identity with an uncharacterised herpesvirus isolated from CSL (Harder et al., 1996; Kennedy-Stoskopf et al., 1986). However, it has recently been established that the PHV-2 isolate used in these studies was in fact derived from a grey seal (*Halichoerus grypus*) (Kennedy-Stoskopf, 2001).

2. Materials and methods

2.1. Animals

CSLs (*Z. californianus*) admitted to The Marine Mammal Center (TMMC: Sausalito, CA, USA) during 1995–1998 were used for this study. This rehabilitation facility admits stranded marine mammals found along the central California coastline (35°59′ to 37°42′N, 121°30′ to 123°05′W). Tissue samples were collected during post-mortem examination and were frozen at −70 °C until required for DNA extraction. Additional analysis was performed on archived frozen sea lion tissues stored at TMMC.

2.2. PCR and DNA sequencing

Representative tissue sampled from tumour masses was used in the sequencing studies. DNA was isolated by phenol/chloroform extraction methods as previously described.
(Maniatis et al., 1989). Two sets of degenerate primers targeting the DNA polymerase (Dpol sense 5'-3': TT(CT) GA(CT) AT(CT) GA(AG) TG and antisense 5'-3': (AG)TC (AGTC)GT (AG)TA (AGT)AT, King et al., 1998) and the terminase (sense 5'-3': TTG TGG ACG AG(AG) (GC)(AGTC)(AC) A(CT)T T(CT)AT and antisense 5'-3': TGT TGG TCG T(AG)(AT) A(AGTC)G C(AGTC)G G(AG)T C, adapted from Hargis et al., 1999) genes were used to amplify corresponding herpesviral genome fragments. An additional PCR using primers (5'-3': CTC CAA ACC TGA TGC TAC and 5'-3': CTG ACA CAC TTC CTT TCC) designed to amplify an EcoRI fragment of PHV-2 (Harder et al., 1996) failed to produce an amplicon with the tumour DNA (data not shown). Fragments were cloned into pGEM-T easy (Promega, Madison, WI) and a minimum of three independent clones was sequenced by the chain termination method (Sanger et al., 1977) using an automated sequencer (Prism, Perkin Elmer, Norwalk, CT). A smaller fragment of the Dpol gene of a PHV-2 isolate (virus isolated from a stranded harbour seal (P. vitulina), Long Island, New York, USA) was also sequenced (Goldstein, T., personal communication) and is included in this study for comparative purposes.

2.3. Phylogenetic analysis

Nucleotide sequences were assembled and analysed using the software package from the University of Wisconsin Genetics Group (GCG). Phylogenetic analysis was performed on alignments (CLUSTAL) using predicted translated amino acids of OtHV-1 with homologous regions of other published herpesviruses. Trees were constructed using the NEIGHBOR program of PHYLIP (version 3.5c, Department of Genetics, University of Washington) and were tested by SEQBOOT and CONSENSE programs to determine the degree of support for the particular tree nodes.

2.4. Detection of OtHV-1 in tissues

DNA was extracted from urogenital tumour tissues collected from 16 cases (15 from the TMMC and 1 individual case from Six Flags Marine World, Vallejo, CA). Additional archival frozen tissues collected on an opportunistic basis at TMMC were also analysed. DNA was extracted as previously described and was subjected to PCR using OtHV-1 specific primers (sense 5'-3': AGATCA AGC CAC AAG CTT CC and antisense 5'-3': GTA GTATCT AGC TGT GCT GC) which were designed to amplify a 348 bp fragment of the OtHV-1 terminase gene. Amplicons were visualised on a 1.2% agarose gel stained with ethidium bromide.

3. Results

3.1. DNA sequencing and phylogenetic analysis

The nucleotide sequence (1377 bp) for the Dpol fragment of OtHV-1 (AF236050) encodes a continuous open reading frame corresponding to amino acid residues 300–748 of the translated transcript of human herpesvirus-8 (HHV-8). A fragment (492 bp: AF236051) of the terminase gene was also sequenced (not shown). Smaller fragments of 165 and
375 bp for the Dpol (AF193617) and terminase (AF193618) genes respectively have been recently reported (Lipscomb et al., 2000).

Phylogenetic analysis was performed on the 448 translated amino acids of the Dpol fragment and equivalent regions from other representative herpesviruses. This analysis clearly grouped OtHV-1 in a cluster containing members of the gammaherpesvirus sub-family. Further analysis was performed using a sub-set of this data where there was overlap with a smaller Dpol fragment generated for PHV-2 (149 amino acid residues at the 3' end of AF236050). This revealed an identical tree topology (Fig. 1) to that produced by analysing

![Fig. 1. A phylogram representing the relationship of OtHV-1 compared with other herpesviruses. This analysis was performed using a region (149 amino acid residues) of the Dpol gene where there was overlap between OtHV-1 and PHV-2. Sequences of viruses (accession numbers shown) are: PHV-2 (phocid herpesvirus-2, Goldstein, T., personal communication), badger HV (Banks, M., personal communication), EHV-2 (equine herpesvirus-2: U20824), MurHV-4 (murine herpesvirus-4: AF105037), macaque rhadinoherpesvirus (AF204166), HHV-8 (human herpesvirus-8: U75698), black rhino herpesvirus (AF287948), BHV-4 (bovine herpesvirus-4: AF318573), HVS (herpesvirus saimiri: X64346), Ateline HV-3 (AF083424), por LHV (porcine lymphotropic HV-1: AF191042, -2: AF191043), alcelaphine HV-1 (AF005370), EBV (Epstein–Barr virus: V01555), HSV-1 (herpes simplex virus-1: D10879), VZV (varicella zoster virus: X04370), CMV (cytomegalovirus: X17403). Significant bootstrap values (>70%) are shown and denote percentage of trees where the viruses to the right of the nodes are placed together.](image-url)
the 448 amino acid residues of OtHV-1. Furthermore, this showed that PHV-2 is distinct from OtHV-1, being grouped with equine herpesvirus-2 (EHV-2) and the newly described badger herpesvirus (Banks, M., personal communication). Additional GCG GAP analysis (Table 1) of the region shared between OtHV-1 and PHV-2 reveals that PHV-2 shares a higher identity to HHV-8 and EHV-2 than to OtHV-1. This further supports the phylogenetic conclusions that OtHV-1 and PHV-2 are separate viruses, derived from distinct lineages. Interestingly, the position of OtHV-1 in the Dpol tree is different to that produced by analysis of the translated amino acids of the terminase fragment (data not shown and Lipscomb et al., 2000). This analysis places OtHV-1 deeper within the gammaherpesvirus subfamily, and has HHV-8 and closely related viruses as immediate relatives of OtHV-1.

3.2. Tissue detection of OtHV-1

OtHV-1 specific PCR of DNA isolated from 16 tumour masses demonstrated the presence of the viral DNA in all cases. As a comparison, archived tissues collected from animals of unknown tumour status were also analysed. Of these, 7/14 (50%) muscle and 4/14 (29%) brain tissue samples were PCR positive for the presence of OtHV-1 genome. Additional positive PCR results indicating the presence of OtHV-1 were obtained for sub-lumbar lymph nodes from 1/2 tumour animals and 6/7 (86%) from animals of unknown tumour status.

4. Discussion

This report describes the partial characterisation of a herpesvirus present in CSL tissue samples. Phylogenetic analysis of the DNA polymerase and terminase gene fragments,
demonstrate that this virus is a member of the gammaherpesvirus subfamily, but is distinct from PHV-2, a previously described gammaherpesvirus isolated from pinnipeds. Based on comparisons with PHV-2, there is sufficient evidence to classify this virus as a novel herpesvirus, tentatively designated Otarine herpesvirus-1 (OtHV-1). Gammaherpesviruses are formally assigned to the subfamily Gammaherpesvirinae containing two genera Rhadinovirus and Lymphocryptovirus. A previous study concluded from the analysis of a small terminase fragment that OtHV-1 is a member of the Rhadinovirus genus (Lipscomb et al., 2000). However, in light of different topology highlighted by the analysis of the Dpol fragment, further sequencing of the OtHV-1 genome, including complete genes and comparison of gene organisation is likely to be required to definitively resolve the classification of OtHV-1.

An OtHV-1 specific PCR was developed to establish a link between the presence of OtHV-1 and urogenital carcinoma in CSL. Viral DNA was evident in all tumours examined, supporting the hypothesis that OtHV-1 may be a factor in oncogenesis. Analysis of additional archival tissues from animals of unknown tumour status, demonstrated OtHV-1 to be relatively common. Viral genome was particularly evident in sub-lumbar lymph nodes. This finding is interesting in light of the fact that sub-lumbar lymph nodes were found to be a consistent site of tumour metastasis in the two previous histological studies (Gulland et al., 1996; Lipscomb et al., 2000).

Although the prevalence of OtHV-1 in the free-ranging sea lion population is unknown, the high incidence of OtHV-1 in these stranded animals suggests that the infection is widespread. This may parallel experiences with other oncogenic gammaherpesviruses such as EBV, which has high prevalence, yet the incidence of EBV-associated neoplasia is relatively uncommon (Oudejans et al., 1997). Since detailed histology was not performed on the animals from which archived tissues were collected, a current study using samples collected from proven tumour-negative animals is underway. Furthermore, work is also underway to further define the tissue distribution, infective properties and natural history of this herpesvirus.

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References


