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Papillomaviruses and herpesviruses: Who is who in genital tumor development of free-ranging Atlantic bottlenose dolphins (*Tursiops truncatus*)?

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ABSTRACT

The number of studies addressing neoplasia in marine mammals has recently increased, giving rise to concern whether such lesions could be reflective of an emerging infectious disease. Eight species-specific viruses, seven papillomaviruses (PVs) and two herpesviruses (HVs) have separately been shown to be associated with genital tumors in Atlantic bottlenose dolphins (*Tursiops truncatus*, Tt): TtPV1-6, as well as HVs provisionally assigned the names DeHV4 and -5 (Delphinid HVs). A definite causal role of these viruses in cell transformation remains to be demonstrated. Concurrent PV- and HV-infection has never been reported in marine mammals.

DNA extractions from biopsies of genital tumors derived from 15 free-ranging Atlantic bottlenose dolphins were selected for molecular examination. Polymerase chain reaction (PCR) analyses revealed the presence of DeHV4, while a serological screening using an antibody-based TtPV enzyme-linked immunosorbent assay (ELISA) demonstrated previous and/or current infection of the HV-positive dolphins with at least one TtPV type. Therefore, care must be taken when drawing conclusions about viral causalities in tumor development, since the "hit and run" and other mechanisms have been described for types of both viral families. This study presents the first evidence of marine mammals having a history of PV- as well as HV-infection and discusses the disputed effects of viral co-infection.

1. Introduction

In humans, viruses are associated with about 15% of the global burden of cancer (Boccardo and Villa, 2007). Concurrent papillomavirus (PV)- and herpesvirus (HV)-infection and the viruses' roles in tumor progression and malignant transformation are the subject of ongoing debate. Human PVs (HPVs) are known to be the causative agents of cancers such as cervical carcinoma, the most common cause of cancer mortality in women in developing countries (Parkin and Bray, 2006) and the second most frequent cancer in women worldwide (Walboomers et al., 1999; Einstein and Goldberg, 2002). In contrast, the role played by human HVs such as Herpes Simplex Virus type 2 (HSV2) as co-factors in such cancers remains unclear.

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Although HSV2 co-infection is regularly found in PV-induced cervical carcinomas, conclusive evidence was reported against a causal role for HV and even against cooperation of both viruses in this cancer (Paavonen and Lehtinen, 1999). However, viruses of both families have been shown to be involved in tumor progression.

PVs are highly prevalent and represent the most frequently sexually transmitted viruses in humans (Garland, 2002). All PVs known to date are associated with benign and/or malignant cutaneous or mucosal lesions, while malignancies are usually caused by infection with so-called high risk PV types. Reports of neoplasia in marine mammals are increasing in number (reviewed in Newman and Smith, 2006), and a high prevalence of orogenital papillomas in free-ranging dolphins and porpoises has been documented (Van Bressem et al., 1996; Bossart et al., 2005; Rehtanz et al., 2010). To date nine classified PVs have been described as being associated with genital tumors in cetaceans (dolphins, porpoises and whales): bottlenose dolphin PV types 1–6 (*Tursiops truncatus* PVs; TtPV1-6) (Rector et al., 2006; Rehtanz et al., 2006; Robles-Sikisaka et al., 2012), with a variant of TtPV3 detected in a penile lesion of an Atlantic white-sided dolphin (*Lagenorhynchus acutus*) (Gottschling et al., 2011); the Burmeister's porpoise PV type 1 (*Phocoena spinipinnis* PV; PsPV1) (Van Bressem et al., 2007); and the harbor porpoise PV types 1 and 3 (*Phocoena phocoena* PV; PphPV 1 and 3). Another completely known cetacean PV is PphPV2, shown to be associated with an esopharyngeal lesion of a harbor porpoise, while three partial sequences of potentially novel PVs have been detected in the Burmeister's porpoise, the harbor porpoise and the killer whale (*Orcinus orca*) (Gottschling et al., 2011). Of all completely classified cetacean PVs, two may represent potential high risk viruses based on E6 protein sequence investigations (PDZ-binding motif XS/TXV/L): TtPV2 (ETEL) (Rehtanz et al., 2006) and PphPV3 (ETFV). Using TtPV virus-like particles (VLPs) (Rehtanz et al., 2009) as the antigen in ELISA studies of free-ranging and captive Atlantic bottlenose dolphins, a recent serologic screening indicated the TtPV-prevalence in this species ranges from 38% to 94% in the monitored populations (Rehtanz et al., 2010). Of these dolphins, 26% had oral and/or genital tumors or had displayed such lesions in the past (in free-ranging as well as captive populations) (Rehtanz et al., 2010). While TtPV2 was isolated from one of those tumors (Rehtanz et al., 2006), transmission electron microscopy provided evidence for the presence of herpes-like viruses in other cases (Bossart et al., 2005).

HVs cause several diseases with a wide range of clinical signs and strategies of replication through different mechanisms, one of which is the development of tumors. The first molecular evidence for the existence of cetacean HVs was obtained from lung and heart tissues of two stranded Atlantic bottlenose dolphins (Blanchard et al., 2001). These infections were believed to have been the cause of death of the animals. Another HV was identified from Atlantic bottlenose dolphin skin lesions which regressed without treatment (Manire et al., 2006), while a Blainville's beaked whale HV was detected in a penile lesion of a stranded whale (Saliki et al., 2006). Several other HV sequences were detected in specimens from genital and oral lesions as well as in blood and spleen samples from other whale and dolphin species (Smolarek Benson et al., 2006; van Elk et al., 2009; Bellière et al., 2010; Maness et al., 2011). A provisional phylogenetic classification of marine mammal HVs detected to date was recently published on the basis of a region of the HV DNA polymerase (Maness et al., 2011). However, currently there is no existing definition of HV-types, -subtypes and -variants on this basis.

The objective of this study was to investigate whether HV- and PV-positive Atlantic bottlenose dolphins, respectively, had a history of infection with viruses of both families, as sometimes observed in humans. Since tumor development resulting from such infections may pose a threat to dolphins, this question is relevant for the future assessment of potential risks for affected populations as well as to the broader issue of PV/HV-co-infection in viral oncogenesis. A secondary objective was the establishment of an internal standard control specifically for marine mammal specimens based on beta-actin polymerase chain reaction (PCR) analysis, as no such control is currently being used in studies aimed to demonstrate the presence of viral nucleic acids.

2. Materials and methods

2.1. Dolphins sampled

Tumor samples were collected from 15 free-ranging Atlantic bottlenose dolphins (*T. truncatus*) with genital sessile papillomas (Rehtanz et al., 2010), captured during the Atlantic Bottlenose Dolphin Health and Risk Assessment (HERA) Project under National Marine Fisheries Service Scientific Research Permit No. 998-1678 issued to Gregory D. Bossart. The animals (identified as "FB" plus their assigned numbers) were captured, examined and released as previously described (Fair et al., 2006) in the estuarine waters near Charleston (CHS), South Carolina, USA in August of 2004 and 2005 ($n = 4$) and the Indian River Lagoon (IRL), Florida, USA in June of 2005 and 2006 ($n = 11$).

2.2. DNA extraction

Specimen tissue was finely minced with a sterile scalpel and digested overnight at 55 °C in 500 μl digestion buffer (1 μg/μl proteinase K, 10 mM Tris, 0.5% SDS, pH 7.4). Deproteinization was performed with phenol, phenol–chloroform–isoamylalcohol and chloroform, followed by ethanol precipitation to recover DNA. Air-dried DNA-pellets were then resuspended in 20–50 μl TE-buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0).

2.3. Polymerase chain reaction studies and preparation for sequencing

All PCR reactions were carried out with an MJ Research PTC-200 thermocycler in a volume of 50 μl with a high-fidelity DNA polymerase containing proofreading activity (Highest-fidelity Pfx DNA Polymerase, Invitrogen, Carlsbad, CA) and primer concentrations of 1 μM each.