Veterinary Microbiology 186 (2016) 150–156

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Short communication

Chelonid herpesvirus 5 in secretions and tumor tissues from green turtles (*Chelonia mydas)* from Southeastern Brazil: A ten-year study

Telma A. Monezi[a,b,*], Dolores U. Mehnert[a], Elisabeth M.M. de Moura[a], Natascha M.G. Müller[a], Patrícia Garrafa[a], Eliana R. Matushima[c], Max R. Werneck[d], Maria I. Borella[b]

[a] University of São Paulo, Institute of Biomedical Sciences, Department of Microbiology, Brazil
[b] University of São Paulo, Institute of Biomedical Sciences, Department of Cell and Developmental Biology, Brazil
[c] University of São Paulo, College of Veterinary Medicine and Animal Science, Department of Pathology, Brazil
[d] Projeto TAMAR-ICMBIO, Ubatuba, São Paulo, Brazil

ARTICLE INFO

Article history:
Received 10 February 2015
Received in revised form 15 February 2016
Accepted 23 February 2016

Keywords:
Brazil
Chelonia mydas
ChHV5
Fibropapillomatosis
Secretions
Ocular fibropapillomatosis

ABSTRACT

Fibropapillomatosis (FP), a neoplastic disease characterized by the formation of multiple tumors affecting different species of sea turtles and, most often, the green turtle (*Chelonia mydas*), is considered one of the major threats to the survival of this species. Recent studies indicate that Chelonid herpesvirus (ChHV5) is the etiological agent of this disease, though its association with anthropogenically altered environments and the immune status of these animals also appears to contribute to disease expression and tumor formation. In this study, tumor biopsy and secretions from green turtles captured off the coast of São Paulo State, Brazil, were used in histological and molecular analyses to detect and characterize circulating ChHV5. In 40.9% of cases, the tumor histopathological findings revealed focal ballooning degeneration with intranuclear inclusion bodies, results which are suggestive of viral infection. ChHV5 was detected using polymerase chain reaction (PCR) on the animals' skin, ocular tumor biopsies, and ocular and oral secretions. The analysis of the detected ChHV5 sequences revealed two distinct genetic sequences together. Phylogenetic analysis indicated that Brazilian samples were similar to ChHV5 samples described for the Atlantic phylogeographic group and are therefore part of the same clade as the Gulf of Guinea and Puerto Rico samples. This similarity suggests a possible flow of the virus between these three regions.

1. Introduction

Fibropapillomatosis (FP) is a neoplastic disease that affects sea turtles—particularly *Chelonia mydas*. It is characterized by cutaneous, ocular, and visceral tumors and is a serious threat to the wild animal survival. It can lead to both emaciation and incapacitation, It prevents the animal from hunting, worsens vision, and may cause organ failure (Smith and Coates, 1938; Jacobson et al., 1989).

The primary cause of the disease is thought to be Chelonid herpesvirus 5 (ChHV5). However, the association between the marine environment and human activity in it is likely to contribute to virus transmissibility and/or to disease expression (including tumor formation), since a high prevalence of FP is common in human-modified environments (Aguirre and Lutz, 2004).

Fibropapillomas can be present on the flippers and the eyes, at the base of the tail, on the carapace and plastron, and in the oral, cervical, groin, and axillary regions. Tumor texture is smooth to verrucous. They can be sessile or pedunculated, and most formations are ulcerated and necrotic (Jacobson et al., 1989). Tumors found in cavitary organs such as the kidneys, liver, and lungs have been classified as myxofibromas or fibromas (Jacobson et al., 1991). Histologically, the tumors can be classified as papillomas, fibropapillomas, or fibromas. Fibropapillomas are intermediate lesions characterized by papillary projection, marked epidermal hyperplasia, fibroblast proliferation in various stages of differentiation, and the presence of collagen bundles in the dermis (Jacobson et al., 1989; Herbst et al., 1999). Squamous epithelial cells are hypertrophic, present cytoplasmic vacuoles, and may include ballooning degeneration containing eosinophilic or basophilic intranuclear inclusion bodies (IIBs) suggestive of viral infection (Jacobson et al., 1991; Herbst et al., 1999).

* Corresponding author at: Universidade de São Paulo, Instituto de Ciências Biomédicas, Departamento de Microbiologia. Av. Prof. Lineu Prestes, 1374, Cidade Universitária, São Paulo, SP, CEP 05508-900, Brazil.
E-mail addresses: telmaamo@usp.br, telmonezi@gmail.com (T.A. Monezi).

http://dx.doi.org/10.1016/j.vetmic.2016.02.020
0378-1135/

Fibropapillomas was first reported close to 80 years ago (Luckes, 1938; Smith and Coates, 1938). Reports have since come to include all sea turtle species; outbreak levels have been reached (Foley et al., 2005; Chaloupka et al., 2008). Global prevalence ranges from 0% to 92%. It has been reported to vary from 0% to 72.5% in Florida (Ehrhart, 1991; Lackovich et al., 1999) and from 1% to 92% in Hawaii (Balazs, 1991). It has averaged 21.5% in Indonesia (Adnyana et al., 1997) and 17% in Africa (Formia et al., 2007). The disease was first reported in Brazil in 1986; prevalence in the country varies by region and year. Average *Chelonia mydas* prevalence was 36.94% between 1986 and 2005; it ranged from 15.41% to 42.11% between 2010 and 2013 (Baptistotte et al., 2001; Baptistotte, 2007; Rossi, 2014).

Since 1998, molecular methods have consistently detected ChHV5 DNA in tumor samples from different sea turtle species from different regions, and partial genetic sequences have been deposited in GenBank (Quackenbush et al., 2001; Ene et al., 2005; Greenblatt et al., 2005). The phylogenetic analysis of these sequences has helped classify the virus within the subfamily Alphaherpesvirinae, part of the recently-created genus *Scutavirus*. These analyses have also helped identify genetic variants in different regions. Herbst et al. (2004) grouped these variants into Pacific and Atlantic branches. Ene et al. (2005) demonstrated the existence of four evolutionary ChHV5 variants in Florida (known as variants A, B, C, and D) that diverged millions of years ago. The prevalence of these Florida variants varies by region. Greenblatt et al. (2005) identified four regional groups: eastern Pacific, western Atlantic/eastern Caribbean, mid-west Pacific, and Atlantic. Samples from Puerto Rico, however, did not fit into any of these branches. A more recent study identified four phylogeographic groups and included the Puerto Rico and the Gulf of Guinea samples within the ChHV5 phylogeny (Patricio et al., 2012). The study concluded that ChHV5's phylogeographic pattern seems to reflect the movements of the host, and that changes to the environment likely caused both current FP outbreaks and the modern evolutionary history of the virus.

In Brazil, PCR studies detected viral polymerase gene sequences that commonly represent human and animal herpesviruses in tumor, blood, and saliva samples, as well as in ocular secretions from animals with FP (Monezi et al., 2006). However, the literature still lacks reports of ChHV5 detection in blood and secretion samples from animals with fibropapillomatosis. In 2013, six partial sequences from Brazilian tumors collected between 2008 and 2010 (two of which were from São Paulo) were added to the world database (Rodenbusch et al., 2014).

Thus, the goals of the present study were to determine whether genetic ChHV5 sequences were present in skin and ocular tumor samples, ocular secretions, or saliva from green turtles captured along the northern coast of São Paulo State from 2001 to 2011, to compare the most current Brazilian samples to the samples described in the literature, and to determine whether inclusion bodies typical of viral infection were present in ocular and skin tumor samples that had been previously analyzed histopathologically.

2. Materials and methods

2.1. Animals and handling methods

Wild *Chelonia mydas* (green turtles) were either rescued or accidentally captured in the Brazilian coastal cities of Ubatuba, São Sebastião, and Angra dos Reis by monitoring teams from the TAMAR Project, run by the Chico Mendes Institute for Biodiversity Conservation (ICMBio). The turtles were sent to the TAMAR Project Rehabilitation Center Base in Ubatuba on the northern coast of São Paulo state, which is recognized as an important sea turtle feeding and refuge area (Gallo et al., 2006).

Each animal was given an identification number. Animals that were injured or diagnosed with fibropapillomatosis were temporarily held at the Rehabilitation Center Base in individual shaded outdoor pools filled with 500–1000 L of water, the temperature of which varied from 20 to 25 °C. Between 100 and 200 g of *Ulva* spp. algae from the region were fed to the animals three to four times per day. These animals were then released after a recovery period. The animals that were not found to have fibropapillomatosis were released into the wild after clinical materials were collected.

Samples for molecular analysis were collected from five animals in 2001, 28 animals between 2005 and 2006, and 22 animals between 2011 and 2012. A total of 47 skin and eye tissue tumor samples were collected, as were 25 ocular secretion samples and 27 saliva samples. Histopathological analyses were performed on the samples from 2011 and 2012. A total of 160 tumor biopsies (154 skin tissue and 6 eye tissue) were performed. Biopsies were also performed on apparently healthy skin from the animals diagnosed with fibropapillomatosis. Normal skin samples from five healthy animals were used as controls.

A veterinarian surgically removed the tumors following the routine procedure used by the TAMAR Project. The animals were handled according to the recommendations from the Ethics Committee on Animal Testing of the Biomedical Sciences Institute of the University of São Paulo (USP) and the USP College of Veterinary Medicine and Animal Sciences.

The tumor tissue biopsies were sectioned and separated into two groups: one for histopathological analysis, and another that was frozen at −20 °C for molecular analysis. Ocular secretions and saliva samples collected using sterile swabs were conditioned in sterile vials containing a 0.01 M phosphate buffer at a pH of 7.6 and frozen to −20 °C until processing.

2.2. PCR amplification and sequencing

The DNA was extracted from tumors and frozen secretions using a Trizol solution (BRL/Life Technologies) and chloroform following the manufacturer's instructions. ChHV5 detection was based on the partial DNA polymerase gene sequence amplification using PCR and nested PCR. The primers and the PCR and nested-PCR conditions were the same as those reported by Lu et al. (2000). In all of the reactions, water samples were included as a negative control.

Products were electrophoresed in 1.5% agarose with ethidium bromide, and bands of interest were sequenced using a BigDye® 238 Terminator v3.1Cycle Sequencing Kit (Applied Biosystems, Cat. No. 239 4337456) and an ABI 3730 automatic sequencer (Applied Biosystems).

2.3. Phylogenetic analysis

Representative sequences from GenBank (www.ncbi.nlm.nih.gov/genbank) were added to investigate the phylogenetic relationships among ChHV5 strains (Table 1, Supplementary information). Nucleotide sequences were aligned using Muscle v3.8.31 (Edgar, 2004) and inspected with SeaView v4.4.0 (Gouy et al., 2010). We selected the General Time Reversible (GTR) model with gamma rates (G) and invariant sites (I) as the best-fit model for our data based on the jModelTest v. 3.7 exhaustive search strategy (Darriba et al., 2012) using PhyML v3.0 (Guindon et al., 2010). To access phylogenetic relationships among ChHV5 strains, we employed a maximum likelihood approach with the GTR + G + I model using the Garli v2.0.1 program (Zwickl, 2006). The support for the maximum likelihood tree was calculated after 1000 non-parametric bootstrap replicates.