INVESTIGATION OF THE NATURAL HISTORY OF CHELONID-FIBROPAPILLOMA-ASSOCIATED HERPESVIRUS INFECTION IN GREEN SEA TURTLES (*CHELONIA MYDAS*)

by

CYNTHIA ANNE PAGE-KARJIAN

(Under the Direction of Nicole Gottdenker)

ABSTRACT

Fibropapillomatosis (FP) is an infectious, neoplastic disease of sea turtles in circumtropical coastal ocean waters worldwide. FP tumors can cause debilitation by hampering turtles' feeding, movement, and vision, and visceral tumors can cause organ failure. Fibropapillomatosis is panzootic in free-ranging green sea turtles (*Chelonia mydas*), and is also a concern in rehabilitating turtles because it requires extensive quarantine measures and complicates prognoses. An alphaherpesvirus, designated chelonid fibropapilloma-associated herpesvirus (CFPHV), is consistently identified in FP tumors, and cell-free tumor extracts successfully transmitted the disease. CFPHV was also identified in some normal turtles via serology and polymerase chain reaction (PCR). Despite numerous studies, the actual pathogenesis of CFPHV, its nature of persistence or latency, and its transmission dynamics are not yet fully understood. The objective of this study was to investigate aspects of CFPHV natural history dynamics in free-ranging and rehabilitating green turtles with and without FP. First, nested PCR was used to identify CFPHV DNA in skin from tumored and non-tumored, free-ranging green turtles, and combined with laser capture microdissection to identify CFPHV DNA

in microscopically separated epidermis and dermis sections of non-tumored skin. A retrospective case series analysis was performed to describe the biology and survival rates of rehabilitating turtles with FP, and evaluate clinical parameters as survivorship predictors. To investigate the relationship between CFPHV loads and clinical disease, and identify potential routes of viral shedding, a quantitative PCR (qPCR) assay was developed, validated, and applied to various biological samples taken from tumored and non-tumored, free-ranging and rehabilitating green turtles. The qPCR data provide relevant, novel evidence for CFPHV DNA localization and mobilization in symptomatic and asymptomatic turtles. CFPHV DNA presence in blood cells may represent a critical viral life cycle phase and transport mechanism, and CFPHV DNA in urine suggests a previously unknown route of transmission. Quantitative CFPHV DNA data also show that, in addition to cutaneous tissues, kidney and nerve cells play a role in CFPHV pathogenesis. Practical application of the presented information will aid in the evaluation of symptomatic and asymptomatic turtles, helping to prevent transmission opportunities among captive turtles and informing management decisions for free-ranging populations.

INDEX WORDS: *Chelonia mydas*, Fibropapillomatosis, Green turtle, CFPHV, Chelonid fibropapilloma-associated herpesvirus, Nested PCR, Quantitative PCR, Rehabilitation

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DEDICATION

This dissertation is dedicated to my parents Pete and Patricia Page, who never doubted me, to my husband Paul Karjian and my dog Little Sister for their endless love and patience, and to my son Noah, for motivating me to try to make a difference in this world. And to the turtles--of course.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Fibropapillomatosis (FP) is a transmissible neoplastic disease that affects sea turtles in tropical and subtropical coastal ocean waters worldwide. FP tumors often cause affected animals to become debilitated by hampering feeding and movement, obscuring vision, or in the case of visceral tumors, leading to organ failure (Balazs 1986; Herbst 1994; Jacobson et al. 1989; Smith & Coates 1938). Fibropapillomatosis has been observed in all hard-shelled marine turtle species, although green turtles (*Chelonia mydas*) appear to be most frequently and severely affected (Aguirre et al. 1999; D'Amato & Moraes-Neto 2000; Harshbarger 1991; Herbst 1994; Limpus & Miller 1994). The disease has become panzootic in green turtle populations over the past 3 decades, with epizootic foci in parts of Florida, Hawaii, and the Caribbean (Chaloupka et al. 2009; Foley et al. 2005; Williams et al. 1994). The epizootic is reportedly declining in Hawaii, but appears to be more stable in Florida, and the disease is also appearing in new localities around the world (Chaloupka et al. 2008; Duarte et al. 2012; Hirama & Ehrhart 2007).

Fibropapillomatosis is characterized by single to multiple cutaneous, flat to raised masses consistent with papillomas, fibromas, or fibropapillomas (Herbst 1994; Herbst et al. 1999). The typical histologic description of FP includes papillary epidermal hyperplasia supported by broad fibrovascular stalks, with a varying ratio of epidermal to dermal proliferation (i.e., Figure 1.1; Herbst 1994). Papillomas are characterized by proliferating epidermis with little or no underlying dermal involvement. Lesions composed predominantly of proliferating dermal components with a relatively normal overlying epidermis are characterized as fibromas. Fibropapillomas are

classified as masses in which hyperplasia is observed in both epidermis and dermis (Herbst 1994). Although the masses are usually found on the soft skin, they may be found anywhere on the turtle's body (Figure 1.2; Herbst 1994). Turtles may also be afflicted with visceral masses (i.e., Figure 1.3), which can occur in all internal organs, and are typically identified histologically as fibromas, myxofibromas, or fibrosarcomas (Herbst 1994; Norton et al. 1990; Work et al. 2004). Visceral tumors tend to develop late in the course of disease, and are generally perceived as a more chronic lesion (Harshbarger 1991; Herbst 1994; Herbst et al. 1999; Jacobson et al. 1989; Lucke 1938). Tumors may also develop in one or both eyes (i.e., Figure 1.4), potentially affecting the cornea, sclera, conjunctiva, and/or periocular skin, including eyelids (Brooks et al. 1994; Flint et al. 2010; Jacobson et al. 1989, 1991). The tumors can grow rapidly, can disrupt a turtle's biological functions including swimming, diving, predator evasion, and feeding activities, and may eventually lead to death (George 1997). In sea turtle rehabilitation centers, FP is a key concern because it requires extensive quarantine measures and complicates prognoses, and in some cases, FP tumors develop after turtles are admitted for other reasons (Page-Karjian et al. 2014).

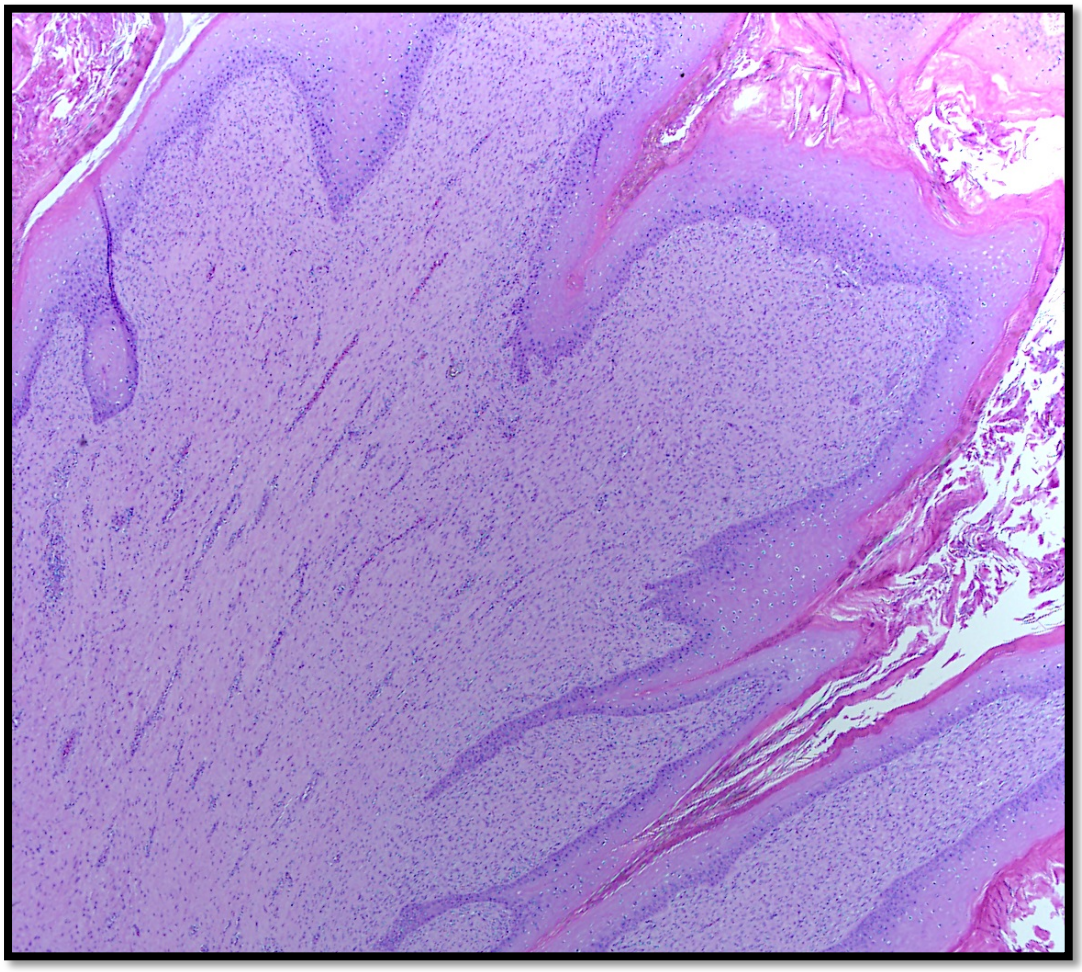

Figure 1.1. Photomicrograph (20X HPF) of a section of cutaneous fibropapilloma removed from a green sea turtle that stranded in Florida. Broad, proliferative, fibrovascular dermal stalks are overladen by hyperplastic epithelium with orthokeratotic hyperkeratosis.

Figure 1.2. Cutaneous fibropapilloma lesions affecting the skin, conjunctivae, and cornea of a juvenile green sea turtle that stranded in eastern Florida, USA (photo credit Georgia Sea Turtle Center)

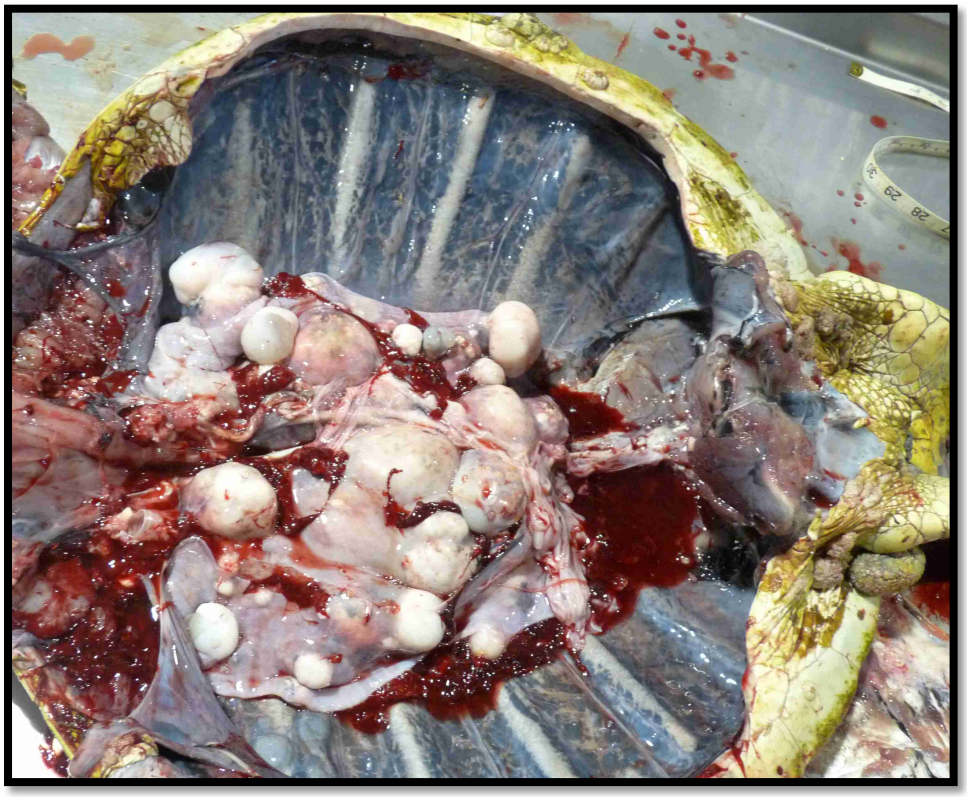

Figure 1.3. Visceral tumors on the right and left lungs of a green sea turtle that stranded in Queensland, Australia (photo credit J. Scott, James Cook University)

Figure 1.4. Ocular tumors on the conjunctiva of a green sea turtle (photo credit Georgia Sea Turtle Center)

FP primarily affects juvenile turtles after they have migrated to near-shore habitats (Ene et al. 2005). There is some evidence that FP occurrence in wild populations may be associated with warm water temperatures, and escalating tumor growth rates during warmer seasons may predispose turtles with FP to further debilitation (Foley et al. 2005; Herbst 1994; Herbst et al. 1995). A positive correlation has been observed between turtles afflicted with FP and shallow/inshore waters, including evidence that turtles develop tumors after they recruit to more coastal habitats (Aguirre et al. 1994, 1998; Ene et al. 2005). A strong association between FP and immune suppression has been reported in captive and wild-caught green turtles, although it is still unclear whether tumor development is the result of altered immune function or the cause of it (Aguirre et al. 1995; Cray et al. 2001; Work & Balazs 1999; Work et al. 2001).

The presumptive etiologic agent most commonly associated with FP is an alphaherpesvirus, designated chelonid fibropapilloma-associated herpesvirus (CFPHV), and recently grouped in the genus *Scutavirus* (Adams & Carstens 2012). This virus has been sequenced but to date has resisted cell culture isolation attempts (Herbst et al. 2004; Lackovich et al. 1999; Lu et al. 1999; Moore et al. 1997; Work et al. 2009). Genetic data suggest that CFPHV was established in sea turtle populations prior to the emergence of FP tumors, and that the virus has co-evolved with its sea turtle hosts (Greenblatt et al. 2005a; Herbst et al. 2004). CFPHV sequences appear to separate into geographic clades (Greenblatt et al. 2005b; Herbst et al. 2004). Two major lineages of the CFPHV sequence variants were identified via phylogenetic analysis, with one clade formed by the 3 Florida variants (A, B, and C) and the Hawaiian variant, and one clade formed by Florida variant D (Herbst et al. 2004). Phylogenetic analysis further indicated that nucleotide sequences of CFPHV variants associated with FP may differ by up to 5.6% (Herbst et al. 2004). Using nucleotide substitution rates that are accepted for other vertebrate herpesviruses, these variants are estimated to have diverged 1.6 - 4.0 million years ago. A 2.2% divergence between a Hawaiian CFPHV variant and the most similar Florida variants was estimated to have occurred 0.6 to 1.6 million years ago (Herbst et al. 2004). Thus, the current FP panzootic is very unlikely to be due to a worldwide spread of a single, emergent, pathogenic viral variant. Instead, environmental or ecological factors are predicted to underlie the current FP disease outbreaks (Herbst et al. 2004).

CFPHV has been consistently associated with FP tumors via multiple molecular and sequencing technologies and was found to be transmissible via cell-free tumor extract, resulting in a commonly perceived causal associative link (Herbst et al. 1995, 2004; Kang et al. 2008; Lackovich et al. 1999; Lu et al. 2000, 2003; Quackenbush et al. 1998, 2001). Identification of

CFPHV DNA in sea turtle tissues has been accomplished by various techniques, including PCR and in situ hybridization (Herbst et al. 1999; Kang et al. 2008; Lackovich et al. 1999; Lu et al. 2000; Quackenbush et al. 1998, 2001). To date, CFPHV DNA has been isolated from the following sea turtle tissues: cutaneous tumors; tumors found on the eye, trachea, lungs, tongue, esophageal mucosa, intestine, kidney, heart, and spleen; scar tissue where an FP tumor was previously removed; and non-tumored tissues including skin, lungs, kidney, heart, spleen, liver, brain, periorbital tissue, conjunctiva, ovary, testis, tongue, gall bladder, intestine, urinary bladder, thyroid (Herbst et al. 1999; Jacobson et al. 1989; Kang et al. 2008; Lackovich et al. 1999; Lu et al. 2000; Quackenbush et al. 1998, 2001). The virus has also been shown to be present, either serologically or through molecular techniques, in some normal animals (Herbst et al. 2008; Lackovich et al. 1999; Lu et al. 2000; Quackenbush et al. 2001). This raises the possibility that there are additional factors – ecologic, immunologic, or microbial – involved in disease development. The actual pathogenesis of CFPHV, its nature of persistence or latency, and transmission dynamics are yet to be determined. The objective of the dissertation research presented here was to investigate aspects of CFPHV natural history dynamics in free ranging and rehabilitating green sea turtles with and without fibropapillomatosis.

CHAPTER 2

PRESENCE OF CHELONID FIBROPAPILLOMA-ASSOCIATED HERPESVIRUS IN TUMORED AND NON-TUMORED GREEN SEA TURTLES (*CHELONIA MYDAS*), AS DETECTED BY POLYMERASE CHAIN REACTION, IN ENDEMIC AND NON-ENDEMIC AGGREGATIONS, PUERTO RICO [1]

[1] Page-Karjian, A., Torres, F., Zhang, J., Rivera, S., Diez, C., Moore, P.A., Moore, D., and C. Brown. 2012. *SpringerPlus* 1:35.
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Abstract

Fibropapillomatosis (FP), a transmissible neoplastic disease of marine turtles characterized by a likely herpesviral primary etiology, has emerged as an important disease in green sea turtles (*Chelonia mydas*) over the past 3 decades. The objectives of this study were to determine the suitability of 3 different chelonid fibropapilloma-associated herpesvirus (CFPHV) gene targets in polymerase chain reaction (PCR) assays of affected tissues; to explore the presence of CFPHV in non-affected skin from turtles with and without tumors; and to better understand tissue localization of the CFPHV genome in a tumor-free turtle by evaluating CFPHV presence in microanatomic tissue sites. Two aggregations of green sea turtles (*Chelonia mydas*) in Puerto Rico were evaluated, with 6 sampling intervals over the 3-year period 2004–2007. Primary and nested PCR for 3 different herpesviral gene targets- DNA polymerase, capsid maturation protease, and membrane glycoprotein B- were performed on 201 skin biopsies taken from 126 turtles with and without external tumors. Laser capture microdissection and nested PCR were used to identify tissue localizations of CFPHV in skin from a normal turtle. Of the turtles sampled in Manglar Bay, 30.5% had tumors; at the relatively more pristine Culebrita, 5.3% of turtles sampled had tumors. All 3 PCR primer combinations successfully amplified CFPHV from tumors, and from normal skin of both tumored and tumor-free turtles. Via nested PCR, the polymerase gene target proved superior to the other gene targets in the positive detection of CFPHV DNA. CFPHV infection may be common relative to disease incidence, supporting the idea that extrinsic and/or host factors could play a transforming role in tumor expression. Laser capture microdissection revealed CFPHV in skin from a tumor-free turtle, harbored in both epidermal and dermal tissues. Identification of CFPHV harbored in a non-epidermal site (dermis) of a tumor-free turtle indicates that virus is latent in a non-tumored host.

Introduction

The sloughing of virally infected epidermal cells from a diseased turtle into the surrounding environment is considered a source of viral shedding. Thus FP tumors are suggested to undergo viral shedding, as evidenced by the presence of CFPHV-associated intranuclear viral inclusions within cells of the epidermal strata germinativum and spinosum of some cutaneous tumors (Jacobson et al. 1989, 1991). In one study, the high prevalence of CFPHV glycoprotein H antibodies in a green turtle population with 0% tumor prevalence suggests that robust antibody responses to natural infection may develop independently of the appearance of cutaneous tumors (Herbst et al. 2008). Because CFPHV's primary target seems to be skin, early or latent infection may be represented by the presence of CFPHV DNA in non-tumored skin from turtles with and without tumors (Quackenbush et al. 2001). There is one report of CFPHV polymerase DNA identified in differentiated epidermal and dermal tissues of tumors sampled from green turtles (Work et al. 2009). To date, however, there are no previous reports using the microanatomic location of CFPHV DNA to investigate potential viral shedding from the skin of non-tumored turtles. In the present study, application of laser capture microdissection permits an innovative, precise way to begin assessing the role that cell type plays in disease processes.

The study described here had 3 objectives. The first was to determine the suitability of various CFPHV gene targets in PCR assays for detection of CFPHV in affected tissues. The second objective was to explore the presence of CFPHV DNA in non-affected skin from both turtles with tumors and tumor-free turtles. The third objective was to use an innovative technology, laser capture microdissection, to evaluate CFPHV presence in epidermal and dermal tissues of normal skin sampled from a tumor-free turtle, in an effort to better identify the tissue location of the CFPHV genome.

Methods and Materials

Animals

In the Culebra archipelago of eastern Puerto Rico, two aggregations of green sea turtles were sampled for tumors over a period of 3 years. One aggregation was sampled in Manglar Bay, a basin on the island of Culebra that has relatively high levels of human activities and wastewater runoff (18-18'13“ N, 065-15°19” W). The other aggregation was sampled at the more pristine cay preserve of Culebrita (18-18°53“ N, 065-13°44” W). Based on telemetry data tracking the movements of turtles found in Manglar Bay, these aggregations were thought to commingle at night at a reef between the two sites (Diez et al. 2010). This is an assumption, however, since transmitters were not deployed on turtles found at Culebrita. According to mitochondrial DNA analysis, Manglar Bay and Culebrita green turtle aggregations recruit from multiple rookeries belonging to all 5 Atlantic and Caribbean Regional Management Units (Velez-Zuazo et al. 2010; Wallace et al. 2010).

A total of 126 green turtles were captured by netting and subsequently released, during 6 different time intervals over the 3-year period 2004–2007. All were classified as juveniles, defined by a curved carapace length < 65 cm (Patricio et al. 2011). Because to date there is no available report on the somatic growth and maturity stages of these aggregations, this age-size classification is arbitrary; however it is based on similar, previously used classifications (Bresette et al. 2010; Chaloupka & Limpus 2001).

Biopsy procedure

Following disinfection (Betadine) of the sampling tissue, tumors and/or normal skin from affected animals were either surgically removed or biopsied using a 6 mm diameter punch. A

punch biopsy of normal, non-tumored skin on the shoulder area was also taken from all animals, whether or not they were affected by FP. Tissues were placed in 10% buffered formalin for 12–24 hours, then cut in half and placed in diethyl pyrocarbonate (DEPC) treated phosphate-buffered saline (PBS, pH 7.6) and kept at 4°C. After transportation to the laboratory (1–2 weeks), they were kept at -80°C until assayed. All procedures were performed under the regulatory authorities of federal and state permit (US NMFS: Permit No. 1253 and PRDRNA 12-EPE-04) and Animal Care and Use Protocols at the University of Georgia.

PCR and sequencing protocol

Up to 25 mg of tissue was minced into small pieces and placed into a 1.5 ml microcentrifuge tube with 200 µl of tissue lysis buffer containing 600 mAU of Proteinase K (Qiagen). Tissues were incubated at 55°C until completely dissolved. Total DNA was extracted using the DNeasy tissue kit (Qiagen). Final concentration was determined by spectrophotometric analysis, using the ratios of absorption at 260 nm versus 280 nm to ensure DNA purity. A final concentration of 0.1-1 µg/µl was used for the PCR reactions. The oligonucleotide primers were designed according to the Hawaii green turtle herpesviral (fibropapilloma) genes, GenBank AF035003, representing a highly conserved region of the herpesviral DNA (Greenblatt et al. 2005b; Quackenbush et al. 1998, 2001). Three different pairs of primers were designed for 3 different genes of the herpesvirus – DNA polymerase catalytic subunit (UL30, pol), capsid maturation protease (UL26), and membrane glycoprotein B (UL27, gB). All primers were manufactured by Integrated DNA Technologies, Inc.

The primary PCR primers and nested PCR primers were designed as follows: the forward and reverse primers for the primary PCR target to DNA polymerase catalytic subunit pol (5’ —

AGC ATC ATC CAG GCC CACA AT CTG— 3’, 5’ —CGG CCA GTT CCG GCG CGT CGA CCA— 3’, respectively) result in an amplification product of approximately 445 bp. These primers were used exactly as described by previous investigators (Lu et al. 2000). The forward and reverse primers for the nested PCR target to DNA polymerase catalytic subunit (5’ —CGG CGA GCC GAA ACG CTC AAG G— 3’, 5’ —TCC GTT CCC CAG CGG GTG TGA A— 3’) result in an amplification product of approximately 364 bp.

The primary and nested PCR primers of capsid maturation protease were designed according to the 3357 to 5006 region of the Hawaiian green turtle herpesviral gene (GenBank AF035003), using Primer3 software (Untergasser et al. 2007). The forward and reverse primers for the primary PCR target to capsid maturation protease (5’—AGA GCG AGG GTT TAG GCT GGA C— 3’, 5’ —CAA TGC CGC CCT TCC TCG TCG G— 3’, respectively) result in an amplification product of approximately 495 bp, and the forward and reverse primers for the nested PCR target to capsid maturation protease (5’ —GAT CAC AAG GAC CGA TGC ACG G— 3’, 5’ —AGC GGT TTC ATC GTA TAT CGC G— 3’, respectively) result in an amplification product of approximately 324 bp.

The primary and nested PCR primers of membrane glycoprotein B were designed according to the 5155 to 7713 region of the Hawaiian green turtle herpesviral gene (GenBank AF035003), using Primer3 software (Untergasser et al. 2007). The forward and reverse primers for the primary PCR target to virion membrane glycoprotein B (5’ —GTG CGC ACT TCC GTA ATC TCG TCC— 3’, 5’ —CAG AGA CGC CAC CTT TAC TCA GGT— 3’, respectively) result in an amplification product of approximately 534 bp, and the forward and reverse primers for the nested PCR target to virion membrane glycoprotein B (5’ —AGT AGG GAA GCA GCT CGT TGT G— 3’, 5’ —CGA CGT AAC GGT ATG GGA GCT G— 3’, respectively) result in

an amplification product of approximately 300 bp.

To ensure that our findings were not the result of contamination, PCRs were run with 2 negative controls- a chicken liver genome template and no genome template. The PCR products were electrophoresed to determine size, along with equivalent plasmid inserts for comparison. Prior to this study, the 3 gene targets were each cloned into plasmids, and the sequences were verified via multiple sequence alignment (Kang et al. 2008). These equivalent plasmid inserts were electrophoresed along with all test samples for size comparison, as positive controls. The PCR products with the same size and corresponding plasmid type were considered a positive product. The PCR products were resolved on 1% agarose gels. For 3 PCR products (one sample of each type of nested amplicon), bands of the appropriate size were excised and purified using the QIAquick Gel Extraction Kit (Qiagen). Capillary (Sanger) DNA sequencing was performed using the BigDye Terminator Kit, and analyzed on a 3730 XL, 96-well capillary electrophoresis DNA sequencing system at the Georgia Genomics Facility at the University of Georgia. Viral sequences were compared to the Hawaiian green turtle herpesvirus, GenBank AF035003, using Clustal multiple sequence alignment (EMBL-EBI 2014) to verify the gene targets according to DNA identity.

Laser capture microdissection protocol

The biopsy sample evaluated was composed of non-tumored skin removed from the shoulder of a turtle without tumors. The tissue was placed in 10% buffered formalin for 1–2 weeks, then placed in a tissue cassette and embedded in paraffin. Paraffin-embedded tissue was cut into 5-10μm sections, 3 of which were mounted on a polyethylene naphthalate, 1.35 μm membrane-slide. The tissue sections were stained using routine hematoxylin and eosin staining

techniques. Epidermal and dermal tissues were microscopically visualized and differentiated, and 4 samples from each of the 2 tissue types were traced and microdissected. Microdissected samples were then cleanly removed using two separate isolation caps, one for each tissue type, which rested on a membrane and thus had no direct contact with the specimen (Molecular Machines & Industries). The membranes, contained within 1.5 ml microcentrifuge tubes, were inundated with 180 µl of tissue lysis buffer and 12 mAU of Proteinase K (Qiagen). Tissues were successively incubated at 56°C and 90°C until completely dissolved. Total DNA was extracted using a DNA FFPE Tissue Kit (Qiagen), and primary and nested PCR for the polymerase gene target were performed, as previously described. The negative control of no genome template was used in these PCR assays; as a positive control, we used the DNA polymerase catalytic subunit pol equivalent plasmid insert as described above. The dermis and epidermis PCR products were resolved on a 1% agarose gel, and bands of the appropriate size were excised, purified using the QIAquick Gel Extraction Kit (Qiagen), and sequenced by the Georgia Genomics Facility at the University of Georgia as previously described. These viral sequences were compared to that of the Hawaiian green turtle herpesvirus, GenBank AF035003, using Clustal multiple sequence alignment (Untergasser et al. 2007) to verify the gene target according to DNA identity.

Results

Number of turtles sampled by location, including numbers of turtles with and without tumors, is presented in Table 2.1. Within 6 sampling sessions over a 3-year period, 126 turtles were captured and a total of 201 skin biopsy samples were taken.

Table 2.1. Number of turtles sampled by location, with and without tumors

Location	Total turtles	Turtles with tumors	Turtles without tumors
Manglar	69	18	51
Culebrita	57	3	54
TOTALS	126	21	105

Three different primer sets were designed for 3 CFPHV genes– DNA polymerase catalytic subunit (UL30, pol), capsid maturation protease (UL26), and membrane glycoprotein B (UL27, gB), and these nested CFPHV PCR targets were confirmed according to gene fragment size. The gene targets were further verified by the results of the Clustal multiple sequence alignments (Untergasser et al. 2007), which were conducted on the 3 nested PCR products- one sample of each type of nested amplicon. Alignment of the sequences revealed that the CFPHV DNA pol PCR product showed 100% (206/206 bp) nucleotide sequence similarity; the capsid maturation protease PCR product showed 99.4% (322/324 bp) nucleotide sequence similarity; and the membrane glycoprotein B PCR product showed 100% (300/300 bp) nucleotide sequence similarity when compared to predicted homologous nucleotide sequences for Hawaiian green turtle herpesvirus, GenBank AF035003 (data not shown).

Results of the primary and nested PCRs for the 3 target sequences, i.e., DNA pol, capsid maturation protease, and membrane glycoprotein B, are shown in Table 2.2. Of the 38 tumor tissues, 89.5% were positive for the polymerase gene target by either primary or nested PCR. Primary PCR for the polymerase gene target was positive for 6 tumors, whereas nested PCR was positive for polymerase for all but 5 tumors (86.8%).

Table 2.2. Number of positive amplification of target gene partial sequences
*P=primary PCR; N=nested PCR

Description of samples			DNA target type*					
			Polymerase		Capsid protease		Glycoprotein B	
Location	Type of tissue	Number	P	N	P	N	P	N
Manglar	Tumor	29	4	24	3	21	1	20
	Skin from turtle with tumor	18	0	8	0	9	0	5
	Skin from normal turtle	71	0	21	1	19	0	17
Culebrita	Tumor	9	2	9	0	2	2	9
	Skin from turtle with tumor	3	0	1	0	1	0	1
	Skin from normal turtle	71	0	13	1	7	1	9

Primary PCR for the capsid maturation protease gene target was positive in 3 tumors; nested PCR was positive for capsid maturation protease in 23 tumors. Primary PCR for the virion membrane glycoprotein B gene target was positive in 3 tumors; nested PCR was positive for glycoprotein B in 29 tumors.

In all but one case, when turtles with tumors were captured, skin from a non-tumored area was also collected. None of these 21 skin biopsies were positive by primary PCR, but 47.6% were positive for CFPHV using the nested PCR technique.

Additionally, skin samples were taken from 105 turtles that had no evidence of tumors, and assayed for presence of CFPHV. Of 142 skin biopsies taken from the 105 normal animals sampled, 32.4% were positive for nucleic acid of the virus by PCR. Of these, only 4.3% were positive using the primary PCR technique, and one of these was positive for both capsid maturation protease and membrane glycoprotein B. Of the 46 skin samples which tested positive

by nested PCR, 39.1% were positive for both polymerase and capsid maturation protease. Three other normal skin samples were only positive via nested PCR for membrane glycoprotein B.

Using laser capture microdissection, DNA extraction, and the DNA pol target nested PCR protocol, CFPHV DNA was detected in both epidermal and dermal tissues from the skin of a non-tumored turtle. Distinct bands were visualized in the approximately 364 bp region of the gel for both epidermal and dermal samples, consistent with the positive control for the CFPHV DNA polymerase gene target. The nested PCR product sequences representing the differentiated dermis and epidermis samples were subjected to Clustal multiple sequence alignment (Untergasser et al. 2007) to verify the gene targets. Comparison of the dermis CFPHV DNA polymerase PCR product showed 96% (288/300 bp) nucleotide sequence similarity; and comparison of the epidermis CFPHV DNA polymerase PCR product showed 98% (331/338 bp) nucleotide sequence similarity when compared to predicted homologous nucleotide sequences for the Hawaiian green turtle herpesvirus DNA polymerase catalytic subunit gene pol (GenBank AF035003). The dermis CFPHV PCR product was noted to be relatively impure, which could explain the low sequence similarity obtained for this product as compared to the other sequences reported here.

Discussion

Of the turtles sampled in Manglar Bay during these 6 sampling sessions, 30.5% had tumors. At the relatively more pristine Culebrita, 5.3% of turtles sampled had tumors. A Fisher's exact test revealed a significant association between proportion of turtles with FP and location (Manglar Bay vs. Culebrita, $p = 0.0005$, $\alpha = 0.05$). Historical records provided by the Puerto Rico Department of Natural Resources indicate that in past surveys tumors were common in

turtles found in Manglar Bay but rare in turtles within the Culebrita aggregation. Specifically, turtles with FP were reported at Manglar Bay with high to medium prevalence, i.e. 57% in 2001–2005 and 30% in 2006–2007 (Diez et al. 2010). In more recent years, however, FP prevalence has been reported to be as low as 0% at Manglar Bay (Patricio et al. 2011). Sampling at Culebrita showed that FP was rare (<1% prevalence) until 2009, when 40% FP disease prevalence was observed in green turtles captured there (Patricio et al. 2011; Velez-Zuazo et al. 2010).

In this study, the tumors were collected immediately into formalin and then after 12–24 hours, changed to RNase-free PBS. This was logistically necessary, due to the tropical working environment- the high ambient temperatures made it impossible to keep ice frozen for the duration of an entire sampling day. Although this technique worked even with brief formalin fixation, in future studies if freezing the tissues is not feasible a more appropriate technique will be employed to conserve the DNA, such as use of 98% ethanol or a ribonucleic acid (RNA) stabilization reagent (i.e., RNAlater, Qiagen).

One aim of this study was to determine the suitability of various CFPHV gene targets in PCR assays for detection of CFPHV in affected tissues. To accomplish this, we tested 3 different gene targets for the CFPHV genome, and applied primary and nested PCR assays to detect the DNA in both tumored and non-tumored tissues. The PCR technique was successful at amplifying the virus from tumors, with polymerase nested PCR performing the most consistently. All but one of the 34 CFPHV-positive tumors yielded a positive result using the nested PCR technique for the polymerase gene target (97.2%). Our results are comparable to other studies that have described the use of nested PCR technique for polymerase gene target to detect CFPHV DNA in tumor tissues. The CFPHV DNA was detected in 100% and 95.7% of tumors examined from green turtles in Hawaii and Florida, respectively (Lackovich et al. 1999; Lu et al. 2000). The

study reported here is an important addition to previous work because it compares 3 different gene targets for CFPHV DNA detection. As expected, the polymerase gene target proved to be superior to the capsid maturation protease and membrane glycoprotein B gene targets in the positive detection of CFPHV DNA. It is vital, however, not to overlook the utility of the alternative gene targets. The one tumor that was negative by nested PCR for polymerase was positive for CFPHV DNA by nested PCR for both capsid maturation protease and membrane glycoprotein B. So, the overall sensitivity of viral detection may be improved in future studies by the use of these additional targets.

Another aim of this study was to explore the presence of CFPHV DNA in non-tumored skin taken from turtles with and without tumors. In addressing this aim, we found that several normal skin samples taken from tumored turtles were CFPHV positive by PCR. Of 21 biopsies taken from normal shoulder skin in animals that had tumors at other sites, 47.6% were positive by nested PCR for the polymerase gene and occasionally the other two DNA targets. These results agree with those of previous reports documenting CFPHV DNA in normal skin from turtles with tumors. In two studies conducted in Hawaiian green turtles with tumors, 57.1% and 93.3% of skin samples were positive by nested PCR for the polymerase gene, respectively (Lu et al. 2000; Quackenbush et al. 1998). In normal skin sampled from tumored green turtles in Australia, 45.5% of samples were positive for the polymerase gene by quantitative PCR (Quackenbush et al. 2001).

The CFPHV DNA was also detected in skin from “normal” turtles, i.e., those without any apparent tumors. There were 105 normal turtles captured and biopsied, and 32.4% of 142 biopsy samples were positive for CFPHV. None of these were positive by primary PCR for the polymerase gene, whereas 34 were positive by the nested PCR technique. These results are

similar to those reported from another part of the world. Of skin samples from 14 normal green turtles in Australia, 21.4% were positive for the CFPHV polymerase gene by quantitative PCR (Quackenbush et al. 2001). However, the findings in our study are also contradictory to some other published reports. In a study on non-tumored, stranded green turtles in Florida, all skin samples were negative by nested PCR for the CFPHV polymerase gene (Lackovich et al. 1999). In two studies conducted in Hawaiian green turtles, all skin samples from normal green turtles were negative by nested PCR (Lu et al. 2000; Quackenbush et al. 1998). The differences in these various studies are not easily explained. Some natural variations can be expected based on the widely different geographic regions, population genetics, and overall environmental considerations. But also, some of the differences in the reported works may be a function of sample size. The work presented here represents a significant contribution to the existing research because the sample size is considerably larger than any previously reported, and may be a more accurate representation of the true nature of the situation.

Separation of dermis and epidermis can be accomplished by gross dissection alone; however, dissection of tissues inherently could lead to sample contamination by unsolicited cell types. Laser capture microdissection allows for precise localization of a specific segment of nucleic acid within a histologic section. As such, this technique was applied to address the third aim of our study: to better identify the precise tissue location of the CFPHV genome in skin sampled from a non-tumored turtle. We employed laser capture microdissection to determine whether CFPHV DNA in a non-tumored section of skin might be harbored in epidermis or dermis, or both. We specifically chose to use skin from a "normal" turtle, and we used a biopsy that had previously been shown to contain CFPHV nucleic acid via PCR. Separating samples from epidermis and dermis yielded surprising results- the viral nucleic acid was present in both

portions. A previous study documents CFPHV DNA in both dermis and epidermis of FP tumors, with higher levels in the dermis (Work et al. 2009). Because viral particles have routinely been observed in epidermal cells of tumored skin, it is generally assumed that tumored skin undergoes viral shedding, at least periodically, and that transmission may occur in this manner. But our findings are notable in that we also found viral nucleic acid in differentiated tissues of normal turtle skin, suggesting there may be viral shedding from non-tumored turtles as well. Additionally, the identification of viral nucleic acid in dermis as well as epidermis raises the possibility that the infection is much more than an epidermal infection, but that there may be systemic cells that also harbor the virus.

The results of this study constitute a meaningful contribution to related evidence that CFPHV infection may be common relative to disease incidence, and support the idea that aspects of the environment and host may play a transforming role in FP disease expression. This idea is further supported by phylogenetic analyses of CFPHV that show evidence of low viral mutability, suggesting coevolution of the virus with marine turtle hosts over millennia and a potential for external factors to affect disease expression (Herbst et al. 2004; Patricio et al. 2012). It is known that turtles develop FP after they recruit to near-shore habitat locations as juveniles (Ene et al. 2005; Herbst et al. 2008). Previous studies show that land use near areas where turtles feed may influence disease rates, with elevated FP incidence grouped in watersheds with high nitrogen footprints (Dailer et al. 2010; dos Santos et al. 2010; Van Houtan et al. 2010). Sites with high FP prevalence, such as the Manglar Bay sampling site in this study, may serve to amplify the disease transmission cycle, since tumors are a known source of viral transmission (Herbst et al. 1995, 1996, 2008). Future studies involving laser capture microdissection should include evaluation of a larger sample size to validate the accuracy of our conclusions. Determining

exactly which circulating cells may be infected could have great value in further understanding of this disease and aid in devising more effective control measures.

CHAPTER 3

FACTORS INFLUENCING SURVIVORSHIP OF REHABILITATING GREEN SEA TURTLES WITH FIBROPAPILLOMATOSIS [1]

[1] Page-Karjian, A., Norton, T.M., Krimer, P., Groner, M., Nelson, S.E., and N.L. Gottdenker. 2014. *Journal of Zoo and Wildlife Medicine.* 45(3):507-519.
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Abstract

Marine turtle fibropapillomatosis (FP) is a debilitating, infectious neoplastic disease that has reached epizootic proportions in several tropical and subtropical populations of green turtles (*Chelonia mydas*). FP represents an important health concern in sea turtle rehabilitation facilities. The objectives of this study were to describe the observed epidemiology, biology, and survival rates of turtles affected by FP (FP+ turtles) in a rehabilitation environment; to evaluate clinical parameters as predictors of survival in affected rehabilitating turtles; and to provide information about case progression scenarios and potential outcomes for FP+ sea turtle patients. A retrospective case series analysis was performed using the medical records of the Georgia Sea Turtle Center (GSTC), Jekyll Island, Georgia, USA, during 2009–2013. Information evaluated included signalment, morphometrics, presenting complaint, time to FP onset, tumor score (0–3), co-morbid conditions, diagnostic test results, therapeutic interventions, and case outcomes. Overall, FP was present in 27/362 (7.5%) of all sea turtles admitted to the GSTC for rehabilitation, either upon admittance or during their rehabilitation. Of these, 25 were green and 2 were Kemp's ridley (*Lepidochelys kempii*) turtles. Of 10 turtles that had only plaque-like FP lesions, 60% had natural tumor regression, all were released, and they were significantly more likely to survive than those with classic FP (P = 0.02 [0.27–0.75, 95% CI]). Turtles without ocular FP were 8 times more likely to survive than those with ocular FP (odds ratio = 8.75, P = 0.032 [1.21–63.43, 95% CI]). Laser-mediated tumor removal surgery is the treatment of choice for FP+ patients at the GSTC; number of surgeries was not significantly related to case outcome.

Introduction

FP represents an important health concern in captive sea turtle management situations, such as rehabilitation, captive rearing, and head start centers; yet the effects of this disease on rehabilitating turtles have not been studied (Stacy et al. 2008; Tristan et al. 2010). The primary objective of this study was to describe the observed epidemiology, biology, and survival rates of turtles affected by FP in a rehabilitation environment. The second objective was to evaluate clinical parameters as predictors of survival in affected turtles in this environment. The third objective was to provide pertinent information about case progression scenarios and potential outcomes for clinicians dealing with sea turtle patients afflicted with FP. To address these objectives, a retrospective case series analysis was performed using the medical records of the Georgia Sea Turtle Center (GSTC), Jekyll Island, Georgia, USA.

Methods and Materials

Case selection

Medical records of all wild-caught sea turtles undergoing rehabilitation at the GSTC between 2009 and 2013 were reviewed to identify fibropapilloma-positive (FP+) cases. The inclusion criteria for FP+ turtles in this study were admission as a sea turtle patient to the GSTC, presentation or development of external tumors, and complete medical records, including all observations and treatments, until time of release or necropsy. FP was diagnosed based on clinical signs, histopathology, and/or advanced imaging technologies.

Case data

The following data were collected from medical records: species and sex (when known); month, county, and state where turtles were found; weight and straight carapace length (SCL); presenting condition; days to onset of tumors; highest tumor score; clinical pathology; ancillary diagnostics; and case outcome.

Presenting complaints included FP, floating, boat strike, debilitated, or stranded on the beach. FP included turtles in which FP was considered the primary presenting condition. Floating included turtles found abnormally buoyant; boat strike included evidence of propeller wounds (single to multiple linear lacerations); debilitation included turtles that were emaciated, weak, and lethargic and had heavy epibiotic loads on their skin; stranded included all other cases. In cases with overlapping presenting complaints, categories were prioritized as follows: boat strike wounds, floating, debilitated, FP, stranded. Individual turtles were designated only one presenting complaint per turtle; however, each turtle was designated one or more co-morbid conditions. Co-morbid conditions during rehabilitation included animals with ongoing floating that continued after presentation, trauma (e.g., bone fracture, boat strike lacerations), metabolic conditions (e.g., emaciation, edema/anasarca, tube fed for >2 weeks), and infection (e.g., osteomyelitis, bacterial dermatitis).

The total number of days before tumors were first observed was recorded for FP+ turtles. Criteria used to categorize FP grossly included single to multiple variably sized, sessile to pedunculated to coalescing masses, adopting a smooth to rugose appearance (Herbst 1994). For turtles that developed FP during rehabilitation, characteristics of tumor onset were recorded (turtle size class and body condition index (BCI = [weight (kg)/SCL^3] x 10,000), month of onset, location tumors first observed; Bjorndal et al. 2000). FP tumor scores (0–3) were assigned to all

patients according to the location, size, and number of tumors (Table 3.1). Tumor scores were designated to reflect the spectrum of FP extent and severity: from non-afflicted (0) to lightly (1; Figure 3.1), moderately (2; Figure 3.2), and heavily (3; Figure 3.3) afflicted (Balazs 1991; Work & Balazs 1999). FP+ turtles whose only FP lesions appeared as smooth, sessile plaques were enumerated, and case files were assessed for diagnostics performed, spontaneous lesion regression, and survivorship.

Table 3.1. Morphologic criteria used to assign tumor scores (0-3) to turtles in this study

	Tumor score			
	0	1	2	3
Number of tumors	--	1-10	>2	>10
Tumor size, range (diameter, cm)		0-1	1-5 OR >5 with a 0-1 cm base	>5
Tumor location	--	Skin or conjunctivae	Skin, shell, conjunctivae, unilateral cornea, previous tumor removal site	Skin, shell, conjunctivae, bilateral corneae, intraocular
Tumor morphology	--	Flat to slightly raised	Raised, proliferative, and/or pedunculated	Severe, aggressive, raised, proliferative/verrucous
Internal tumors	No	No	No	Yes

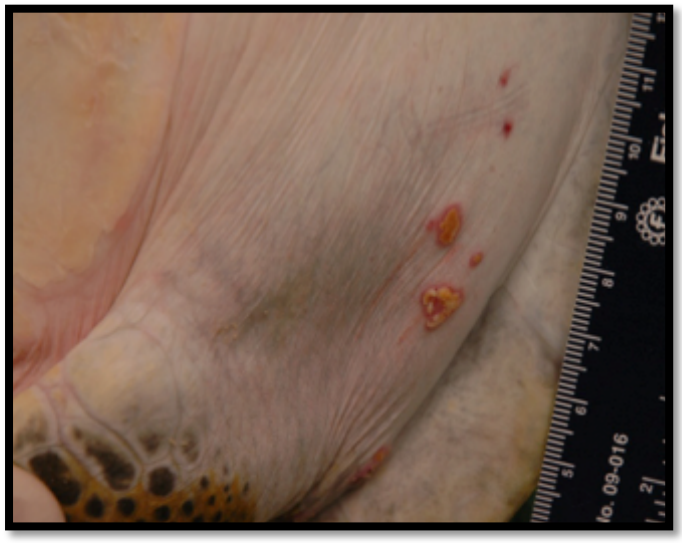

Figure 3.1. Example of FP tumor score 1: 1-10 flat to slightly raised lesions that are 0-1 cm in diameter and located on the skin (photo credit Georgia Sea Turtle Center)

Figure 3.2. Example of FP tumor score 2: >2 raised, proliferative lesions that are 1-5 cm in diameter and located on the skin and shell (photo credit Georgia Sea Turtle Center)

Figure 3.3. Example of FP tumor score 3: >10 severe, aggressive, proliferative, verrucous lesions that are >5 cm in diameter and located on the skin, shell, conjunctivae, and corneae (photo credit Georgia Sea Turtle Center)

Clinical pathology parameters were evaluated for blood samples collected from the dorsal cervical sinus of all FP+ turtles using a sodium-heparinized syringe. Routine complete blood counts, including packed cell volume, manual differential, and estimated leukocyte count (manual technique), were performed "in house" by a certified veterinary technician trained in reptilian hematology. Plasma biochemical values, including albumin, total protein, uric acid, calcium, potassium, globulins, phosphorus, blood urea nitrogen, cholesterol, glucose, chloride, sodium, aspartate transaminase, triglyceride, and creatine kinase, were performed by Idexx Laboratory on a Beckman AU5400 Chemistry System random access chemistry analyzer (Beckman Coulter, Inc.). Clinical pathology values at the most severe stage of tumor score were used for all FP+ turtles.

Additional diagnostics used to diagnose or rule out FP were evaluated, including biopsy and histopathology, PCR, endoscopy, and imaging techniques, including computed tomography

(CT) scan, magnetic resonance imaging (MRI), radiography, and ultrasound. Histologic criteria used to diagnose FP included presence of papillary epidermal hyperplasia supported on broad fibrovascular stromal stalks, often with orthokeratotic hyperkeratosis and a varying ratio of epidermal to dermal proliferation (Herbst 1994). Regressing tumors were histologically characterized by focal epidermal hyperplasia bordered and infiltrated by moderate to marked numbers of lymphocytes and macrophages. The PCR assay used was a research assay for the DNA polymerase (pol; UL30) gene segment of chelonid fibropapilloma-associated herpesvirus-5 (CFPHV5), employed by the University of Florida, College of Veterinary Medicine, Zoological Medicine and Wildlife Disease Laboratory; positive results were confirmed via sequencing (Ackermann et al. 2012). Therapeutic interventions reported included routine treatments of FP+ turtles and CO^2 laser-mediated tumor removal surgery.

Case outcomes included natural death, euthanasia, release, or long-term captivity. Total duration of rehabilitation was recorded for all turtles. Long-term patient follow-up data were obtained from some records, including satellite tracking of turtles fitted with a transmitter prior to release.

Patient care

Before admission as a patient to the GSTC, all turtles were recovered by U.S. southeastern state stranding network officials and transported to the GSTC for medical attention. Turtles' identities were determined using flipper and/or internal passive transponder identification numbers, if available. All FP+ turtles were housed in 3 m diameter tanks (1.1 m depth), which were partitioned in half (two turtles per tank), with independent water supplies (water temperature 25°C; 30 ppt salinity). All tanks were fitted with independent life support

systems with filtration (bead) and disinfection (ozone); water quality parameters were tested frequently (temperature, pH and salinity twice daily, and all other parameters weekly), and the systems were routinely flushed. Natural light periodicity ranged from 8 hr in the winter to 14 hr in the summer. Green turtles were fed a 1% body weight diet consisting of a mixture of seafood (Atlantic mackerel, herring, squid, shrimp), greens (romaine lettuce, cucumber, green bell pepper), and/or elemental gelatin. A predominantly vegetarian diet (that also included gelatin) was fed to all green turtles that would consume it. For more critical and/or emaciated turtles, initial feedings consisted of primarily seafood; as rehabilitation progressed and the turtles' weight stabilized, they were transitioned to a more vegetarian diet, with seafood limited to medication delivery or not at all. All patients were regularly supplemented with oral multivitamins (Mazurit Vita-Zu^{TM}) avian tablets [1/2 tablet q. 24 hr]; this dosage includes 209 IU vitamin A, 6.25 IU vitamin E, 6.25 IU vitamin C, and 6 mg vitamin B1/thiamine and calcium with vitamin D (150 mg q. 24 hr). Patients were also routinely supplemented with iron if anemia was observed; either injectable iron dextran (10 mg/kg SC) for inappetent turtles or oral ferrous sulfate (10 mg/kg) for turtles regularly eating on their own (Carpenter et al. 2001). Frequency of iron administration was based on hematocrit and plasma iron levels and ranged from q. 24 hr to q. 7 days. Turtles with FP were physically quarantined from other animals housed at the GSTC. Extensive sanitary husbandry techniques were routinely used to maintain quarantine and prevent potential disease transmission via fomites or personnel.

Routine therapeutic and diagnostic treatments

Typical therapeutic and diagnostic treatments of turtles with FP involve nutritional support (tube feeding) and fluid therapy, treatment of secondary infections or other conditions,

stress minimization during captivity, complete blood counts and plasma biochemistries, imaging techniques and endoscopy to detect internal tumors, and surgical excision of tumors. For turtles that underwent surgical excision of tumors, all tumors were removed in the first surgery; thus, any subsequent surgeries were to remove tumor re-growths or new tumors. Other treatments were administered as necessary, including cleaning and debridement of external traumatic injuries and correction of floating abnormalities (lung injury or gastrointestinal impaction). Routine therapies administered to all rehabilitating patients in this study included Betadine (Purdue Products LP) scrub and 70% ethyl alcohol, used regularly to clean the carapace and plastron, and silver sulfadiazine cream (Par Pharmaceutical, Inc.) applied to areas of skin and/or shell irritation, using aseptic techniques. After light debridement of the carapace and plastron, other topical treatments were applied to patients as needed, including Medihoney™, fresh honeycomb, Gentamicin-impregnated bone cement (Jorgensen Labs), and Doxirobe™ (Pfizer). Some FP+ patients were also intermittently treated with antivirals, including oral acyclovir ([80 mg/kg, q. 24 hr] Zovirax™), oral SHaNa Vet™ [25–30 mg/kg, q. 24 hr], topical SHaNa Vet™ cream ([q. 1 week] Animal Necessity), and/or topical gangciclovir ophthalmic gel in certain turtles with ocular FP ([q. 7 days] Zirgan™ by Bausch & Lomb).

Epidemiologic and statistical analyses

The following associations were evaluated using Fisher exact and odds ratios for categorical data: number of tumor removal surgeries (1/>1) versus case outcome (survived/died), ocular tumor status versus case outcome, and tumor morphology (plaques/classic FP) versus case outcome. Means were calculated for the following continuous data: weight, SCL, and BCI measurements at admission, time to tumor onset, total time in rehabilitation, time before tumor

re-growth, and hematology and plasma biochemistry values (at peak FP disease severity).

Percentages were calculated for categorical parameters (i.e., species, year and location found, presenting complaints, co-morbid conditions, case outcomes). Ancillary diagnostic tests were enumerated and assessed for usefulness in their ability to detect or rule out FP. Tumor locations on the FP+ turtles' bodies (i.e., front versus back, head/neck, eyes, front flippers, inguinal skin, shell(s), tail/tail base, shell-skin interface, internal, wound site) were categorized and enumerated. Statistical analyses were performed using JMP statistical software (v.10, SAS Institute, Inc.).

Results

Signalment, demographics, and morphometrics

From 2009 to 2013, a total of 362 turtles were admitted to the GSTC: 123 green turtles (*C. mydas*), 187 loggerhead turtles (*Caretta caretta*), and 52 Kemp's ridley (*Lepidochelys kempii*). Over- all, FP was present in 27/362 (7.5%) of all sea turtles admitted to the GSTC for rehabilitation, either upon admittance or during their rehabilitation. Of these, 25 were green turtles and 2 were Kemp's ridley turtles. No loggerhead turtles were diagnosed with FP. For all remaining statistical analyses, only green turtles were considered in this study. By year, there were 4 FP+ cases in 2009, 3 in 2010, 6 in 2011, 6 in 2012, and 6 in 2013. Turtle sexes were not statistically assessed, due to an overwhelmingly large number of ''unknown'' sexes. The FP+ turtles were recovered in near shore waters of Georgia (7/25 or 28%) and Florida (18/25 or 72%) in the following counties: Camden, Glynn, Brevard, Nassau, Duval, St. John's, and Indian River. Mean (±SE) weights and SCLs at admission of FP+ turtles are reported in Table 3.2. The highest FP prevalence was in the 30–34.9 cm size class (9/25, 36%; Figure 3.4).

Table 3.2. Morphometrics, hematology and plasma biochemistry data at time of peak fibropapillomatosis (FP) severity in green turtles affected by FP. Numbers reported in each category are average and standard error.

	n	FP+ green turtles
Morphometrics		
Straight carapace length (cm)	24	36.9 ± 1.5
Weight (kg)	25	6.16 ± 0.84
Hematology		
Packed cell volume (%)	21	26.28 ± 2.11
Heterophils (%)	21	48.43 ± 3.01
Lymphocytes (%)	21	40.86 ± 3.0
Monocytes (%)	21	6.86 ± 1.14
Eosinophils (%)	21	3.81 ± 0.9
Estimated total white blood cell count	21	3,933.33 ± 597.26
Plasma biochemistry		
Total protein (g/dl)	25	3.06 ± 0.25
Albumin (g/dl)	22	1.25 ± 0.11
Globulin (g/dl)	22	2.27 ± 0.17
Albumin/globulin (A:G) ratio	22	0.54 ± 0.02
Uric acid (mg/dl)	22	1.39 ± 0.26
Calcium (mg/dl)	22	5.7 ± 0.36
Potassium (mEq/dl)	22	4.36 ± 0.24
Phosphorus (mg/dl)	22	8.81 ± 1.18
Calcium/phosphorus (Ca:P) ratio	22	0.79 ± 0.09
Blood urea nitrogen (BUN; mg/dl)	19	46.79 ± 6.88
Cholesterol (mg/dl)	19	127.32 ± 15.77
Glucose (mg/dl)	24	95.67 ± 7.7
Chloride (mEq/dl)	19	117.68 ± 2.55
Sodium (mEq/dl)	22	162.41 ± 8.83
Aspartate transaminase (AST; U/L)	22	221.23 ± 20.97
Triglyceride (mg/dl)	17	126.29 ± 25.84
Creatine kinase (U/L)	22	3,103.91 ± 964.46

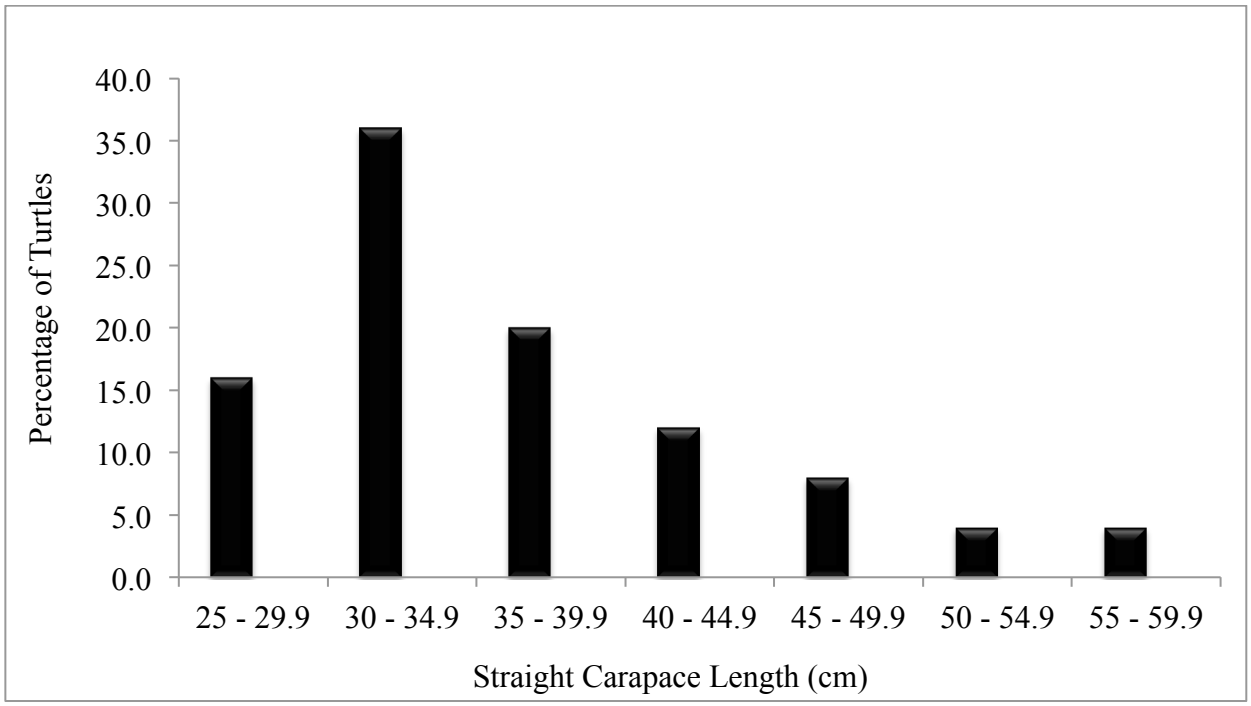

Figure 3.4. Straight carapace length (cm) of turtles affected by fibropapillomatosis (FP), reported as percentages of turtles per size class

Presenting complaints and co-morbid conditions

The presenting complaints of the FP+ turtles included floating (8/25, 32%), stranding (5/25, 20%), boat strike (5/25, 20%), FP (4/25, 16%), and debilitation (3/25, 12%). Although 9/25 (36%) of the FP+ turtles presented with tumors, FP was only considered as a primary presenting complaint in 4 turtles. Co-morbid conditions observed in FP+ turtles include ongoing floating (14/25, 56%), emaciation (9/25, 36%), skin infection (8/25, 32%), osteomyelitis (6/25, 24%), boat strike lacerations (5/25, 20%), being tube fed for more than 2 weeks (5/25, 20%), edema (4/25, 16%), and bone fracture (4/25, 16%). Causes of floating were diagnosed in 10/14 (71.4%) floating turtles: gastrointestinal impaction (6/10 or 60%); lung tear (2/10 or 20%); and unilateral pneumonia and spinal fracture (1/10 or 10% each).

Tumor onset

Thirteen of the 25 FP+ turtles (52%) presented with FP, including turtles that developed tumors in <1 week. Twelve of 25 (48%) developed tumors <1 week after entering rehabilitation: 1–10 weeks (7/25 or 28%), 31–40 weeks (4/25 or 16%), and 11–20 weeks (1/25 or 4%); no turtles developed FP between 21 and 30 weeks (Figure 3.5). Of these 12, mean (±SE) time to onset of tumors was 87 days ± 28 days, and ranged from 0 to 259 days. Most of the 12 turtles that developed FP in rehabilitation were in the smaller size classes (9/12 in the 30–34.9 cm size class or below), whereas 8/13 of the turtles that presented with FP were in relatively larger size classes (35–39.9 cm size class or above). Mean BCI did not significantly differ between the two groups: turtles that developed FP during rehabilitation had a mean BCI of 1.06 (± SE 0.07) versus a mean BCI of 1.15 (± SE 0.04) for turtles that presented with FP. Turtles that developed FP in rehabilitation usually did so during the warmer months (10/12 or 83.3% first grew tumors between April and September), and tumors were often first observed in the inguinal region(s) of the turtles' bodies (7/12 or 58.3%).

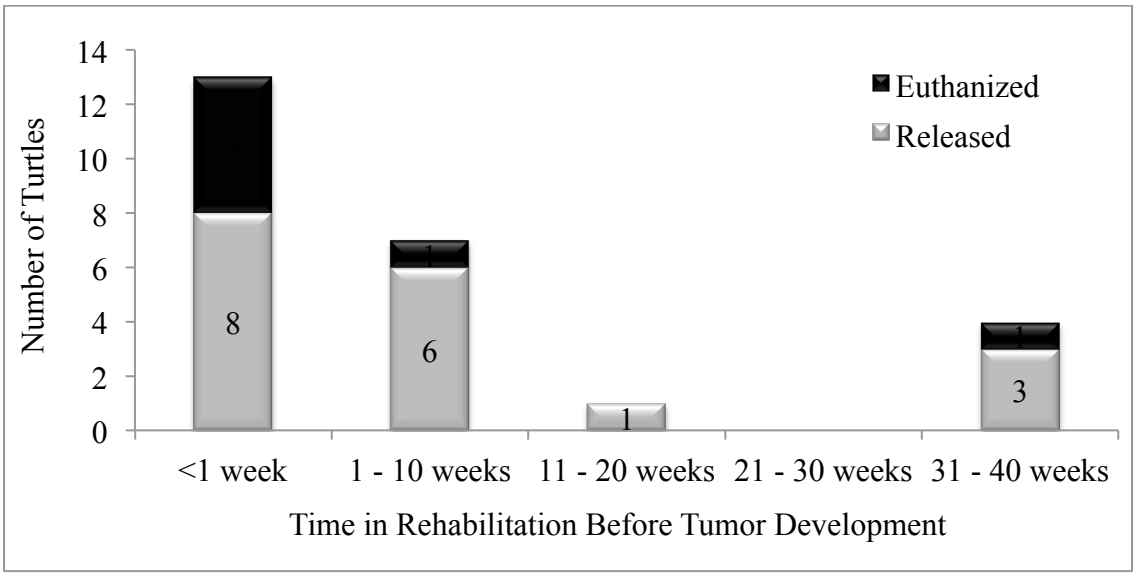

Figure 3.5. Time to tumor expression in weeks, measured as time in captivity until time of tumor expression, related to outcome (euthanized/released)

Tumor score and location

Tumor score 2 represented the majority (14/25 or 56%) of FP+ patients, followed by tumor score 1 (6/25 or 24%) and tumor score 3 (5/25 or 20%). The front flipper(s) and shoulders were most commonly afflicted with FP, followed by the inguinal region(s), plastron, and eyes (Table 3.3). Sixteen of the 25 FP+ turtles (64%) had tumors on both the dorsoanterior and the posterior portions of their bodies; 5/25 (20%) turtles had tumors only on the front of their bodies (e.g., face, eye(s), neck, front flippers and shoulders, anterior shell), and 4/25 (16%) had tumors only on the posterior parts of the their bodies (e.g., inguinal region(s), tail, cloaca, posterior shell). Turtles without ocular FP were 8 times more likely to survive than those with ocular FP (odds ratio = 8.75, P = 0.032 [1.21–63.43, 95% CI]).

Table 3.3. Tumor locations on turtles affected by fibropapillomatosis (FP)

Tumor location	Number with FP in this location	% with FP in this location
Front flipper(s)	19	76
Inguinal region(s)	16	64
Anterior and posterior	16	64
Scutes and seams	15	60
Eye(s)	9	36
Neck	8	32
Tail/tail base	7	28
Anterior only	5	20
Posterior only	4	16
Head/face	3	12
Internal	2	8
Wound site	1	4

Plaque-like FP lesions

Histopathology and PCR were not performed in all cases. Of the 10 turtles with only plaque-like lesions grossly, 7 were evaluated histologically and all of these were confirmed to be FP+. Four of these 10 turtles were evaluated using PCR, and all 4 were positive for CFPHV5. All 4 turtles that tested positive by PCR were also confirmed FP+ histologically (Herbst 1994). Six of 10 had natural tumor regression, all were released, and plaque-positive turtles were significantly more likely to survive than those with classic FP ($P = 0.02$ [0.27–0.75, 95% CI]).

Clinical pathology, antemortem diagnostics, and necropsy results

Mean (±SE) hematology and plasma biochemistry values for FP+ green turtles are reported in Table 3.2. Complete necropsies were performed in all 7 of the FP+ turtles that were euthanized/ died, revealing internal tumors in two turtles. For one of these turtles, the internal tumors were diagnosed via antemortem CT, which precipitated the decision to euthanize.

Antemortem radiographs, performed in all 25 FP+ patients, suggested internal tumors in the other turtle with internal FP. Antemortem diagnostic assays to evaluate for internal tumors were used in several other FP+ patients, including CT (6/25 or 24%), MRI (1/25 or 4%), and endoscopy (4/25 or 16%). No internal tumors were diagnosed via MRI or endoscopy. Overall, biopsy and histopathology were used as diagnostic tools in 16/25 (64%) cases, and PCR was used in 7/25 (28%). Skin lesions were positively diagnosed as FP via histopathology in 13/25 (52%) of turtles with tumors. Of the 13 turtles that were positive for FP by histopathology, 5 were also positive for CFPHV5 via PCR. Of the two tumor samples that were negative by PCR, one was diagnosed histologically as regressing FP and one was histologically negative for FP in a turtle that had been histologically FP+ on previous biopsies.

Tumor removal

Of the FP+ turtles in this study, 13/25 (52%) underwent one or more CO_2 laser-mediated surgeries to remove FP tumors. The number of tumor removal surgeries was not significantly related to case outcome. Overall, 5/13 (38.5%) of the turtles that had tumors surgically removed experienced FP regrowth within an average of 36 days (±SE 4.7) after the surgery. Of the 23 total FP lesions that were surgically removed from 13 turtles, 14/23 (60.9%) resulted in tumor regrowth and 9/23 (39.1%) did not.

Case outcomes

The mean (±SE) total time in rehabilitation was 228 days (± 61) for FP+ turtles. Of the 25 FP+ cases examined in this study, 18 (72%) were released after treatment and supportive care, including 5 (20%) released with mild cutaneous tumors and 13 (52%) released tumor free, 5

(20%) euthanized due to FP, 2 (8%) dying in captivity, and 2 (8%) living in permanent captivity after being transferred to public aquarium facilities.

Discussion and Conclusions

The FP+ patient signalment in this study is consistent with the current understanding that FP primarily affects juvenile green sea turtles after they have migrated to near-shore habitats (Aguirre et al. 1994; Aguirre et al. 1998; Ene et al. 2005; Fick et al. 2000; Herbst 1994). Turtles that developed FP in rehabilitation were in smaller size classes than turtles that presented with FP, further supporting this observation. Interestingly, the mean SCL of FP+ turtles in this study fits within a size class previously reported to have the highest FP prevalence (Hirama & Ehrhart 2007). The demographics of FP+ cases seen by the GSTC, however, do not completely represent the FP demographics in southeastern U.S. sea turtle populations: many sick or injured sea turtles likely die/end up as prey items for other animals and thus are not admitted into rehabilitation at all.

In this study, several of the turtles had FP when first encountered, and several others developed tumors shortly after entering rehabilitation (i.e., 1–2 weeks). It may be reasonable to assume that these turtles were already infected with CFPHV when they entered the GSTC, since the shortest time to tumor development in one study of experimental transmission of cell-free tumor extracts was 14.6 weeks post-inoculation (Herbst et al. 1995). A subset of turtles developed tumors after spending 11–40 weeks in rehabilitation; these cases may fit with the idea that in captive turtles, FP tumor development may be due to either viral recrudescence of latent CFPHV infections or recent viral infection in debilitated turtles with underlying health problems (Stacy et al. 2008; Work et al. 2009, 2001). Due to these findings, quarantine measures are

recommended when housing green turtles, including physical separation of green turtles from other sea turtle species, and a separate quarantine section (including an independent water filtration system or facility) for any green turtles with signs of FP. More research is needed on the CFPHV replication, latency, and transmission cycles to help develop better diagnostic tools for differentiating between infected and uninfected green turtles. Water temperatures were consistent across tanks for the turtles in this study and remained relatively constant regardless of season (23°C in winter to 27°C during summer). Regardless of this fact, it was observed that most of the turtles that developed FP in rehabilitation did so during the warmer months; this agrees with previous reports correlating tumor outbreak to warm seasons and water temperatures (Foley et al. 2005; Herbst 1994; Herbst et al. 1995; Lafferty et al. 2004). Lowering tank water temperatures by 2–5°C after tumor removal surgery may help to prevent or reduce the likelihood of tumor regrowth. Other measures that may help to prevent tumor re-growth include administration of lysine ([50 mg/ kg q. 24 hr] CVS Pharmacy Inc.) and the antivirals and neutraceuticals described above as needed. The lack of a significant difference in BCI between turtles that presented with FP and those that developed FP in rehabilitation may reflect the fact that none of these turtles was considered healthy; all were receiving treatment for other ailments in addition to FP.

The findings presented here with regard to tumor location on FP+ turtles' bodies are consistent with previous reports that cutaneous tumors may be found anywhere on the soft skin but also occur on the plastron and carapace (Herbst 1994; Jacobson et al. 1989; Work et al. 2004). With regard to front versus rear of the turtle, no predilection of tumor site was observed. Previous studies have reported greater total numbers of tumors present on either the anterior or the posterior parts of FP+ turtles' bodies (Adnyana et al. 1997). Exact number of tumors per site

was not available in some of the case records. Thus, it was not possible to compare total number of tumors per bodily site in this study. Of the 25 FP+ turtles, 9 (36%) had ocular tumors; this is similar to results of another study, in which 47.6% of FP+ turtles had ocular tumors (Hirama & Ehrhart 2007). Importantly, FP+ turtles with ocular tumors had a poorer prognosis than FP+ turtles without ocular tumors. In cases with severe ocular FP (i.e., bilateral corneal tumors, intraocular tumor(s)), euthanasia may be warranted upon presentation. Turtles with less severe ocular FP, on the other hand, (i.e., unilateral corneal, uni-/bilateral conjunctival) should be considered candidates for treatment if materials and trained personnel are available. In turtles that developed FP in rehabilitation, the most common site that tumors were first observed was the inguinal region. Complete and serial physical examinations of rehabilitating sea turtles should thus include close observation of these regions, in particular for new tumor growth.

Histopathologic results were consistent with the typical histologic appearance of FP, as described above (Herbst 1994; Herbst et al. 1999). Turtles with only plaque-like tumor lesions were confirmed to be FP+ via histopathology and PCR, quelling any suspicions that lesions with this appearance are not truly FP. Of the 7 histologically FP-positive cases, 3 were diagnosed as regressing FP and 4 were diagnosed as active FP; thus, not all plaque-like FP lesions should be considered to be regressing tumors (Bennett et al. 1999). Spontaneous lesion regression was observed in more than half of the turtles with only plaques, whereas complete tumor regression without the aid of tumor removal surgery was not observed in any of the FP+ turtles that had pedunculated or verrucous-appearing lesions (incomplete regression was observed in one turtle with pedunculated tumors). Furthermore, the presence of only plaque-like FP lesions was significantly associated with survivorship in rehabilitating turtles with FP.

Hematologic values are useful parameters that can indicate the health status of sea turtles. Blood parameter values can be affected by many factors, including age, gender, season/climate, nutrition, circulating hormones, and body hydration (Aguirre et al. 1995; Campbell 2006; Duguy 1970; Stamper et al. 2005). Plasma biochemistry values in sea turtles may be related to their physiologic state but also can indicate chronic stress or pathologic conditions (Aguirre et al. 1995; Campbell 2006). Relative to the in-house reference intervals for healthy turtles used at the GSTC, FP+ turtles tended towards mild anemia, mild hyperglobulinemia with concurrent mild decrease in the A:G ratio, mild hypocalcemia and concurrent mild decrease in the calcium to phosphorus ratio, mild to moderate increased blood urea nitrogen (likely due to increased catabolism from feeding a high protein diet), and mild hypernatremia. These changes varied by turtle but overall are consistent with anemia of chronic disease and antigenic stimulation and are compatible with the clinical presentation for FP. The results are consistent with those from other studies, in which green turtles with FP were found to have anemia, monocytosis, eosinophilia, hypoproteinemia, hypocalcemia, hypoalbuminemia, and hyperglobulinemia (Adnyana et al. 1997; Aguirre & Balazs 2000; Aguirre et al. 1995). It has been suggested that turtles with moderate to severe FP suffer from chronic inflammation, which would agree with some of the blood parameter patterns reported here (i.e., anemia, hypoproteinemia, hyperglobulinemia; Aguirre et al. 1995; Cray et al. 2001). Hypocalcemia and hyperphosphatemia are common findings in rehabilitating turtles; supplemental calcium can be given to improve the Ca:P ratio, including daily oral supplementation, or when more severe, injectable supplementation, which is appropriate for turtles with ionized calcium concentrations, 0.7 mg/dl (calcium gluconate [100 mg/kg i.m. or i.c. q. 8 hr] or calcium lactate/calcium glycerophosphate [10 mg/kg s.c. or i.m. as needed]; Carpenter et al. 2001; Norton 2005; Norton et al. 1990; Stringer et al. 2010; Swimmer

2000; Varela 1997). Natural sunlight is very beneficial for normalizing Ca:P ratios, and a more vegetarian diet will also help to lower blood phosphate concentrations (Stringer et al. 2010). Additional supplementation with vitamins D and E may help to normalize the biochemical parameters as well. It is important to note that most of these patients were in rehabilitation for primary reasons other than FP, as outlined in the above discussion of case presentations and co-morbid conditions. These other health issues, as well as factors of living in captivity, represent confounding variables in consideration of the blood parameter results shown here.

Radiography is a useful screening tool for detection of internal disease processes in sea turtles but was not a reliable method of diagnosing internal tumors in these cases (Croft et al. 2004; Straub & Jurina 2001). Diagnostic CT and MRI were useful in this study for directing the course of therapy of FP+ patients, including diagnosis of internal tumors, via provision of high-quality cross-sectional images (Croft et al. 2004). Although laparoscopy is widely regarded as a valid method for diagnosing internal tumors, it was not used as a primary diagnostic tool in these cases, particularly because many of the turtles were debilitated upon arrival at the GSTC. CT was often chosen over laparoscopy because it was easily accessible, does not require anesthesia, and is more accurate in identifying small tumors in the dorsal lungs and kidneys, areas often missed by laparoscopy. If suspicious lesions are noted, follow-up laparoscopy and biopsy are recommended.

Development of FP is probably a multifactorial process involving factors of CFPHV pathogen life stage and virulence, turtle host immune response to infection, and environmental variations, including climate change and anthropogenic perturbation (Herbst et al. 1995, 1999). Therefore, FP case progression can vary widely from one turtle to another. For example, one case in this study describes a juvenile female green turtle found stranded on a beach with

plastron wounds and rehabilitated for 37 weeks before FP was first observed. The tumors were aggressive, proliferative, and recurrent (tumor score 3), and addressed with multiple surgeries, extensive diagnostics, and various therapeutics. Eventually the turtle was euthanized due to severe FP and poor prognosis. In contrast, another case involved a turtle that stranded with moderate to severe FP (tumor score 2), which was successfully treated via one tumor removal surgery, quickly recovered, and was released after only 9 weeks in rehabilitation. The authors recommend that triage criteria for green turtles with FP be based on tumor score (Table 3.1): certain turtles with tumor score 3 should be considered outright euthanasia candidates; turtles with tumor score 1 or 2 should be treated on a case-by-case basis, taking into consideration concurrent conditions, available treatment options, case progression, and quarantine capabilities.

Rehabilitation of FP+ turtles frequently entails extensive investment of resources and personnel. Regularly observed complications in FP+ turtles include poor nutritional condition and general health upon admission and other opportunistic infections (bacterial, viral, fungal, or parasitic; Stacy et al. 2008; Work et al. 2003). The data in this study suggest that approximately 10% (12/123) of rehabilitating green turtles may be expected to develop FP within about 90 days. Thus, whenever possible, green turtles should be rehabilitated and released within 90 days to help circumvent FP development during rehabilitation. Fully rehabilitating turtles to health should take precedence, however; in particular, some traumatic injuries can take months to years to fully heal. Additionally, seasonality is another factor to consider when planning a release date; turtles at the GSTC are not typically released if seawater temperatures are <18°C. A commonly held tenet states that sea turtles should not be released with any FP tumors; however, this approach can result in considerable delays in rehabilitation (Mader 2006). In addition to placing a substantial burden on facilities and limiting the number of turtles that can be treated, prolonged

captivity of FP+ patients can exacerbate cycles of tumor removal and regrowth. An acceptable alternative rehabilitation outcome is reduction of tumor burden and rehabilitation to a clinically stable condition suitable for release. Candidates for release with mild cutaneous FP must be carefully selected, taking into account tumor score and progression. One example from this study involves a juvenile female green turtle found floating and edematous with no tumors, which then developed FP after 8 weeks in rehabilitation. This turtle was treated with tumor removal surgery and supportive care, fitted with a satellite transmitter, and released with mild cutaneous FP. Tracking data revealed that the turtle's location was actively transmitted for 57 days as she moved normally within her natural range (SEATURTLE.org). This turtle re-stranded 10 months later, however, with large external tumors. The turtle was re-released after another tumor removal surgery and supportive care.

Currently, euthanasia candidacy and timing of release for FP+ turtles are determined by the attending veterinarian, according to clinical findings, medical opinion, and U.S. Fish and Wildlife Service permitting conditions. In general, the most consistently applied reasons for euthanasia of FP+ patients are bilateral tumor involvement of the corneas (resulting in complete or near complete blindness), aggressive tumor recurrence, and/or presence of internal tumors (i.e., tumor score of 3; Table 3.1). Criteria for release should give utmost consideration to the animal's ability to survive in its current condition. This study provides valuable information that will assist managers and veterinary staff to make informed animal welfare decisions and maximize rehabilitation resources.

This study was performed retrospectively. This method of case analysis has certain inherent disadvantages, such as a reliance on the accuracy of written records, incomplete data, difficulty controlling bias and confounders due to lack of randomization and blinding, and

difficulty establishing causal associations. Due to these issues, the results presented here are observational and, at best, hypothesis generating (Hess 2004). The results of this study provide a logical summary of many aspects of FP+ cases in rehabilitation, suggest important parameters to evaluate in prospective FP+ cases, and provide useful predictors for possible case outcomes (i.e, tumor score, presence of ocular FP, plaque-like FP lesions only). Future research should include epidemiologic surveys using larger sample sizes and comparisons between different sea turtle rehabilitation facilities.

CHAPTER 4

QUANTIFYING CHELONID FIBROPAPILLOMA-ASSOCIATED HERPESVIRUS IN SYMPTOMATIC AND ASYMPTOMATIC REHABILITATING GREEN SEA TURTLES [1]

[1] Page-Karjian, A., Norton, T.M., Ritchie, B., Brown, C.C., Mancia, C., Jackwood, M., and N.L. Gottdenker. Submitted to *Endangered Species Research*, 2/6/2015.

Abstract

Fibropapillomatosis (FP), the most important infectious disease of sea turtles, is characterized by cutaneous and in some cases systemic tumors, and is associated with chelonid fibropapilloma-associated herpesvirus (CFPHV). Despite extensive research on FP, the pathogenesis of CFPHV remains poorly understood, particularly regarding asymptomatic infections. Here, we provide evidence for detectable CFPHV DNA in biological samples from symptomatic and asymptomatic turtles. Using a probe-based qPCR assay for CFPHV, we evaluated the relationship between CFPHV viral nucleic acid loads and clinical disease in rehabilitating green turtles (*Chelonia mydas*), and investigated potential routes of CFPHV shedding. Samples of tissue, blood, urine, and feces were collected from 67 green turtles at 3 rehabilitation facilities in the southeastern USA. Turtles were divided into 3 study groups: clinical signs of FP (n = 23); history of FP but no clinical signs (n = 13); and no known history of FP (n = 31). Via qPCR, CFPHV DNA was reliably detected in FP tumors, non-tumored skin, blood, urine, cloacal swabs, and plasma from green turtles in all 3 groups. Our results provide novel evidence for CFPHV DNA in blood cells, which may represent a critical phase of the CFPHV life cycle and provide a mechanism for viral transport, and documents that viral DNA can be detected in the urine of symptomatic and asymptomatic turtles. Using qPCR, CFPHV gene copies can be quantified in various samples for evaluation of subclinical patients, helping prevent transmission opportunities among captive turtles and informing management decisions for free-ranging turtle populations.

Introduction

DNA tumor viruses such as chelonid fibropapilloma-associated herpesvirus (CFPHV) tend to establish long-term persistent intracellular infections (Moore & Chang 2010). Genomic analyses of reptilian herpesvirus sequences indicate that CFPHV should be grouped among the *Alphaherpesvirinae* (Davison et al. 2009; McGeoch et al. 2005, 2006), however, CFPHV also harbors some genes that are typical of *Beta-* and *Gammaherpesvirinae* (Ackermann et al. 2012). In general, alphaherpesviruses tend to be epitheliotropic and neurotropic, and beta- and gammaherpesviruses tend to exhibit tropism for lymphocytes (Davison 2007). Regardless of subfamily, most herpesviruses establish a post-inoculation systemic infection accompanied by a cell-associated viremia. In infected individuals, viremia is usually indicative of viral replication, and can be detectable during either the primary infection event or during viral reactivation following a period of latency (Hamprecht et al. 1998).

Herpesvirus is commonly transmitted after induction of latent infections, with periodic viral shedding following stressful factors such as concomitant disease, malnutrition, environmental stress such as temperature changes (high or low), movement of animals, introduction of new animals to an established collection, or breeding activity. Virus shedding may or may not be associated with concurrent signs of disease (Ritchie 2006). A horizontal route of transmission by CFPHV was demonstrated when cell-free cutaneous tumor extract inoculates were shown to transmit FP to uninfected turtles (Herbst et al. 1995). The primary target of CFPHV seems to be skin, and CFPHV may be transmitted by sloughing of virally infected epidermal cells into the environment (Herbst et al. 1995, 1999). However, an 83.6% seroprevalence of CFPHV glycoprotein H antibodies was observed in a green turtle aggregation that had no signs of FP tumors (Herbst et al. 2008). This finding suggests that there may be

alternate viral transmission cycles involving virus replication and shedding from tissues other than cutaneous tumors. Determining how CFPHV is transmitted in apparently healthy FP-free populations could have major implications in designing effective prevention and disease control strategies (Herbst et al. 2008). Recently, a nested PCR assay identified CFPHV DNA in 32.4% of skin samples taken from tumor-free, wild-caught green turtles from Puerto Rico, suggesting that these asymptomatic turtles may be capable of shedding viral DNA from normal skin and infecting others (Page-Karjian et al. 2012). Such asymptomatic turtles may remain disease-free, or may be subclinical during a transient viral incubation phase. Intermittent shedding by subclinically infected turtles, whether via epithelial shedding or other routes, could explain spontaneous infections in established turtle colonies and suggest an additional mechanism for viral transmission between turtles under natural conditions.

The pathogenesis of CFPHV is poorly understood, particularly with regards to detection of viral DNA in asymptomatic turtles. For example, because the virus has not been replicated in cell culture, data are lacking regarding viral loads in various fluid and tissue samples during the course of infection with CFPHV-associated disease. Viral load data are routinely used in the clinical management of human and animal herpesvirus–associated diseases, particularly for the prevention, diagnosis and monitoring of herpevirus-associated malignancies and lymphoproliferative disorders, and are thus of great potential relevance for sea turtle FP (Gartner & Preiksaitis 2010; Stanton et al. 2013). Quantitative PCR technology is a flexible, rapid, sensitive, specific, and quantitative method for the detection of pathogen nucleic acid that can be used to measure viral DNA in diverse clinical and research settings (Mackay 2004). Using qPCR, viral DNA kinetics can be evaluated and compared for symptomatic and asymptomatic infected individuals, helping to predict disease outbreaks, explain differences in various disease

states, and improve transmission risk management (e.g., Yamamoto et al. 2014).

Sea turtle rehabilitation facilities offer a unique opportunity to access and observe wild sea turtles in a controlled setting. In this study, we 1) developed and validated a qPCR assay for detection and quantification of CFPHV DNA, in order to 2) evaluate the relationship between CFPHV DNA loads and clinical disease in rehabilitating green turtles; and 3) investigate potential routes of CFPHV DNA shedding by symptomatic and asymptomatic turtles.

Methods and Materials

Study design and biological sample collection

Sixty-seven green turtles were divided into 3 study groups based on clinical signs and history at the time of sampling: Group A- turtles with clinical signs of FP (n = 23); Group B- turtles with a history of FP but with no clinical signs (i.e., turtles that previously had FP tumors surgically removed; n = 13); and Group C- turtles with no known history of FP (n = 31). Biological samples were collected from juvenile green turtles at 3 rehabilitation facilities in the southeastern United States- the Georgia Sea Turtle Center, Jekyll Island, GA; the Loggerhead Marinelife Center, Juno Beach, FL; and The Turtle Hospital, Marathon, FL. As negative controls, samples were collected from one green turtle with no known history of FP raised in a captive breeding facility where FP has not been observed in over 30 years, and from 2 adult female freshwater turtles (*Trachemys scripta elegans*) raised in a captive breeding facility in the southeastern USA. This research was conducted with the approval of the University of Georgia Office of the Vice President for Research Institutional Animal Care and Use Committee (IACUC), permit #A2012 10-011-Y1-A0, and with USA state permits to collect, transport and store biological samples from green turtles: a scientific collecting permit (#29-WJH-13-44) and a

Special Purpose Importation permit (#S2-WJH-13-2) from the Georgia Department of Natural Resources, Wildlife Resources Division; and a Marine Turtle Permit (#149) and authorization to receive and/or transport sea turtle biological samples from the Florida Fish and Wildlife Conservation Commission.

Blood

Venipuncture sites were swabbed with sterile povidone iodine followed by 70% alcohol prior to needle insertion. Using heparinized, 3 or 6 mL syringes and 21-25 gauge, 1-1.5 inch needles (depending on the turtle's size), 3-6 mL of blood was collected aseptically into lithium heparin (LiH) tubes from the dorsal cervical sinus of each turtle. Aliquots (0.2 – 1.0 mL) of fresh whole blood were placed into 1.5 mL cryotubes. The remaining blood samples were centrifuged in the LiH tubes at 2,500 rpm for 10 minutes. Separated plasma samples were then placed into 1.5 mL cryotubes. Blood and plasma samples were stored at -80°C for up to 3 months prior to analysis.

FP tumors

CO_2 laser-mediated surgery was used to remove FP tumor samples from FP+ turtles. Varying levels of power, pulse rate, and hand piece size were used depending on surface area extent and depth of FP. All tumors were removed in one surgical procedure per turtle, and procedures lasted a maximum of 2.5 hours. A 2-5 cm diameter portion of each tumor sample was placed into a tissue cassette and then placed into 10% buffered formalin for up to 10 days, followed by routine preparation for histological examination (University of Georgia, Veterinary Diagnostic and Investigational Laboratory [UGA VDIL]). The prepared histological slides were

evaluated to confirm the morphologic diagnosis of FP. Remaining tumor samples were placed into either 1.5 mL cryotubes or Whirlpak® bags and stored at -80°C for up to 3 months prior to analysis.

Non-tumored skin biopsies

Following preparation of the biopsy site with Betadine® scrub and 70% ethyl alcohol, and subcutaneous injection of 2% lidocaine solution (5 mg/kg mixed 1:1 with sodium bicarbonate; PennVet), skin biopsies were aseptically collected from the left or right shoulder using a sterile, disposable 4 mm biopsy punch. Healing was generally rapid (<2 weeks); biopsy sites were routinely cleaned until healed, using silver sulfadiazine cream on the wounds as needed. Skin biopsy samples were sectioned in half using a sterile scalpel blade (No. 10): half of each sample was placed into a 1.5 mL cryotube and stored at -80°C for up to 3 months prior to analysis; the other half was placed in a tissue cassette and then placed into 10% buffered formalin for up to 10 days, followed by routine preparation for histological examination (UGA VDIL). The prepared histological slides were evaluated microscopically to confirm the absence of FP.

Urine

Urine samples (1-30 mL) were opportunistically and aseptically collected into sterile 4 oz urine cups as turtles were handled for rehabilitation purposes (i.e., physical therapy, shell treatment, diagnostic imaging, medication administration, etc.), then transferred into 60 mL Eppendorf tubes. Tubes were sealed with Parafilm® and stored at -80°C for up to 3 months prior to analysis.

Feces

Fecal samples (up to 1 g) were opportunistically collected from the turtles' tanks within 4 hours after defecation, placed into Whirlpak® bags, and stored at -80°C for up to 3 months prior to analysis.

Oral and cloacal swabs

Oral and cloacal mucosal swabs were collected by swabbing buccal and cloacal mucosae with separate, sterile cotton-tipped swabs for 5-10 seconds, using moderate pressure to collect epithelial cells. Swab tips were immediately placed into cryotubes and stored at -80°C for up to 3 months prior to analysis.

Nucleic acid extraction

Genomic DNA was extracted from each sample using the appropriate kits (Qiagen): DNeasy Blood and Tissue Kit for blood, non-tumored skin, and FP samples; MinElute Virus Spin Kit for plasma, oral swabs, and cloacal swabs; QIAamp DNA Stool Mini Kit for feces; and QIAamp Viral RNA Kit for urine. To normalize samples prior to qPCR, the concentration of the extracted DNA (µg/µl) in each sample was measured using absorbance spectrophotometry (Nanodrop), and ratios of absorption at 260 nm versus 280 nm were evaluated to ensure DNA purity. Extracted DNA was stored at -20°C for up to 3 months prior to qPCR analysis.

CFPHV-specific qPCR development

To quantify CFPHV DNA loads in various green turtle biological samples, we developed a probe-based qPCR assay based on the highly conserved DNA polymerase region (UL30) of the CFPHV genome. Reaction efficiency and precision were demonstrated using a plasmid standard curve.

Primer and probe design and optimization

Prospective primers were designed based on a consensus sequence using all currently available sequence data of the UL30 region of the CFPHV genome (GenBank). Consensus sequence development, performed using Clustal Omega multiple sequence alignment software (EMBL-EBI) and CLC Bio Main Workbench 6 (Qiagen), selectively included UL30 sequences isolated from green turtle isolates and excluded UL30 sequences isolated from other sea turtle species to increase assay specificity. Sequences used in consensus development are presented in Table 4.1.

Table 4.1. GenBank accession numbers of CFPHV UL30 sequences used in qPCR consensus sequence development

Sequence identifier	GenBank accession number
Fibropapilloma-associated turtle herpesvirus from Hawaii DNA polymerase (pol) gene	AY390420.1
Fibropapilloma-associated turtle herpesvirus from Puerto Rico DNA polymerase (pol) gene	AY390421.1
Fibropapilloma-associated turtle herpesvirus from California DNA polymerase (pol) gene	AY390422.1
Fibropapilloma-associated turtle herpesvirus DNA polymerase gene	AY395516.1
Fibropapilloma-associated turtle herpesvirus strain FL_var_A polymerase (UL30) gene	AY646888.1
Fibropapilloma-associated turtle herpesvirus strain FL_var_C polymerase (UL30) gene	AY646889.1
Fibropapilloma-associated turtle herpesvirus strain FL_var_D polymerase (UL30) gene	AY646890.1
Fibropapilloma-associated turtle herpesvirus strain FL_var_A polymerase (UL30) gene	AY646891.1
Fibropapilloma-associated turtle herpesvirus strain FL_var_B polymerase (UL30) gene	AY646892.1
Fibropapilloma-associated turtle herpesvirus strain HA_variant polymerase (UL30) gene	AY646893.1
Fibropapilloma-associated turtle herpesvirus strain FL var C polymerase (UL30) gene	AY646894.1
Fibropapilloma-associated turtle herpesvirus isolate T1 polymerase gene	HM348895.1
Fibropapilloma-associated turtle herpesvirus isolate T3 polymerase gene	HM348896.1
Fibropapilloma-associated turtle herpesvirus isolate T4 polymerase gene	HM348897.1
Fibropapilloma-associated turtle herpesvirus isolate T8 polymerase gene	HM348898.1
Fibropapilloma-associated turtle herpesvirus isolate T2b polymerase (pol) gene	HQ000006.1
Fibropapilloma-associated turtle herpesvirus isolate T8b polymerase (pol) gene	HQ000007.1
Fibropapilloma-associated turtle herpesvirus isolate PR2_cm_2009 DNA polymerase (UL30) gene	JN580279.1
Fibropapilloma-associated turtle herpesvirus isolate PR3_cm_2010 DNA polymerase (UL30) gene	JN580280.1
Fibropapilloma-associated turtle herpesvirus isolate PR6_cm_2006 DNA polymerase (UL30) gene	JN580283.1

The forward and reverse primer sequences (Table 4.2) selected using Primer3Plus software (Rozen & Skaletsky 2000) amplify a 173 bp fragment of the CFPHV UL30 gene. A primer matrix was used to determine optimal primer concentrations; 320 nM for both the forward and reverse primers were chosen, based on low cycle quantification (C_q)-value (Bustin et al. 2009) and reduced primer concentration (Stratagene 2004). Assay specificity was improved via use of a dual-labeled hydrolysis probe unique to CFPHV UL30 DNA, developed using Primer3Plus software (Rozen & Skaletsky 2000). The selected probe was synthesized with a 6-FAM (Fluorescein) fluorescent dye and a ZEN internal quencher (Table 4.2). Probe concentration (320 nM) selection was the lowest yielding acceptable detection. Primers and probe were manufactured by Integrated DNA Technologies (IDT). A melt curve analysis performed using SYBR Green chemistry (95°C for 10 min, 60°C to 95°C incremented stepwise by 5°C per 30 s) was used to confirm amplification of a single product and ensure the absence of amplification artifacts (Bustin & Nolan 2004). Capillary sequencing, the BLAST algorithm (Altschul et al. 1990), and Clustal Omega multiple sequence alignment software (EMBL-EBI) were used to confirm qPCR product identity during assay development (100% sequence identity to CFPHV-5 partial genome; GenBank HQ878327.2). To demonstrate the presence of amplifiable DNA, primers developed and optimized for *C. mydas* β-actin and GAPDH DNA (Table 4.2; GenBank AY373753.1 and FJ234450.1, respectively) were applied to all samples and included in all qPCR runs.

Table 4.2. Primers and probe sequences used in qPCR assay for CFPHV UL30 and *C. mydas* gene segments

Sequence identifier	Sequence
CFPHV UL30, forward primer	5'-AACGCTTGCTTTTGGACAAG-3'
CFPHV UL30, reverse primer	5'-CCAGCGGGTGTGAATAAAAT-3'
CFPHV UL30, hydrolysis probe	5'-6-FAM-TGGCCATCA-ZEN-AGCTGACGTGCA-3'
C. mydas β-actin, forward primer	5'-TGGTACAGTCTCCCATTCCA-3'
C. mydas β-actin, reverse primer	5'-AGGCATACAGGGACAACACA-3'
C. mydas GAPDH, forward primer	5'- TCTGGGATAGGTTGGGAGTC-3'
C. mydas GAPDH, reverse primer	5'- TCCTAGGCGATACTGCCTCT-3'

Construction of a CFPHV plasmid DNA standard curve for qPCR

The CFPHV UL30 standard curve was constructed by first cloning a 173 bp fragment amplified with the qPCR primers described above into the pGEM-T Easy Vector (Promega). The plasmid was linearized using the EcoRV enzyme, and the product obtained was sequenced to confirm identity. The 3,173 bp plasmid containing the target CFPHV gene has a molar mass of 2.06×10^6 g/mole. The plasmid was diluted in nuclease-free water (Qiagen) to yield 2.92×10^{10} copies/µl. Ten-fold serial dilutions of the diluted plasmid were used as qPCR templates to generate a standard curve, and were included in each run of the qPCR assay.

qPCR reaction conditions and data interpretation

Quantitative PCR reactions (20 µl) were conducted in 96-well 0.2 µl PCR plates (Agilent Technologies) containing 10 µl SensiFAST^{TM} Probe Lo-ROX (Bioline), 0.8 µl (320 nM) each of forward and reverse CFPHV UL30 primers (IDT), 0.2 µl (320 nM) of fluorescent probe (IDT),

and 8.2 µl genomic or plasmid DNA. All qPCR reactions were carried out using a MX3000 qPCR instrument (Stratagene) and the following reaction conditions: 10 min at 95°C, 40 cycles of 30 s at 95°C, 1 min at 55°C, and 1 min at 72°C. Samples and plasmid DNA standards were assayed in duplicate along with four negative (no template) controls (nuclease-free distilled water; Qiagen). As recommended in the MIQE guidelines (Bustin et al 2009), extensive laboratory precautions were taken to avoid assay contamination by genomic or plasmid DNA. These precautions included physical separation of all steps of DNA sample preparation, qPCR reaction setup, thermal cycling, and PCR product analysis procedures; use of PCR-dedicated containers, pipette tips with aerosol filters, and certified reagents; and regular and thorough cleansing of all surfaces using a DNA/RNA degrading solution (70% bleach). Any reagents suspected of contamination were immediately disposed of, and any steps suspected of cross-contamination or DNA carry-over were repeated using fresh reagents. Quantitative PCR data were analyzed with the Stratagene MXPro qPCR software (Agilent Technologies, version 3.2). Tested samples were considered positive if duplicates had mean copy number equal to or higher than the assay's analytical LOD. Using the coefficient of determination (R^2) values, reaction efficiency and precision were calculated for all qPCR assays. To help increase C_q value accuracy, an adaptive baseline threshold was generated for each assay run via MXPro software. Regression analysis of the standard curve (Larionov et al. 2005) was used to determine the number of CFPHV DNA copies for each *C. mydas* DNA sample. CFPHV UL30 copy numbers were calculated per µg of gDNA extracted from each biological sample.

qPCR validation

qPCR efficiency and precision

To determine qPCR assay efficiency and precision, 3 separate plasmid dilution series containing 3 to 3 x 10^{10} copies were constructed as described above and each curve was tested in replicates of 10. R^2 values were calculated using C_q-values among plasmid curves and runs. Efficiency values were calculated using the formula $E = 10^{(-1/slope)}$ and the slope of each standard curve, with ideal assay efficiency at 100% and target efficiency between 90 and 110%. The C_q of each dilution was plotted and data were fitted to a straight line to determine the linear dynamic range of each curve.

Sensitivity and specificity

An assay's lower LOD, also known as analytical sensitivity, is the lowest concentration that can be detected with reasonable certainty (Bustin et al. 2009). Here, the LOD was determined by applying qPCR to 42 replicates of low gene copy number (1, 5, 50, and 500 copies) plasmid DNA. Analytical specificity was determined by testing samples from 3 alphaherpesviruses (bovine herpesvirus 3 (BHV-3), and phocine herpesvirus 1 and 2 (PhHV-1, PhHV-2)) for cross-reactivity with our qPCR primers and probe. Additional CFPHV UL30 qPCR assay specificity was conferred via qPCR testing of biological samples collected from a captive-raised green turtle from a well-established, captive turtle collection in which FP has not been documented, as well as samples from 2 adult female freshwater turtles (*T. scripta elegans*).

Repeatability

Repeatability was determined by a single person using qPCR to test 3 CFPHV DNA-positive samples each of FP, non-tumored skin, blood, plasma, urine, and cloacal swab samples spanning the qPCR assay linear operating range on the Stratagene platform (Friedman et al. 2014). Samples were tested in replicates of 8, and an intra-assay CV of 0 to 20% was considered acceptable (Pfaffl 2004).

Sequence analysis

To confirm qPCR results, randomly selected CFPHV DNA-positive qPCR products constituting approximately 33% of the CFPHV DNA-positive qPCR products were sequenced using capillary (Sanger) sequencing technology (Genewiz) and 5 µl of 5mM forward CFPHV UL30 primer. Selected qPCR products were purified with the QIAquick Gel Extraction Kit (Qiagen). Sequence data for CFPHV-DNA-positive samples were analyzed using FinchTV DNA trace viewer software (Geospiza, Inc.) and compared to existing DNA sequences in the National Center for Biotechnology Information (NCBI) GenBank database using the BLAST algorithm (Altschul et al. 1990). Aligned sequences with ≥97% identity to the sample sequence were considered a match.

Statistical Analysis

For each of the 3 study groups, the number of CFPHV DNA-positive assays was enumerated according to sample type, and mean CFPHV DNA copy number (number of CFPHV DNA copies/µg sample) and gDNA concentration (µg gDNA/ul) were calculated for each sample type in each group. Mean CFPHV DNA copy numbers in each group were tested for

normality via the Shapiro-Wilkes test, and then compared between the 3 study groups for blood, non-tumored skin biopsies, urine and cloacal swab samples using the Kruskal-Wallis test for non-parametric data, with α set at 0.05. Post-hoc pairwise comparisons were performed on statistically significant results using the Mann-Whitney U-test for non-parametric data with a Bonferroni correction. FP tumor, plasma and fecal samples were not analyzed in this way due to a lack of sufficient sample sizes in the 3 study groups. Additionally, data from groups B and C were combined for each sample type (except for urine and plasma- sample sizes were too small for these variables), and the means were compared to the mean copy numbers from group A using the 2-tailed Mann-Whitney U-test for non-parametric data with α set at 0.05. This type of analysis allowed us to effectively evaluate differences in the mean viral copy numbers between FP+ and tumor-free turtles. The measure of agreement between various biologically related sample types was calculated using the unweighted Kappa statistic: relationships were assessed between blood and urine, blood and non-tumored skin biopsies, urine and cloacal swabs, and whole blood and plasma samples. Statistical analyses were performed using IBM SPSS Statistics for MacIntosh, version 22.0.

Results

qPCR assay validation and optimization

Reaction efficiency, coefficients of determination, and linear dynamic range

The CFPHV pol plasmid standard curves (e.g. Figure S1) had reaction efficiencies ranging from 94.2 to 111.4% (mean = 97.53 ± SD 5.62), and coefficients of determination (R^2) ranging from 0.924 to 0.999 (mean = 0.986 ± SD 0.02; Dataset S1). The assay was determined to have a dynamic range between 50 and ~2 billion gene copies, based on reaction efficiency.

Sensitivity and specificity

The qPCR assay was able to detect low plasmid copy numbers ranging from 16.7% (7/42) for 1 gene copy, 50% (21/42) for 5 gene copies, 85.7% (36/42) for 50 gene copies, and 100% (42/42) for 500 gene copies (Dataset S2). Based on a 50% certainty cutoff as defined by OIE (2009), the minimum limit of detection (LOD) of our qPCR assay was 50 gene copies per reaction. Therefore, only unknown samples whose average quantities were ≥50 gene copies were considered positive. Presence of amplifiable DNA was demonstrated by amplification of *C. mydas* β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) DNA in all samples included in the study. The primers and probe developed for qPCR were specific to the CFPHV DNA polymerase (UL30) gene target: qPCR primers were unable to amplify DNA from other closely related herpesviruses (BHV-3, PhHV-1, PhHV-2) assayed. Template controls (i.e., nuclease free distilled water; Qiagen) and biological controls (i.e., known negative samples from captive-reared green and freshwater turtles) did not amplify (Dataset S3). Additionally, melt curve analysis showed a single amplified product when we used SYBR green I chemistry (Bioline) to test our chosen primers with the qPCR assay (Figure S2; Bustin & Nolan 2004). The amplified and sequenced PCR product was identified as 'CFPHV-5' (GenBank HQ878327.2; 100% pairwise identity).

Repeatability

For each biological sample type that tested positive for CFPHV DNA, the overall mean coefficients of variation (CV) for samples of relatively low, medium and high viral DNA copy number were 12.41% for FP tumors; 13.97% for non-tumored skin; 14.42% for blood; 13.68% for plasma; 18.18% for urine; and 13.86% for cloacal swabs (Dataset S4).

Viral DNA detection in biological samples

Biological samples were collected from n = 67 rehabilitating green turtles: group A (turtles with clinical signs of FP, n = 23); group B (turtles with a history of FP but with no clinical signs, n = 13); and group C (turtles with no known history of FP, n = 31). A total of 351 DNA extracts from 67 individual sea turtles were screened for CFPHV by qPCR (Dataset S5). In group A, 21/23 turtles (91.3%) had at least one CFPHV DNA-positive sample via qPCR; FP tumor samples were not available for the 2 turtles with no CFPHV DNA-positive samples. Overall, of the 44 turtles that did not have grossly visible FP at the time of sampling, 20 turtles had at least one CFPHV DNA-positive tissue, blood, or urine sample (7 in group B; 13 in group C), and 24 had all samples negative for CFPHV DNA via qPCR. Table 4.3 shows mean extracted gDNA concentrations (μg gDNA/μl), percentage of positive samples, and CFPHV DNA copy numbers (number of CFPHV DNA copies/μg sample) for each sample type within the 3 study groups. CFPHV DNA was not identified in oral swab or fecal samples.

Table 4.3. Summary of qPCR results for tumor, skin, blood, plasma, urine, mucosal swabs, and fecal samples taken from tumored and tumor-free rehabilitating green turtles

Group A	FP+ Turtles (n = 23)							
	FP tumors	Non-tumored skin	Whole blood	Plasma	Urine	Cloacal swab	Oral swab	Feces
Mean [gDNA] (µg/µl) (± SD)	0.053 (±0.036)	0.034 (±0.03)	0.028 (±0.01)	0.043 (±0.02)	0.125 (±0.03)	0.051 (±0.05)	0.049 (±0.02)	0.033 (±0.02)
Number (%) positive via qPCR	18/18 (100%)	10/12 (83.3%)	7/21 (33.3%)	2/21 (9.5%)	4/6 (66.7%)	6/16 (37.5%)	0/17 (0%)	0/10 (0%)
Mean copy number / µg DNA (± SD)	3.24×10^8 ($\pm 5.44 \times 10^8$)	22,722 (±27,255)	1,737 (±1,504)	682 (±363)	3,482 (±3,663)	3,084 (±4,959)	---	---
Group B	Turtles with a history of FP (n = 13)							
		Non-tumored skin	Whole blood	Plasma	Urine	Cloacal swab	Oral swab	Feces
Mean [gDNA] (µg/µl) (± SD)		0.016 (±0)	0.016 (±0.01)	0.048 (±0.02)	0.115 (±0.02)	0.052 (±0.04)	0.035 (±0.02)	0.015 (±0.01)
Number (%) positive via qPCR		1/1 (100%)	6/13 (46.2%)	1/13 (7.7%)	3/5 (60%)	3/13 (23.1%)	0/5 (0%)	0/2 (0%)
Mean copy number / µg DNA (± SD)		2,316 (±0)	5,141 (±5,375)	207 (±0)	699 (±709)	79 (±26)	---	---
Group C	Turtles with no known history of FP (n = 31)							
		Non-tumored skin	Whole blood	Plasma	Urine	Cloacal swab	Oral swab	Feces
Mean [gDNA] (µg/µl) (± SD)		0.025 (±0.01)	0.034 (±0.02)	0.046 (±0.02)	0.122 (±0.02)	0.041 (±0.02)	0.031 (±0.01)	0.033 (±0.02)
Number (%) positive via qPCR		6/27 (22.2%)	8/31 (25.8%)	2/31 (6.5%)	1/9 (11.1%)	4/31 (12.9%)	0/26 (0%)	0/23 (0%)
Mean copy number / µg DNA (± SD)		5,463 (±6,587)	6,567 (±9,493)	806 (±707)	449 (±0)	2,056 (±2,262)	---	---

No significant differences in CFPHV DNA copy numbers between the 3 study groups were found for whole blood, skin biopsy or cloacal swab samples. Statistical comparisons between study groups were not possible for urine samples due to small sample sizes. All samples

were not available for all turtles. Measures of agreement between sample types within individual turtles are given in Table 4.4.

Two sample types compared	n	+/+	-/-	+/-	-/+	Level of Agreement (%)	Kappa (±SE; 90% CI)
Blood, urine	18	6	13	0	0	100	1 (±1; 1 – 1)
Blood, non-tumored skin	38	8	20	1	9	73.7	0.44 (±0.15; 0.15 – 0.74)
Blood, plasma	65	5	44	16	0	75.4	0.3 (±0.15; 0 – 0.6)
Urine, cloacal swabs	20	3	10	5	2	65	0.22 (±0.24; 0 – 0.69)

Table 4.4. Measures of agreement between qPCR data from various biological sample types for individual turtles

qPCR product sequence data

Using the BLAST algorithm (Altschul et al. 1990), 16/20 (80%) GenBank sequences that were used to develop the consensus sequence on which our qPCR was based (Table 4.1) were matched with ≥97% identity to our qPCR product sequences. Sequence data were obtained for 30 samples that were positive for CFPHV DNA via qPCR: 6 FP tumor samples; 6 non-tumored skin samples; 7 whole blood samples; 2 plasma samples; 4 urine samples; and 5 cloacal swab samples. All sequences obtained were from biological samples taken from different individual turtles; multiple sample types were not sequenced from the same turtle. CFPHV DNA-positive samples of FP tumors, non-tumored skin, blood, plasma, urine and cloacal swab samples matched several aligned DNA sequences with ≥97% identity; GenBank accession numbers are provided in Table S1.

Discussion

Via qPCR, CFPHV DNA was amplified from FP tumors, non-tumored skin, blood, urine, cloacal swabs, and plasma sampled from green turtles in all 3 study groups. Although several previous studies have identified CFPHV DNA in FP tumors and non-tumored skin (Alfaro-Nunez et al. 2014; Lackovich et al. 1999; Lu et al. 2000; Page-Karjian et al. 2012; Quackenbush et al. 2001), here we provide novel findings regarding CFPHV DNA presence in blood, urine, cloacal swabs, and plasma from turtles with and without tumors. This study provides critical information for a deeper understanding of the complex CFPHV pathogenesis in symptomatic and asymptomatic turtles.

In general, CFPHV DNA copy numbers in FP tumors were several orders of magnitude greater than those seen in any other sample types; this observation lends further support to the hypothesis that CFPHV is highly associated with the formation of FP tumors. Our finding that 83.3% of non-tumored skin samples from FP+ turtles (i.e., turtles with FP) tested positive for CFPHV DNA is similar to a previous study, in which 88% of FP+ green turtles had non-tumored skin samples that tested positive for CFPHV DNA by qPCR (Alfaro-Nunez et al. 2014). Our finding that skin samples from 7/28 (25%) of tumor-free turtles were positive for CFPHV DNA via qPCR is also similar our previous study, in which 32.4% of skin samples from tumor-free turtles were positive for CFPHV DNA via nested PCR (Page-Karjian et al. 2012). Although significant differences in viral DNA copy numbers in non-tumored skin samples were not observed between the 3 study groups, the relatively large mean viral DNA copy number for non-tumored skin samples in FP+ turtles (22,722 copies/μg DNA) compared to that of tumor-free turtles (5,014 copies/μg DNA) supports the proposed epitheliotropic nature of CFPHV (Herbst 1994; Jacobson et al. 1989, 1991).

Herpesviruses are capable of both local and disseminated infections within a host (Baron et al. 1996). Circulating CFPHV DNA (DNAemia) was observed in whole blood samples taken from turtles in all 3 study groups, and numbers of positive samples were similar between the 3 groups (Table 4.3), as well as between tumored (7/21 or 33.3%) and tumor-free turtles (14/44 or 31.8%). These data suggest that CFPHV, like many other known herpesviruses, may infect a subset of leukocytes and become detectable in peripheral whole-blood samples during lytic replication. When linked to infectious virus, the presence of DNAemia could suggest a critical mechanism for viral transport to the skin from sites of initial infection or latency. If CFPHV behaves similarly to other herpesviruses, a potential sequence of events is that herpesvirus DNAemia in asymptomatic turtles is observed during a transient period after primary infection when viral progeny disseminate through lymphatics to infect cells in close contact with the bloodstream, such as lymphocytes. In asymptomatic and symptomatic turtles, DNAemia may be observed during a less transient secondary phase usually lasting several days, when virus is released directly into the bloodstream and comes into contact with the capillary system of all body tissues (Baron et al. 1996). Although statistically significant differences in viral DNA copy numbers were not observed between study groups, a trend towards higher DNA copy numbers in the blood is readily observable in the tumor-free turtles (mean ± SD CFPHV DNA copy number for groups B and C: 5,956 ± 7,757) as compared to the FP+ group (mean ± SD CFPHV DNA copy number for group A: 1,737 ± 1,504). This trend may be explained by taking into account host immune defenses: circulating interferon and immune responses may account for waning viral DNAemia in symptomatic patients. Any immune responses may be too late, however, to prevent seeding of virus into the target organ(s) and sites of shedding. The outcome of CFPHV infection in various cell types likely depends on interactions between virus proteins and host

factors at the cellular level and is modulated by the innate and adaptive immune responses of the infected host (Baron et al. 1996). Thus, depending on the immune status of the infected individual, it is possible that DNAemia precedes tumor development. The mean CFPHV DNA copy number in skin biopsy samples from tumor-free turtles (groups B and C) is slightly less than in whole blood samples from tumor-free turtles, and the Kappa statistic (K = 0.44) for whole blood and skin biopsy samples suggests that skin and blood viral DNA loads are related. These data indicate that in subclinical turtles, DNAemia may be related to the presence of CFPHV DNA in the skin, and therefore the multi-centric nature of FP tumors could be attributable to circulating virus in infected cells within the bloodstream (e.g., circulating tumor precursor cells, lymphocytes; Pellett et al. 2006).

Overall, CFPHV DNA was detected in only 5 plasma samples in relatively low DNA copy numbers, and the low Kappa statistic between whole blood and plasma samples indicates poor agreement. These findings suggest that, like many other herpesviruses (e.g., equine herpesvirus-1), CFPHV is strongly cell-associated and does not replicate outside of host cells while in the bloodstream (Lunn et al. 2009), further supported by the fact that all 5 plasma samples that were positive for CFPHV DNA by qPCR were visibly hemolyzed and presumed have contained lysed blood cells.

Four of 6 (66.7%) of the symptomatic turtles (group A) had urine samples that were positive for CFPHV DNA by qPCR, and 4/14 (28.6%) asymptomatic turtles (groups B and C) had CFPHV DNA-positive urine samples. These findings show that symptomatic and asymptomatic CFPHV DNA-positive turtles are capable of shedding CFPHV DNA into their environment via urine, and suggests, for the first time, that turtles are capable of shedding viral DNA by a route other than via skin cells. A related sea turtle herpesvirus, lung-eye-trachea

disease-associated virus, has been shown to remain infectious in seawater for up to 120 hours (Curry et al. 2000). Thus, CFPHV DNA presence in urine represents a potential route of environmental CFPHV transmission, and warrants further investigation, including the documentation of CFPHV in water and determining how long CFPHV can persist outside the host within the marine environment. Statistical comparison between the study groups was not possible due to small sample sizes, however the mean viral DNA copy number in the urine of FP+ turtles (group A) is an order of magnitude larger than that observed in groups B or C. This suggests that FP+ turtles are likely to shed relatively greater quantities of CFPHV DNA in their urine than tumor-free turtles. The perfect level of agreement between whole blood and urine samples (K = 1) implies that turtles with CFPHV circulating in their blood (DNAemia) may also be likely to shed CFPHV DNA in their urine. If the virus infected the renal epithelium, progeny could be released to the urine through damage to the renal epithelium, allowing infected cells to pass into the glomerular filtrate. Another explanation is that the kidneys, urinary bladder epithelium, and/or cloacal epithelium are sites of CFPHV DNA persistence (Baron et al. 1996). The small number of urine samples in this study limits our conclusions, however. Although one benefit of working with rehabilitating turtles is that certain samples are more easily collected, sea turtle urine can be very difficult to obtain under the best circumstances. This fact makes the data presented here all the more valuable as an indication of the natural history of CFPHV-associated disease, and as a basis for future research.

A few cloacal swab samples were positive for CFPHV DNA in each study group, with a clear trend of a greater proportion of positive samples in group A (44.4%), followed by groups B (23.1%) and C (12.9%). The relatively low prevalence of CFPHV DNA in cloacal swabs and the complete lack of CFPHV DNA in fecal samples and oral swabs suggest that positive results did

not result from environmental contamination of samples. Future studies of CFPHV in biological samples taken from wild-caught green turtles, however, should include simultaneous assessment of environmental samples for viral DNA in addition to the biological samples to ensure the absence of environmental contamination.

Certain characteristics of CFPHV DNA detection observed in our study are also typical of other types of herpesvirus infections. For example, in humans infected with Kaposi's sarcoma-associated herpesvirus (KSHV), the gammaherpesvirus also known as human herpesvirus-8 (HHV-8), viremia is associated with tumor burden and Kaposi's sarcoma (KS) disease progression, and is thought to be an early manifestation of the pathophysiologic events leading up to KS lesion development in immunosuppressed individuals (Pellett et al. 2006; Engels et al. 2003). Our data suggest a similar scenario with CFPHV DNAemia. Our identification of CFPHV DNA in green turtle urine samples is also similar to the situation reported for KSHV: KSHV DNA has been detected in urine samples from both symptomatic and asymptomatic humans, and urine is a proposed vehicle for KSHV transmission (Beyari et al. 2004; Santos-Fortuna & Caterino-de-Araujo 2003). Cytomegalovirus, a betaherpesvirus also called human herpesvirus-5 (HHV-5), is also shed in the urine by symptomatic and asymptomatic infected patients (Canon et al. 2011; Yamamoto et al. 2014). Another betaherpesvirus, elephant endotheliotropic herpesvirus-1 (EEHV-1), was shown to be detectable in blood via qPCR in symptomatic and asymptomatic Asian elephants (*Elephas maximus*), and in some cases EEHV-1 DNAemia episodes were coincident with detection of EEHV-1 DNA in urine samples (Stanton et al. 2013). Interestingly, there is evidence of a genetic basis for these clinical similarities, as the CFPHV-5 genome is thought to combine genes typical of the alpha-, beta-, and gammaherpesvirinae: phylogenetic analysis of the CFPHV-5 genome revealed at least

4 genes atypical for alphaherpesviruses that each have well defined homologues in the genomes of beta- or gammaherpesviruses. While none of these gene products is known to have an essential role in viral replication, each one apparently plays a biological relevant role in either pathogenesis or immune responses (Ackermann et al. 2012). Expression of a combination of these genes may be related to the complex pathophysiology of CFPHV. Atypical genes have also been reported to occur among the mardiviruses, which comprise tumorigenic avian alphaherpesviruses such as Marek's disease virus (Afonso et al. 2001; McGeoch et al. 2006; Tulman et al. 2000).

Using guidelines outlined by the World Organization for Animal Health (OIE 2009) and the minimum information for publication of qPCR experiments (Bustin et al. 2009), the qPCR assay presented here reliably detected and quantified CFPHV DNA in various green turtle biological samples. The assay has high analytical sensitivity and specificity for CFPHV DNA and is repeatable and robust. DNA-based PCR assays are not capable of determining if a disease agent or infection is viable, and therefore function only as a proxy for an infectious organism. CFPHV viral particles have been previously demonstrated via electron microscopy (EM) in FP tumor samples (Jacobson et al. 1991). The concentration of viruses has to be high enough in biological fluid samples to allow detection using EM, however, and tissue samples must be large enough to contain area(s) of infection and permit EM preparation (Goldsmith & Miller 2009). In the current study, fluid sample volumes were too small to permit detection of viral particles via EM, and skin biopsy samples were too small to permit DNA extraction for qPCR and EM preparation. Using the BLAST algorithm (Altschul et al. 1990), 16/20 (80%) GenBank sequences that were used to develop the consensus sequence on which our qPCR was based (Table 4.1) were matched with ≥97% identity to our qPCR product sequences. This verifies the

assay's ability to accurately isolate and amplify the targeted CFPHV DNA sequences, and indicates its high level of gene target specificity. As molecular diagnostics become more affordable, sea turtle health experts can use qPCR to monitor CFPHV gene copies and thereby detect early signs of viral presence in blood, urine, and tissue samples. Identification of asymptomatic turtles with CFPHV DNA in tissues via qPCR can help to determine quarantine status and avoid transmission opportunities among rehabilitating turtles. Additionally, using this qPCR will allow researchers to evaluate quantifiable CFPHV DNA loads and how they relate to free-ranging green turtle disease and mortality, immunity, and certain environmental variables such as water temperature and quality. Subclinical CFPHV infections are of great epidemiological importance, as they may provide a reservoir that allows the disease to persist even in smaller aggregations of turtles, and can constitute major sources of viral dissemination through the population (Baron et al. 1996). Further studies evaluating the kinetics of CFPHV DNA loads in blood, urine and skin samples from turtles prior to, during and after FP tumor development are needed to answer this question. Given that green turtles are protected under the Endangered Species Act and recovery of their populations is of global concern, an enhanced ability to assess the health status of captive and free-ranging sea turtles will enable biologists and veterinarians to better manage population restoration and rehabilitation actions.

CHAPTER 5

POTENTIAL SITES OF CHELONID FIBROPAPILLOMA-ASSOCIATED HERPESVIRUS LATENCY AND PERSISTENCE IN GREEN SEA TURTLES

[1] Page-Karjian, A., Norton, T.M., Ritchie, B., Whitfield, J., and N.L. Gottdenker. To be submitted to *Journal of Wildlife Diseases*.

Abstract

Chelonid fibropapilloma-associated herpesvirus (CFPHV) is considered to be a major contributing etiologic agent of marine turtle fibropapillomatosis (FP). Evidence from previous studies predicts that CFPHV DNA is unequally distributed within an infected individual turtle, with large concentrations of viral DNA in cutaneous tumor tissues. The objective of this study was to measure and compare CFPHV DNA quantities in skin, blood, urine, major organs, and nervous tissue samples of tumored and non-tumored, free-ranging green turtles (*Chelonia mydas*) to provide information on anatomic localization of CFPHV DNA during two different disease states. Via quantitative PCR for the CFPHV UL30 gene segment, CFPHV DNA was identified in tumors, skin, heart, kidney, nerves, blood, and urine sampled from 5 tumored and 5 non-tumored, juvenile green turtles that stranded in Florida, USA. For the 8 turtles (5 with FP, 3 tumor-free) with samples that tested positive for CFPHV UL30, viral DNA copy numbers per cell were: 0.04-372.4 copies/cell in cutaneous tumors; 0.004 copies/cell in non-tumored skin; 0.001-0.3 viral copies/cell in nerve tissues, blood and urine samples, and 0.004-0.09 viral copies/cell in organ tissues. Frequently co-occurring sites of CFPHV DNA localization in individual turtles include tumor and kidney (n = 4); and tumor and urine, kidney and urine, and kidney and brachial plexus (n = 2 each). These findings support previous research that identified CFPHV DNA in normal skin, blood and urine samples, and implicate several candidate non-tumored anatomic sites of CFPHV DNA localization and potential mobilization that may be involved in CFPHV latency, replication and shedding. Although the actual nature of CFPHV persistence or latency is yet to be determined, the data presented here offer valuable insight about CFPHV pathogenesis and transmission dynamics in symptomatic and asymptomatic green turtles.

Introduction

Chelonid fibropapilloma-associated herpesvirus (CFPHV) is a key contributing etiologic agent of marine turtle fibropapillomatosis (FP), as shown by a consistent association of the virus with FP tumors via various molecular diagnostic techniques such as PCR. As with most other herpesviruses, CFPHV infections are thought to be host-specific and lifelong (Herbst et al. 2004), with established latent infections sporadically interrupted by episodes of viral reactivation and potential replication- thereby allowing intermittent virus transmission over many years (Alfaro-Nunez et al. 2014; Young & Rickinson 2004). In herpesviral diseases that induce tumors, latent viral proteins are thought to be responsible for tumorigenesis (Young & Rickinson 2004). Although CFPHV DNA polymerase (pol, UL30) sequences have been detected via PCR in nearly every tested fibropapilloma and fibroma (Alfaro-Nunez et al. 2014; Lackovich et al. 1999; Lu et al. 2000; Page-Karjian et al. 2012; Quackenbush et al. 1998, 2001), several data sets indicate that most CFPHV genomes in visible tumors are not replicating. Rather, it is hypothesized that FP tumors carry small loci of productive CFPHV infection, with a background latent infection in other tumor tissues and possibly other anatomic sites (Greenblatt et al. 2004).

Evidence from various studies predicts that CFPHV DNA is unequally distributed within an infected individual turtle, with large concentrations of viral DNA in cutaneous tumor tissues (Alfaro-Nunez et al. 2014; Lu et al. 2000; Quackenbush et al. 2001). Widespread tissue distributions of CFPHV DNA have been found using PCR, particularly in tumored turtles. Specifically, CFPHV DNA was identified via nested PCR in kidney, liver, lung, spleen, heart, brain, periorbital tissues, nerve, ovary, testis, tongue, gall bladder, urinary bladder, thyroid, and intestine samples taken from turtles with FP (Lu et al. 2000; Quackenbush et al. 1998). To date,

however, quantitative CFPHV DNA data are lacking for internal tissues and specific nervous tissues samples, particularly those from non-tumored turtles. Here, we use quantitative PCR (qPCR) to compare viral DNA loads in skin, blood, urine, major organs, and various nervous tissues to provide pertinent information regarding anatomic localization of CFPHV DNA in tumored and non-tumored free-ranging green turtles (*Chelonia mydas*).

Methods and Materials

Animals and biological samples

Ten juvenile green turtles (5 with tumors, 5 tumor-free) that stranded in eastern Florida, USA during March-July 2014 were stored frozen (-20°C) for a maximum of 9 months, and were subsequently transported on ice and necropsied at the University of Georgia, College of Veterinary Medicine in Athens, GA USA. For all turtles, demographic data were collected from the Florida Fish and Wildlife Conservation Commission Sea Turtle Stranding and Salvage Network stranding forms, and morphologic data (e.g., weight, straight and curved carapace lengths) were collected prior to each necropsy. Body condition indices (BCI) were calculated as BCI = [weight (kg)/SCL^3] x 10,000 (Bjorndal et al. 2000). At the beginning of each necropsy, biological samples were collected aseptically. First, up to 3 mL of whole blood was collected from the heart of each turtle using a 6 mL syringe and a sterile, 21 gauge, 1.5-inch needle. Tissue samples were then carefully dissected and collected using fresh, sterile, disposable #11 scalpel blades and clean forceps for each sample. Tissue samples collected include 1-5 cm^3 samples of cutaneous and visceral tumors, non-tumored skin from the right or left shoulder, major organs (e.g., heart, liver, lung, thymus, spleen, kidney, small intestine, urinary bladder, adrenal gland), and nervous tissues (e.g., brain, optic and spinal nerves, brachial and sacral plexuses). Tissue

samples were individually placed into sterile Whirlpak® bags and stored at -80°C for up to 2 weeks prior to DNA extraction. When available, up to 35 mL of urine was collected from the bladder and/or renal pelvises. Full necropsies were conducted after the sample collection process was complete. For the 5 tumored turtles, tumor number, location, size, and morphology were recorded prior to and during necropsy, and tumor scores (0-3) were assigned using the FP tumor scoring system illustrated in Table 3.1.

This research was conducted with the approval of the University of Georgia Office of the Vice President for Research Institutional Animal Care and Use Committee (IACUC), permit #A2012 10-011-Y1-A0, and with USA state permits to collect, transport and store biological samples from green turtles: a scientific collecting permit (#29-WJH-13-44) and a Special Purpose Importation permit (#S2-WJH-13-2) from the Georgia Department of Natural Resources, Wildlife Resources Division; and a Marine Turtle Permit (#149) and authorization to receive and/or transport sea turtle biological samples from the Florida Fish and Wildlife Conservation Commission.

Nucleic acid extraction

Genomic DNA (gDNA) was extracted from each sample using the appropriate kits (Qiagen): DNeasy Blood and Tissue Kit for blood, non-tumored skin, FP, and organ tissue samples; and QIAamp Viral RNA Kit for urine. To normalize samples prior to qPCR, the concentration of the extracted DNA (µg/µl) in each sample was measured using absorbance spectrophotometry (Nanodrop), and ratios of absorption at 260 nm versus 280 nm were evaluated to ensure DNA purity. Extracted DNA was stored at -20°C for up to 2 weeks prior to qPCR analysis. Negative controls (nuclease free water) were included in each DNA extraction reaction.

qPCR assay

Quantitative PCR protocol specifications from a recently developed qPCR assay were followed (described in Chapter IV). The assay uses singleplex primers designed to target a highly conserved region of the DNA polymerase gene (UL30) in the CFPHV genome. All PCR assays were run using a StepOne™ Real-Time PCR System (Life Technologies) at the University of Georgia Poultry Diagnostic and Research Center. A conservative approach was taken with regards to positive result acceptance to help decrease the risk of false positive results. All PCR data were confirmed by testing samples with qPCR at least twice, and all samples were tested in triplicate in each assay. To demonstrate the presence of amplifiable DNA, primers developed and optimized for *C. mydas* β-actin DNA (Table 4.2; GenBank AY373753.1) were applied to all samples and included in qPCR runs. Quantitative PCR data were analyzed with the StepOne™ Real-Time PCR System Software (Life Technologies, version 2.2.3). Samples were considered positive if triplicates had a mean copy number equal to or higher than the assay's analytical limit of detection. Reaction efficiency and precision were calculated for all qPCR assays using the coefficient of determination (R^2) values. An adaptive baseline threshold was generated for each assay run via the StepOne™ software to help enhance C_q value accuracy. The number of CFPHV DNA copies for each DNA sample was determined using regression analysis of the standard curve (Larionov et al. 2005), and CFPHV UL30 copy numbers were calculated per μg of gDNA extracted from each biological sample. Viral copy number per cell was calculated for each samples according to the following formula: 0.1 μg DNA per 20,000 cells (Greenblatt et al. 2004). All positive viral amplicons were confirmed by nested PCR and capillary (Sanger) sequencing. To prepare for sequencing, qPCR amplicons of the appropriate size (approximately 173 bp) were cut from 2% agarose gel using a sterile scalpel blade and

purified using QIAquick columns (Qiagen) according to manufacturer's instructions, then sequenced in both directions using the BigDye Terminator Kit and analyzed on a 3730 XL, 96-well capillary electrophoresis DNA sequencing system at Genewiz, Inc. Any amplicons that lacked sequence verification were considered false positives and were excluded from the analysis. Two negative controls (nuclease free water) were included in each qPCR assay and sequencing reaction to help rule out contamination.

Statistical and sequence analysis

Means and standard deviations were calculated for SCL, SCW, weight, BCI, and viral DNA copy number data, which were then compared between tumored and non-tumored turtles using the 2-tailed Mann-Whitney U-test for non-parametric data with α set at 0.05. All statistical analyses were performed using IBM SPSS Statistics for MacIntosh, version 22.0. Sequence data for all CFPHV DNA-positive samples were analyzed using FinchTV DNA trace viewer software (Geospiza, Inc.) and compared to existing DNA sequences in NCBI GenBank using the BLAST algorithm (Altschul et al. 1990).

Results

Animals

Demographic and stranding data (e.g., morphometrics, age class, sex, month and location found, primary cause of death) for tumored and non-tumored turtles are presented in Table 5.1.

Table 5.1. Demographic and stranding data for the tumored and non-tumored green turtles that were sampled during necropsy

	Turtles with FP (n = 5)	Turtles without FP (n = 5)
Sex	Female: 4 Male: 1	Female: 3 Male: 2
Mean (±SD) straight carapace length (cm)	45.7 ± 4.4	30.54 ± 4.37
Mean (±SD) straight carapace width (cm)	35.9 ± 3.89	24.52 ± 2.76
Weight (kg)	10.52 ± 2.89	3.43 ± 1.15
Mean (±SD) BCI	1.14 ± 0.41	1.18 ± 0.13
Month found	April: 4 June: 1	March: 2 April: 1 May: 1 November: 1
Primary cause of death	Debilitation/FP: 3 Boat strike: 2	Fisheries interaction: 2 Floating: 2 Boat strike: 1

All 10 turtles were classified as juveniles based on morphometric data. Statistical differences were found between tumored and non-tumored turtles for SCL ($p = 0.01$), SCW ($p = 0.01$), and body weight ($p = 0.01$), with the tumored turtles being larger and heavier. The mean BCI for tumored turtles was lower than that of non-tumored turtles, although BCI values did not statistically differ between the 2 groups ($p > 0.05$).

Of the 5 tumored turtles, tumors were located on the inguinal regions (n = 4), base of the front flippers (n = 3), neck (n = 3), eye (n = 2), front and hind flippers (n = 2 each), and kidney (n = 1). One of the tumored turtles was categorized as tumor score 1; the other 4 turtles were categorized as tumor score 3 (Table 3.1).

Biological samples and qPCR results

Overall, gDNA was extracted from 155 biological samples, including 6 tumor, 10 non-tumored skin, 10 skeletal muscle, 74 organ tissue, 44 nervous tissue, 9 whole blood, and 2 urine samples. Samples that tested negative for CFPHV DNA included all skeletal muscle, lung, liver, thymus (n = 10 each), urinary bladder (n = 9), small intestine, brain (n = 8 each), adrenal gland, and spinal nerve (n = 7 each) samples. CFPHV DNA was identified in 17/82 (20.7%) samples taken from tumored turtles, and in 6/75 (8%) samples taken from non-tumored turtles. Quantitative PCR results for CFPHV DNA-positive samples are summarized in Table 5.2. Additional detailed information on viral copy number per μg DNA in individual samples is provided in Tables S2 and S3.

CFPHV UL30 copy numbers per cell ranged between: 0.04-372.4 copies/cell in cutaneous tumors; 0-0.004 copies/cell in non-tumored skin; and 0-0.3 copies/cell in non-cutaneous, non-tumored tissues. In nerve tissues alone, CFPHV UL30 copy number ranged between 0-0.3 viral copies/cell, and 0-0.09 viral copies/cell in organ tissues alone. In blood and urine samples, CFPHV UL30 copy number ranged from 0 to 0.3 viral copies/cell, with the highest copy number (0.067 copies/cell) in a urine sample taken from a turtle with tumor score 3. The highest individual copy number per cell in non-tumored tissues (0.32 viral copies per cell) was found in an optic nerve sample taken from a non-tumored turtle. Additional detailed information on viral copy number per cell in individual samples is provided in Tables S4 and S5.

In individual turtles, CFPHV DNA was identified concurrently in multiple anatomic sites. Frequently co-occurring sites include tumor and kidney (n = 4); and tumor and urine, kidney and urine, and kidney and brachial plexus (n = 2 each). In one tumored turtle, CFPHV DNA was identified in 2 different nervous tissue sites (brachial plexus and optic nerve), as well

as cutaneous tumor, kidney, and urine. In another tumored turtle, CFPHV DNA was concurrently identified in cutaneous and visceral tumors, sacral plexus, kidney, blood, and urine samples. In 3 non-tumored turtles, CFPHV DNA was concurrently identified in blood, heart, kidney, optic nerve, and brachial plexus samples (n = 1 each).

Table 5.2. Mean gDNA concentrations (µg/µl), number and percentage of positive samples, mean viral copy numbers per µg DNA, and mean viral DNA copy number per cell for tissue, blood, and urine samples taken from tumored and non-tumored turtles

Tumored turtles (n = 5)				
	Mean [gDNA] (µg/µl)	Number (%) qPCR + for CFPHV DNA	Mean viral DNA copy number / µg DNA (range)	Mean number of viral DNA copies / cell (range)
Cutaneous tumor	0.014	5/5 (100%)	2.5×10^7 ($7.9 \times 10^3 – 7.4 \times 10^7$)	126 (0.42 – 372)
Visceral tumor	0.044	1/1 (100%)	3.6×10^2	0.002
Urine	0.134	2/2 (100%)	6.8×10^3 ($1.2 \times 10^2 – 1.3 \times 10^4$)	0.03 (0.001 – 0.07)
Kidney	0.02	4/5 (80%)	6.0×10^3 ($7.8 \times 10^2 – 1.8 \times 10^4$)	0.03 (0.004 – 0.09)
Optic nerve(s)	0.013	1/4 (25%)	2.3×10^3	0.02
Whole blood	0.003	1/4 (25%)	5.5×10^3	0.03
Brachial plexus	0.014	1/5 (20%)	9.6×10^3	0.05
Sacral plexus	0.008	1/5 (20%)	2.5×10^3	0.01
Non-tumored skin	0.014	1/5 (20%)	8.6×10^2	0.004
Non-tumored turtles (n = 5)				
	Mean [gDNA] (µg/µl)	Number (%) qPCR + for CFPHV DNA	Mean viral DNA copy number / µg DNA (range)	Mean number of viral DNA copies / cell (rsnge)
Heart	0.042	2/5 (40%)	1.6×10^3 ($1.5 \times 10^3 – 1.7 \times 10^3$)	0.008 (0.007 – 0.008)
Kidney	0.008	1/5 (20%)	9.7×10^3	0.05
Optic nerve(s)	0.007	1/5 (20%)	6.1×10^4	0.3
Brachial plexus	0.006	1/5 (20%)	1.6×10^3	0.008
Whole blood	0.02	1/5 (20%)	1.1×10^3	0.005

DNA sequence data

DNA sequences isolated from the qPCR-positive amplicons matched with 100% identity to the GenBank sequences shown in Table 5.3. A sample CFPHV UL30-positive amplicon sequence chromatogram is given (Figure S3). There were no differences observed between sequence identities of amplicons of tumored versus non-tumored turtles.

Table 5.3. GenBank sequences that matched with 100% identity to qPCR amplicons that tested positive for CFPHV DNA UL30

Sequence identifier	GenBank accession number
Hawaiian green turtle herpesvirus DNA polymerase catalytic subunit (pol)	AF035003.2
Florida green turtle herpesvirus DNA polymerase (pol) gene	AF035004.1
Olive ridley turtle herpesvirus DNA polymerase gene	AF049904.1
Green turtle herpesvirus polymerase gene	AF239684.2
Chelonid herpesvirus 5 DNA polymerase gene	AF299107.1
Chelonid herpesvirus 5 DNA polymerase gene	AF299108.1
Chelonid herpesvirus 5 DNA polymerase gene	AF299109.1
Fibropapilloma-associated turtle herpesvirus from Hawaii DNA polymerase (pol) gene	AY390420.1
Fibropapilloma-associated turtle herpesvirus from Puerto Rico DNA polymerase (pol) gene	AY390421.1
Fibropapilloma-associated turtle herpesvirus DNA polymerase gene	AY395516.1
Fibropapilloma-associated turtle herpesvirus unique long region containing UL9-UL30 genes, genomic sequence	AY644454.1
Fibropapilloma-associated turtle herpesvirus strain FL_var_A polymerase (UL30) gene	AY646888.1
Fibropapilloma-associated turtle herpesvirus strain FL_var_D polymerase (UL30) gene	AY646890.1
Fibropapilloma-associated turtle herpesvirus strain HA_variant polymerase (UL30) gene	AY646893.1
Fibropapilloma-associated turtle herpesvirus isolate T1 polymerase gene	HM348895.1
Fibropapilloma-associated turtle herpesvirus isolate T3 polymerase gene	HM348896.1
Fibropapilloma-associated turtle herpesvirus isolate T4 polymerase gene	HM348897.1
Fibropapilloma-associated turtle herpesvirus isolate T2b polymerase (pol) gene	HQ000006.1
Fibropapilloma-associated turtle herpesvirus isolate T8b	HQ000007.1

polymerase (pol) gene	
Chelonid herpesvirus 5, partial genome	HQ878327.2
Fibropapilloma-associated turtle herpesvirus isolate PR2_cm_2009 DNA polymerase (UL30) gene	JN580279.1
Fibropapilloma-associated turtle herpesvirus isolate PR6_cm_2006 DNA polymerase (UL30) gene	JN580283.1
Chelonid herpesvirus 5 isolate 14010SP DNA polymerase gene	JN938584.1
Chelonid herpesvirus 5 isolate 12910SP DNA polymerase gene	JN938585.1
Chelonid herpesvirus 5 isolate 02409ES DNA polymerase gene	JN938586.1
Chelonid herpesvirus 5 isolate 03210BA DNA polymerase gene	JN938588.1

Discussion

Herpesviruses exhibit a complex replication cycle involving the nucleus and cytoplasm of host target cells (Grimm et al. 2012; Pellet & Roizman 2007), and tumor growth can be an inadvertent effect of these complex replication strategies. Persistent viral infections are those in which the virus is not cleared but remains in specific cells of infected hosts, and can include stages of silent and productive viral infection that do not involve killing or excessively damaging host cells. Latent viral infection is a type of persistent infection wherein the virus lies dormant within a small portion of host cells (Boldogh et al. 1996). Long-term, persistent, intracellular infections with intermittent periods of latency and recrudescence are characteristic of DNA tumor herpesviruses such as CFPHV (Moore & Chang 2010).

Others have theorized that latent CFPHV transcripts are responsible for FP tumor growth (Greenblatt et al. 2004; Work et al. 2009). For example, histologic hallmarks of productive herpesvirus infection such as herpesvirus-like intranuclear inclusions and ballooning cellular degeneration were only observed in the dermis and epidermis of 2% of naturally occurring FP tumors assayed by microscopy (Herbst et al. 1995, 1999; Jacobson et al. 1989, 1991). Higher

levels of CFPHV UL30 DNA were observed in the dermis as compared to the epidermis, suggesting that while vegetative viral production occurs in keratinocytes, a majority of the CFPHV viral genomes within tumors resides in fibroblasts integrated either into host DNA or as episomes (Ganem 2006; Work et al. 2009). This infection pattern is also seen in Kaposi's sarcoma-associated herpesvirus (KSHV), a gammaherpesvirus that is present in latent form in >96% of tumor cells, while <1–3% of cells appears to be productively infected at some stage of tumor development (Blasig et al. 1997; Dupin et al. 1999; Katano et al. 2000). Since it is known that with some types of herpesvirus-induced neoplasia, infection remains latent with only a fraction of viral genes expressed in proliferating transformed cells while virion production occurs in other tissues (Butel 2000), there may be alternative tissues besides cutaneous tumors involved in CFPHV latency, replication and shedding. This hypothesis is supported by the data presented herein, with large quantities of CFPHV DNA identified in all cutaneous tumor samples, and relatively smaller CFPHV DNA quantities identified in various nerve and tissue samples.

CFPHV is a member of the subfamily *Alphaherpesvirinae* (Greenblatt et al. 2005a; Lackovich et al. 1999; Quackenbush et al. 2001; Yu et al. 2001). Members of this subfamily are known for their ability to undergo intracellular replication during different stages of host cellular differentiation, a capability that allows them to infect a wide variety of host cell types including highly differentiated, non-dividing cells such as neurons. In mammals, alphaherpesviruses (e.g., herpes simplex and varicella zoster viruses in humans, equine herpesvirus-1 (EHV-1) in horses) are acquired early in life and establish lifelong latent infections in neurons from which viral reactivation occurs periodically (Cohrs & Guilden 2011; Paillot et al. 2008). With this study we have identified potential sites of CFPHV persistent and/or latent infection that include optic nerves and nerves of the brachial and sacral plexuses. The optic nerve samples are likely

composed of retinal ganglia cell axons and glial cells. Specific nerves that may be included in the brachial plexus samples are the ventral branches of cranial nerve 6, cranial nerves 7 and 8, the inferior brachial nerve, superficial and deep radial nerves, and the supracoracoideus, subscapular, axillary, radial, ulnar, and median nerves. The sacral plexus samples may include branches from spinal nerves 17-21, including the crural, femoral, obturator, ischiadicus, sciatic, peroneal, and tibial nerves (Wyneken 2001). We previously observed that the inguinal regions, front flippers, and eyes and are some of the most common locations to observe FP tumors in juvenile green turtles (Table 3.3), and that cutaneous tumors are most frequently first observed in the inguinal regions on turtles that develop FP in captivity (Page-Karjian et al. 2014). The CFPHV DNA localization data presented here implies that in some cases, cutaneous tumors in these locations may be related to herpesviral reactivation from optic nerves and/or nerves within the brachial and sacral plexuses.

Another highly represented site of CFPHV DNA localization in tumored and non-tumored turtles identified via qPCR is the kidney: CFPHV UL30 amplicons were present in kidney samples of 4/5 (80%) of the tumored turtles and in 1/5 (20%) of the non-tumored turtles. In 2 tumored turtles, CFPHV DNA was concurrently identified in renal tissues and urine samples, with lower viral copy numbers in urine than in kidney samples. These findings support our previous hypothesis that CFPHV DNA may be shed via the renal-urinary tract by green turtles with and without FP. Renal-urinary excretion of viral DNA is observed in herpesviruses in other species, including KSHV of humans (Santos-Fortuna & Caterino-de-Araujo 2005), Lucke tumor herpesvirus of leopard frogs (Carlson et al. 1994), and elephant endotheliotropic herpesvirus-1 (EEHV-1; Stanton et al. 2013). The fact that CFPHV DNA was not identified in any urinary bladder samples, even in those turtles that had kidney and/or urine samples that

tested positive for CFPHV, further implicates the kidneys, rather than the urinary bladder epithelium, as a potential site of viral DNA persistence and/or secretion.

Two turtles, one with tumors and one tumor-free, had blood samples that tested positive for CFPHV DNA via qPCR. This finding supports our previous qPCR data that identified CFPHV DNA in approximately 1/3 of blood samples taken from tumored and non-tumored green turtles (Chapter IV). Such DNAemia may be due to CFPHV infection of peripheral blood mononuclear cells (PBMCs), and may be observed during a viral replicative phase in active or reactivated infections (van der Meulen et al. 2000). Thus during phases of viral replication, CFPHV DNA may be more likely to be detected throughout the body. It is unclear however whether the identification of CFPHV DNA in 2 heart tissue samples, one from a tumored and one from a non-tumored turtle, is related to CFPHV DNA presence in the blood or nerves, or whether the heart is a site of viral persistence in some turtles.

In previous studies, CFPHV DNA was not identified in any of 19 (Quackenbush et al. 1998) or 28 (Lu et al. 2000) normal-appearing skin and organ tissue samples taken from non-tumored turtles. Differences in assay sensitivity may explain the fact that here we identified CFPHV DNA in samples from non-tumored turtles: our qPCR assay has a higher sensitivity (50 viral copies or 9.3×10^{-6} pg of herpesviral DNA) than previously used nested PCR assays (0.1 pg herpesviral DNA; Lu et al. 2000; Quackenbush et al. 1998) and therefore can detect very small amounts of viral DNA. Of the 5 tumored turtles that tested positive for CFPHV DNA in this study, the mean viral DNA copy number for non-tumored tissues (excluding blood and urine) was 5,060 viral copies/µg DNA, while the mean viral copy number for non-tumored tissues of 3 non-tumored turtles was 14,975 viral copies/µg DNA. Although these averages are not statistically different ($p > 0.05$), the relatively higher viral DNA copy number observed in the

tumored turtles may be related to a sequestering of CFPHV genomes within tumored tissues, thereby leading to reduced viral copy numbers in non-tumored tissues. The high viral DNA copy numbers observed in cutaneous tumor tissues (mean ± SD = $2.5 \times 10^7 \pm 3.4 \times 10^7$ viral copies/μg DNA) lends further support to this hypothesis.

As shown in Table 5.2, viral copy numbers per cell can also be estimated and compared. In a previous study that used qPCR for CFPHV UL30, a small range was reported for viral copy number in cutaneous tumors (2-20 copies/cell). In this study we found a large range of viral copy number in cutaneous tumors (0.04-372.4 copies/cell), a difference that may reflect which part of the tumor was sampled, since other researchers have shown wide variability in viral copy numbers depending on tumor sample location (Greenblatt et al. 2004; Work et al. 2009). Similarly to previous reports (Quackenbush et al. 2001), we observed a relatively small range of viral copy number in non-cutaneous, non-tumored tissues (0-0.3 copies/cell).

In this study, CFPHV DNA was found in relatively few anatomic sites as compared to previous studies that used nested PCR to evaluate for CFPHV DNA in green turtle tissues taken during necropsy (Lu et al. 2000; Quackenbush et al. 1998). For example, we found that 8/63 (12.7%) of non-tumored organ tissue samples taken from tumored turtles were positive for CFPHV DNA. Compare this to nested PCR results reported by Lu et al. (2000) and Quackenbush et al. (1998), who identified CFPHV DNA in >80% and 28% of non-tumored tissue/organ samples taken from tumored turtles, respectively. A plausible explanation for these differences between studies is our use of fresh, sterile scalpel blades and handles to obtain each individual sample, which would presumably help decrease the risk of cross-contamination between samples. Comparatively lower CFPHV DNA prevalence in the samples analyzed here could also be attributable to a small sample size, or to DNA degradation from freezing and

thawing the carcasses prior to necropsy. These considerations may also help to explain the comparatively low number of non-FP skin samples that tested positive for CFPHV DNA by qPCR.

Although CFPHV UL30 is part of the beta temporal expression group (expressed during productive or lytic infection), the qPCR assay presented here detects gDNA of the CFPHV UL30 gene independent of virus life stage. Further studies are needed to differentiate specific infected cell types and to determine which stage of the viral life cycle is being identified (i.e., latency versus replication). True differentiation between viral latency and replication will require sequencing and experimental verification of CFPHV latency-associated transcripts, followed by development of validated immunohistochemical and reverse transcriptase qPCR (RT-qPCR) assays with subsequent application to fresh or properly preserved tissue samples taken from sea turtles with and without FP. Further studies should also aim to include a larger sample size; although not logistically possible in this study due to unpredictable carcass availability and limited carcass storage options, a larger sample size would have helped to strengthen the hypotheses postulated here. The actual pathogenesis and nature of CFPHV persistence or latency are yet to be determined, however, the data presented here give relevant information regarding the anatomic localization and mobilization of CFPHV DNA in symptomatic and asymptomatic green turtles. Such qPCR data on biological samples of free-ranging sea turtles are particularly valuable because they are logistically difficult, time-consuming, and costly to obtain. With this study we provide important evidence for anatomic sites of CFPHV DNA localization in symptomatic and asymptomatic green turtles, and offer insight about CFPHV pathogenesis and transmission dynamics.

CHAPTER 6

CONCLUSIONS

The dissertation research data presented here allow us to better understand certain aspects of CFPHV natural history dynamics in free ranging and rehabilitating green sea turtles with and without fibropapillomatosis. Using nested PCR for 3 different CFPHV gene targets to test skin and tumor samples from tumored and non-tumored, free ranging green turtles in Puerto Rico, CFPHV DNA was identified in 32.4% of clinically normal turtles. This result suggests the presence of a subpopulation of asymptomatic viral carriers in an FP-endemic turtle aggregation. The CFPHV DNA polymerase (UL30) gene target was shown to be the most reliable and sensitive of 3 tested gene targets, and comparative PCR data showed that a greater number of CFPHV DNA-positive samples were identified when the more sensitive nested PCR assay was used. With a retrospective case series analysis of rehabilitating green turtles with FP, we describe the occurrence and case progression scenarios of FP in a rehabilitation setting. Case evidence-based suggestions are given to assist wildlife and zoo veterinarians in clinical decision-making for turtles with FP, including prognostic indicators such as the presence of ocular tumors, cutaneous plaque-like FP lesions only, and number of tumor removal surgeries.

A quantitative PCR (qPCR) diagnostic assay for the CFPHV UL30 gene target was developed, validated, optimized, and then applied to various biological samples taken from rehabilitating and free ranging juvenile green turtles with and without FP. The qPCR assay was shown to be sensitive, specific, robust, and repeatable. The qPCR data provide evidence supporting an etiologic role of CFPHV in the FP disease pathogenesis, and suggest that

approximately 10-25% of juvenile green turtles from an FP-endemic region (eastern Florida, USA) have CFPHV DNA on their skin. We provide, for the first time, qPCR evidence for CFPHV DNAemia in approximately 1/3 of rehabilitating green turtles, including evidence that the viral DNA is cell-associated within the blood. Identification of CFPHV DNAemia suggests circulating blood as a viral transport mechanism that may be related to the multi-centric nature of FP tumors, and that also appears to be related to the presence of CFPHV DNA in urine. Identification of CFPHV DNA in urine and kidney samples suggests that the kidneys may be a site of CFPHV localization or persistence, and indicates for the first time the potential for a horizontal route of CFPHV DNA shedding other than via sloughing of infected skin cells. CFPHV DNA presence in nerve samples dissected from tumored and non-tumored turtles suggests that, like other alphaherpesviruses, CFPHV DNA is neurotropic and may localize to nerve tissues during latent stages.

More research is needed to improve our understanding of the CFPHV natural history and pathogenesis within infected green turtles. The qPCR data provided here would be enhanced by similar surveys using larger sample sizes and repeated measures taken for individual turtles, as well as paired microscopic verification of all qPCR findings. Future investigations including controlled infectivity trials are needed to help verify renal-urinary excretion as a route of CFPHV shedding. More work is also needed to determine if CFPHV particles can persist in the marine environment. Immunohistochemistry assays could help define proposed anatomic sites of virus localization and behavior. FP pathogenesis would be clarified by identifying a full array of comparative gene expression (transcriptomics) in non-tumored versus severely tumored green turtles. Application of a reliable, widely available serologic test for CFPHV exposure would also improve our understanding of the status of CFPHV infection in free ranging turtle populations.

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APPENDICES

Page

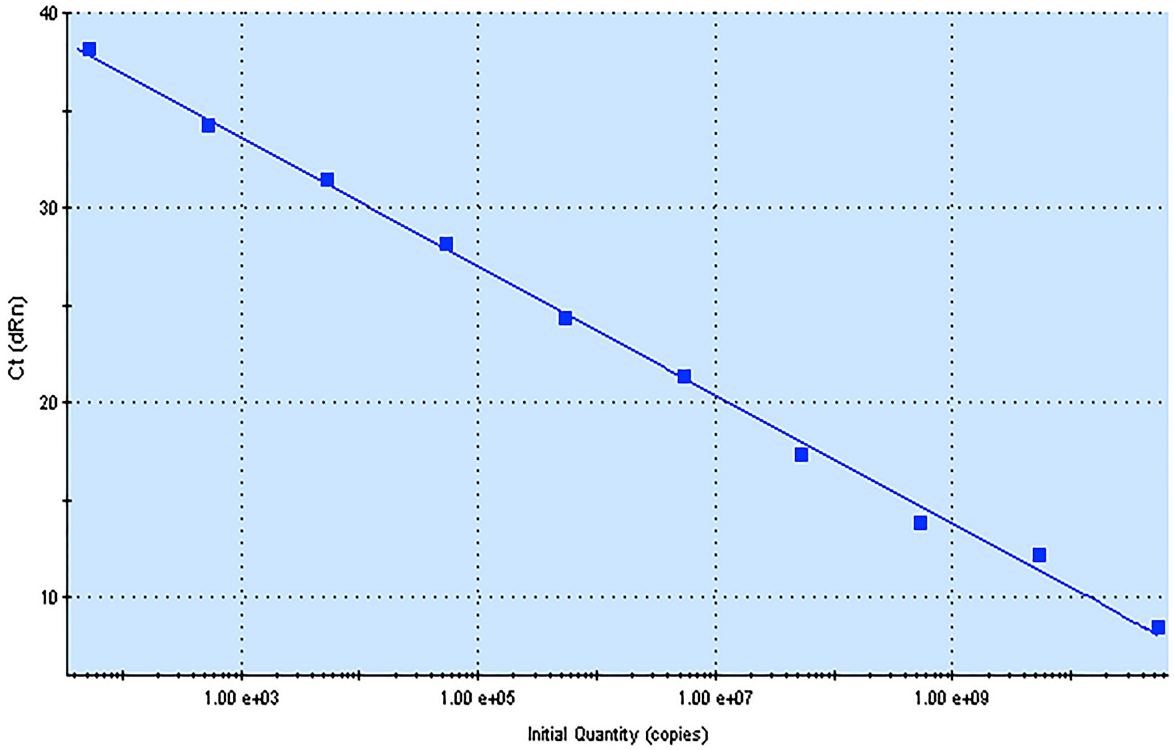

Figure S1. Example of a CFPHV pol plasmid standard curve used in the qPCR assays presented here

Table S1. GenBank sequence information that matched with ≥97% identity to sequences isolated from CFPHV DNA-positive samples of FP tumors, non-tumored skin, blood, plasma, urine and cloacal swabs

Sequence identifier	GenBank accession number
Hawaiian green turtle herpesvirus thymidine kinase (UL23), membrane-associated protein (UL24), minor capsid protein (UL25), capsid maturation protease (UL26), virion scaffolding protein (UL26.5), virion membrane glycoprotein B (gB), DNA cleavage/packaging protein (UL28), single-stranded DNA-binding protein (UL29), DNA polymerase catalytic subunit (pol), nuclear phosphoprotein (UL31), DNA cleavage/ packaging (UL32), DNA cleavage/packaging protein (UL33), membrane-associated phosphoprotein (UL34), basic phosphorylated capsid protein (UL35) genes, complete cds; and very large tegument protein (UL36) gene	AF035003.2
Florida green turtle herpesvirus DNA polymerase (pol) gene, partial cds	AF035004.1
Loggerhead turtle herpesvirus DNA polymerase (pol) gene, partial cds	AF035005.1
Olive ridley turtle herpesvirus DNA polymerase gene, partial cds	AF049904
Olive ridley turtle herpesvirus DNA polymerase gene, partial cds	AF049904.1
Green turtle herpesvirus polymerase gene, complete cds	AF239684.2
Chelonid herpesvirus 5 DNA polymerase gene, partial cds	AF299107.1
Chelonid herpesvirus 5 DNA polymerase gene, partial cds	AF299108.1
Chelonid herpesvirus 5 DNA polymerase gene, partial cds	AF299109.1
Chelonid herpesvirus 5 DNA polymerase gene, partial cds	AF299110.1
Fibropapilloma-associated turtle herpesvirus from Hawaii DNA polymerase (pol) gene	AY390420.1
Fibropapilloma-associated turtle herpesvirus from Puerto Rico DNA polymerase (pol) gene	AY390421.1
Fibropapilloma-associated turtle herpesvirus from California DNA polymerase (pol) gene	AY390422.1
Fibropapilloma-associated turtle herpesvirus DNA polymerase gene	AY395516.1
Fibropapilloma-associated turtle herpesvirus UL region containing UL9-UL30 genes	AY644454.1
Fibropapilloma-associated turtle herpesvirus strain FL_var_A polymerase (UL30) gene	AY646888.1
Fibropapilloma-associated turtle herpesvirus strain FL_var_C polymerase (UL30) gene	AY646889.1
Fibropapilloma-associated turtle herpesvirus strain HA_variant polymerase (UL30) gene	AY646893.1
Fibropapilloma-associated turtle herpesvirus strain FL var C	AY646894.1

polymerase (UL30) gene	
Fibropapilloma-associated turtle herpesvirus isolate T1 polymerase gene	HM348895.1
Fibropapilloma-associated turtle herpesvirus isolate T3 polymerase gene	HM348896.1
Fibropapilloma-associated turtle herpesvirus isolate T4 polymerase gene	HM348897.1
Fibropapilloma-associated turtle herpesvirus isolate T8 polymerase gene	HM348898.1
Fibropapilloma-associated turtle herpesvirus isolate T2b polymerase (pol) gene	HQ000006.1
Fibropapilloma-associated turtle herpesvirus isolate T8b polymerase (pol) gene	HQ000007.1
Chelonid herpesvirus 5, partial genome	HQ878327.2
Fibropapilloma-associated turtle herpesvirus isolate PR2_cm_2009 DNA polymerase (UL30) gene	JN580279.1
Fibropapilloma-associated turtle herpesvirus isolate PR3_cm_2010 DNA polymerase (UL30) gene	JN580280.1
Fibropapilloma-associated turtle herpesvirus isolate PR6_cm_2006 DNA polymerase (UL30) gene	JN580283.1
Chelonid herpesvirus 5 isolate 14010SP DNA polymerase gene, partial cds	JN938584.1
Chelonid herpesvirus 5 isolate 12910SP DNA polymerase gene, partial cds	JN938585.1
Chelonid herpesvirus 5 isolate 02409ES DNA polymerase gene, partial cds	JN938586.1
Chelonid herpesvirus 5 isolate 13110SP DNA polymerase gene, partial cds	JN938587.1
Chelonid herpesvirus 5 isolate 03210BA DNA polymerase gene, partial cds	JN938588.1

Dataset S1. CFPHV pol plasmid qPCR standard curve reaction efficiency and coefficient of determination (R^2) data used in qPCR assay validation

Dilution Series #	qPCR Run #	Efficiency	R^2
1	1-1	98.6	0.996
1	1-2	106	0.998
1	1-3	103	0.999
1	1-4	111.4	0.977
1	1-5	102.7	0.962
1	1-6	105.3	0.986
1	1-7	94.8	0.992
1	1-8	90.3	0.992
1	1-9	91.4	0.998
1	1-10	91.7	0.991
2	2-1	97.2	0.991
2	2-2	94.2	0.999
2	2-3	90.8	0.998
2	2-4	108	0.928
2	2-5	96.3	0.992
2	2-6	93.1	0.997
2	2-7	92.8	0.996
2	2-8	94.1	0.994
2	2-9	91.1	0.996
2	2-10	92.6	0.975
3	3-1	100.7	0.997
3	3-2	98.8	0.994
3	3-3	104	0.924
3	3-4	101.8	0.996
3	3-5	93.7	0.993
3	3-6	92.2	0.989
3	3-7	97	0.989
3	3-8	97.6	0.986
3	3-9	97.2	0.991
3	3-10	97.6	0.966
	Mean	97.533	0.986
	Std Dev	5.620	0.019

Dataset S2. Sensitivity data used in qPCR validation

qPCR run #	CFPHV copy #	Replicate #	*Cq*
1	1	1	32.54
2	1	2	33.64
3	1	3	No Cq
4	1	4	No Cq
5	1	5	36.66
6	1	6	No Cq
7	1	7	No Cq
8	1	8	No Cq
9	1	9	No Cq
10	1	10	No Cq
11	1	11	No Cq
12	1	12	No Cq
13	1	13	No Cq
14	1	14	No Cq
15	1	15	No Cq
16	1	16	No Cq
17	1	17	No Cq
18	1	18	No Cq
19	1	19	No Cq
20	1	20	No Cq
21	1	21	No Cq
22	1	22	No Cq
23	1	23	No Cq
24	1	24	No Cq
25	1	25	No Cq
26	1	26	No Cq
27	1	27	39.51
28	1	28	No Cq
29	1	29	38.42
30	1	30	No Cq
31	1	31	No Cq
32	1	32	No Cq
33	1	33	38.78
34	1	34	No Cq
35	1	35	No Cq
36	1	36	No Cq
37	1	37	No Cq
38	1	38	No Cq
39	1	39	No Cq
40	1	40	39.6

41	1	41	No Cq
42	1	42	No Cq
	Total # positive by qPCR:		7

qPCR run #	CFPHV copy #	Replicate #	*C*q
1	5	1	33.29
2	5	2	30.12
3	5	3	37.83
4	5	4	36.17
5	5	5	38.79
6	5	6	No Cq
7	5	7	39.63
8	5	8	No Cq
9	5	9	No Cq
10	5	10	No Cq
11	5	11	No Cq
12	5	12	39.43
13	5	13	No Cq
14	5	14	No Cq
15	5	15	No Cq
16	5	16	No Cq
17	5	17	No Cq
18	5	18	No Cq
19	5	19	No Cq
20	5	20	No Cq
21	5	21	39.27
22	5	22	38.32
23	5	23	No Cq
24	5	24	No Cq
25	5	25	38.14
26	5	26	No Cq
27	5	27	38.27
28	5	28	37.23
29	5	29	36.36
30	5	30	36.16
31	5	31	No Cq
32	5	32	39.07
33	5	33	39.03
34	5	34	No Cq
35	5	35	No Cq
36	5	36	38.17

37	5	37	No Cq
38	5	38	No Cq
39	5	39	35.87
40	5	40	36.07
41	5	41	36.47
42	5	42	36.05
	Total # positive by qPCR:		21

qPCR run #	CFPHV copy #	Replicate #	*C*q
1	50	1	31.87
2	50	2	No Cq
3	50	3	36.49
4	50	4	37.25
5	50	5	39.46
6	50	6	35.86
7	50	7	37.85
8	50	8	38.14
9	50	9	37.28
10	50	10	36.65
11	50	11	38.79
12	50	12	No Cq
13	50	13	39.13
14	50	14	38.94
15	50	15	36.47
16	50	16	38.91
17	50	17	38.49
18	50	18	No Cq
19	50	19	35.36
20	50	20	39.39
21	50	21	37.36
22	50	22	37.49
23	50	23	36.39
24	50	24	36.56
25	50	25	34.25
26	50	26	33.46
27	50	27	32.49
28	50	28	No Cq
29	50	29	32.62
30	50	30	32.09
31	50	31	37.93
32	50	32	37.8

33	50	33	37.3
34	50	34	No Cq
35	50	35	No Cq
36	50	36	31.85
37	50	37	32.56
38	50	38	38.54
39	50	39	32.2
40	50	40	32.75
41	50	41	32.74
42	50	42	32.88
	Total # positive by qPCR:		36

qPCR run #	CFPHV copy #	Replicate #	*C*q
1	500	1	27.22
2	500	2	26.93
3	500	3	35.06
4	500	4	34.35
5	500	5	34.05
6	500	6	32.9
7	500	7	34.14
8	500	8	35
9	500	9	32.6
10	500	10	34.14
11	500	11	36.15
12	500	12	35.32
13	500	13	35.37
14	500	14	35.77
15	500	15	33.5
16	500	16	35.73
17	500	17	36.51
18	500	18	36.89
19	500	19	32.94
20	500	20	32.44
21	500	21	32.75
22	500	22	33.33
23	500	23	32.54
24	500	24	32.93
25	500	25	31.45
26	500	26	30.25
27	500	27	32.96
28	500	28	28.16

29	500	29	28.4
30	500	30	30.4
31	500	31	34.53
32	500	32	34.77
33	500	33	34.4
34	500	34	30.09
35	500	35	31.96
36	500	36	31.23
37	500	37	30.39
38	500	38	35.06
39	500	39	28.95
40	500	40	28.99
41	500	41	28.79
42	500	42	28.85
	Total # positive by qPCR:		42

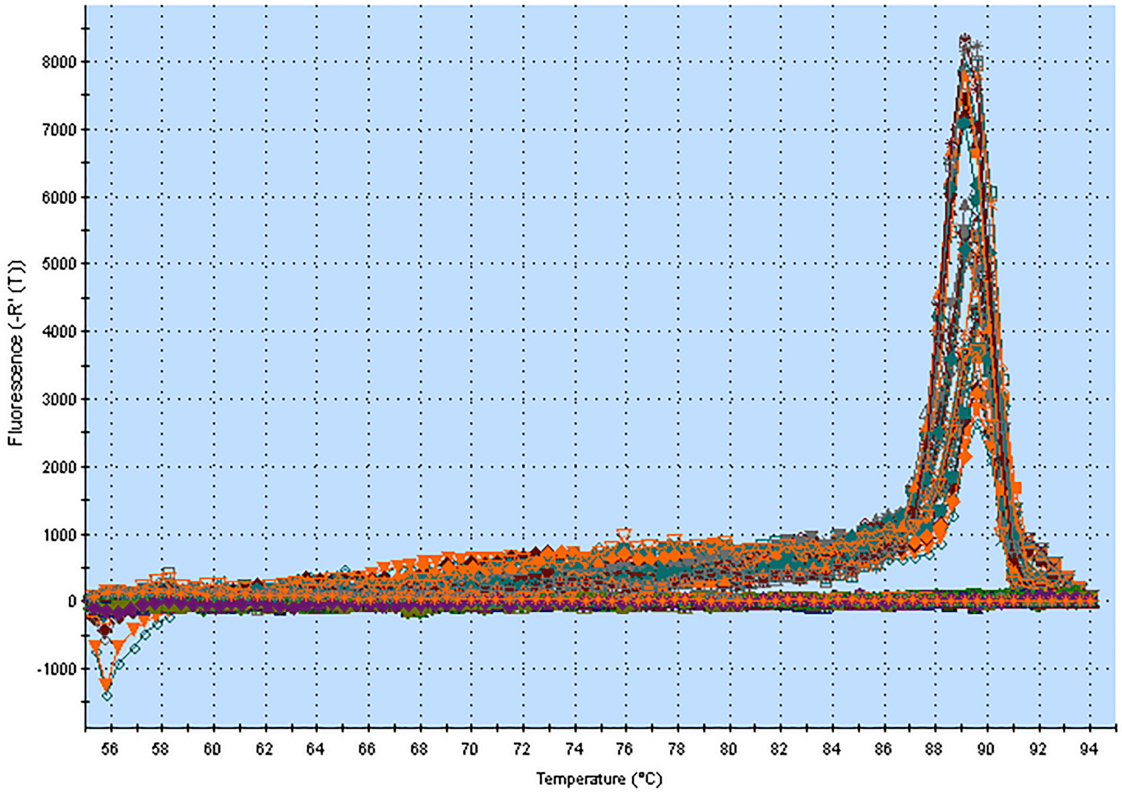

Figure S2. qPCR melt curve analysis showing a single amplified product when qPCR UL30 primers were tested using SYBR green I chemistry

Dataset S3. Specificity data used in qPCR assay validation

Sample ID	Sample Type	CFPHV UL30 *C*q
BHV-3	Purified plasmid	No *C*q
PhHV-1	Purified plasmid	No *C*q
PhHV-2	Purified plasmid	No *C*q
T. scripta elegans 1	Blood	No *C*q
T. scripta elegans 1	Plasma	No *C*q
T. scripta elegans 1	Skin	No *C*q
T. scripta elegans 1	Oral swab	No *C*q
T. scripta elegans 1	Cloacal swab	No *C*q
T. scripta elegans 1	Feces	No *C*q
T. scripta elegans 2	Blood	No *C*q
T. scripta elegans 2	Plasma	No *C*q
T. scripta elegans 2	Skin	No *C*q
T. scripta elegans 2	Oral swab	No *C*q
T. scripta elegans 2	Cloacal swab	No *C*q
T. scripta elegans 2	Feces	No *C*q
C. mydas neg control	Blood	No *C*q
C. mydas neg control	Plasma	No *C*q
C. mydas neg control	Skin	No *C*q
C. mydas neg control	Oral swab	No *C*q
C. mydas neg control	Cloacal swab	No *C*q
C. mydas neg control	Feces	No *C*q
C. mydas neg control	Urine	No *C*q

Dataset S4. Repeatability data used in qPCR assay validation

Sample Type: FP tumors			Sample Type: Non-FP skin		
Sample	Replicate #	CFPHV Copy #	Sample	Replicate #	CFPHV Copy #
Low copy #	1	23294	Low copy #	1	177
Low copy #	2	21176	Low copy #	2	200
Low copy #	3	22668	Low copy #	3	218
Low copy #	4	23937	Low copy #	4	197
Low copy #	5	17740	Low copy #	5	189
Low copy #	6	18230	Low copy #	6	220
Low copy #	7	29967	Low copy #	7	226
Low copy #	8	19382	Low copy #	8	197
Mean		22049.25	Mean		203
Std Dev		3950.04	Std Dev		16.886
CV(%)		0.179	CV(%)		8.318
Med copy #	1	1413211	Med copy #	1	23243
Med copy #	2	1293505	Med copy #	2	21087
Med copy #	3	1403622	Med copy #	3	25497
Med copy #	4	1413211	Med copy #	4	23504
Med copy #	5	945701	Med copy #	5	24150
Med copy #	6	808625	Med copy #	6	19047
Med copy #	7	1347439	Med copy #	7	15860
Med copy #	8	1320197	Med copy #	8	27658
Mean		1243188.875	Mean		22505.75
Std Dev		233063.0458	Std Dev		3739.34
CV(%)		18.747	CV(%)		16.615
High copy #	1	244640017	High copy #	1	41549
High copy #	2	226987722	High copy #	2	35069
High copy #	3	231671631	High copy #	3	46626
High copy #	4	238067536	High copy #	4	49898
High copy #	5	222398511	High copy #	5	51271
High copy #	6	223917845	High copy #	6	33899
High copy #	7	169378649	High copy #	7	54498
High copy #	8	325622473	High copy #	8	41831
Mean		235335548	Mean		44330.125
Std Dev		43056308	Std Dev		7525.108
CV(%)		18.296	CV(%)		16.975

Mean	12.40735	Mean	13.96950
CV(%)	03	CV(%)	416

Sample Type: Blood			Sample Type: Plasma		
Sample	Replicate #	CFPHV Copy #	Sample	Replicate #	CFPHV Copy #
Low copy #	1	326	Low copy #	1	640
Low copy #	2	315	Low copy #	2	471
Low copy #	3	360	Low copy #	3	397
Low copy #	4	280	Low copy #	4	623
Low copy #	5	375	Low copy #	5	586
Low copy #	6	288	Low copy #	6	555
Low copy #	7	269	Low copy #	7	431
Low copy #	8	260	Low copy #	8	422
Mean		309.125	Mean		515.625
Std Dev		42.367	Std Dev		96.726
CV(%)		13.706	CV(%)		18.759
Med copy #	1	2515	Med copy #	1	1157
Med copy #	2	2358	Med copy #	2	1081
Med copy #	3	3495	Med copy #	3	1381
Med copy #	4	3052	Med copy #	4	1096
Med copy #	5	2576	Med copy #	5	1419
Med copy #	6	3356	Med copy #	6	1118
Med copy #	7	2102	Med copy #	7	834
Med copy #	8	3157	Med copy #	8	924
Mean		2826.375	Mean		1126.25
Std Dev		505.896	Std Dev		200.318
CV(%)		17.899	CV(%)		17.787
High copy #	1	10785	High copy #	1	1550
High copy #	2	10702	High copy #	2	1400
High copy #	3	11299	High copy #	3	1438
High copy #	4	9344	High copy #	4	1498
High copy #	5	12340	High copy #	5	1540
High copy #	6	13569	High copy #	6	1529
High copy #	7	12257	High copy #	7	1478
High copy #	8	10486	High copy #	8	1615
Mean		11347.75	Mean		1506
Std Dev		1322.278	Std Dev		67.834
CV(%)		11.652	CV(%)		4.504

Mean	14.41899		13.683192
CV(%)	741	Mean CV(%)	6

Sample Type: Urine			Sample Type: Cloacal swabs		
Sample	Replicate #	CFPHV Copy #	Sample	Replicate #	CFPHV Copy #
Low copy #	1	220	Low copy #	1	543
Low copy #	2	319	Low copy #	2	411
Low copy #	3	224	Low copy #	3	606
Low copy #	4	227	Low copy #	4	484
Low copy #	5	271	Low copy #	5	515
Low copy #	6	235	Low copy #	6	558
Low copy #	7	278	Low copy #	7	518
Low copy #	8	209	Low copy #	8	574
Mean		247.875	Mean		526.125
Std Dev		37.722	Std Dev		59.989
CV(%)		15.218	CV(%)		11.402
Med copy #	1	1574	Med copy #	1	2822
Med copy #	2	1715	Med copy #	2	2940
Med copy #	3	1141	Med copy #	3	3169
Med copy #	4	1045	Med copy #	4	3042
Med copy #	5	1602	Med copy #	5	3212
Med copy #	6	1418	Med copy #	6	3105
Med copy #	7	1726	Med copy #	7	4161
Med copy #	8	1096	Med copy #	8	3042
Mean		1414.625	Mean		3186.625
Std Dev		283.023	Std Dev		412.766
CV(%)		20.007	CV(%)		12.953
High copy #	1	7512	High copy #	1	6092
High copy #	2	7076	High copy #	2	7731
High copy #	3	7521	High copy #	3	8108
High copy #	4	4710	High copy #	4	6610
High copy #	5	6220	High copy #	5	8220
High copy #	6	5075	High copy #	6	5848
High copy #	7	4742	High copy #	7	9291
High copy #	8	6567	High copy #	8	9103
Mean		6177.875	Mean		7625.375
Std Dev		1193.819	Std Dev		1314.568
CV(%)		19.324	CV(%)		17.239

Mean	18.18312	Mean	13.86484
CV(%)	977	CV(%)	961

Dataset S5. DNA concentration and CFPHV copy number (raw qPCR data) for various biological samples taken from rehabilitating green turtles

Sample Type: FP tumor			
Turtle ID	Study Group	[DNA] ug/ul	CFPHV copy#
1	A	0.118	290491257
2	A	0.06	498969330.6
3	A	0.0412	736427473.3
4	A		
5	A		
6	A		
7	A		
8	A	0.0526	8703736.8
9	A	0.0034	32467545.2
10	A	0.0773	1626.3
11	A	0.0384	63488097.5
12	A	0.0482	53373143
13	A	0.0124	72567.5
14	A	0.0302	8883013.5
15	A	0.018	2653889.6
16	A	0.0311	2031752904
17	A	0.0556	605312608
18	A	0.048	481119.4
19	A	0.0458	794234.8
20	A		
21	A	0.0793	295668501.4
22	A	0.15	1207452703
23	A	0.0463	560786.2
		n	18

Sample Type: Non-FP skin			
Turtle ID	Study Group	[DNA] ug/ul	CFPHV copy#
1	A	0.0854	23242.8
2	A	0.0116	40608.6
3	A		
4	A	0.0247	14042.9
5	A		
6	A		
7	A		
8	A		
9	A	0.0114	245.5
10	A		

11	A	0.0171	576.8
12	A		
13	A		
14	A		
15	A		
16	A	0.0139	71414.4
17	A	0.0161	0
18	A	0.0271	4504.3
19	A	0.0176	65708.4
20	A	0.0604	0
21	A		
22	A	0.0271	3482.3
23	A	0.0929	3392.7
24	B	0.0163	2316.2
25	B		
26	B		
27	B		
28	B		
29	B		
30	B		
31	B		
32	B		
33	B		
34	B		
35	B		
36	B		
37	C	0.018	0
38	C	0.0145	0
39	C		
40	C		
41	C		
42	C	0.0166	0
43	C	0.0192	0
44	C	0.0139	0
45	C	0.0202	0
46	C	0.0224	0
47	C	0.0281	0
48	C		
49	C	0.0242	0
50	C	0.0498	0
51	C	0.0214	648.9
52	C	0.0213	0
53	C	0.0299	0

54	C	0.0131	0
55	C	0.0244	0
56	C	0.0171	8351.9
57	C	0.0401	0
58	C	0.0469	603.5
59	C	0.0545	17374.8
60	C	0.0135	0
61	C	0.0171	0
62	C	0.0163	0
63	C	0.0115	0
64	C	0.05	4738.6
65	C	0.0186	0
66	C	0.0207	1060.3
67	C	0.0223	0
		n	40

Sample Type: Whole blood			
Turtle ID	Study Group	[DNA] ug/ul	CFPHV copy#
1	A		
2	A		
3	A	0.0288	0
4	A	0.0427	180
5	A	0.0185	0
6	A	0.0427	1295.6
7	A	0.007	1243.7
8	A	0.0192	0
9	A	0.0167	176.5
10	A	0.0251	0
11	A	0.016	0
12	A	0.0193	0
13	A	0.0267	0
14	A	0.0363	0
15	A	0.0118	0
16	A	0.03	0
17	A	0.0141	0
18	A	0.0448	0
19	A	0.043	0
20	A	0.052	0
21	A	0.0474	3607
22	A	0.005	1730.4
23	A	0.0426	3922
24	B	0.0178	2514.9

25	B	0.0479	1521.3
26	B	0.0235	0
27	B	0.0061	0
28	B	0.005	0
29	B	0.0209	0
30	B	0.007	0
31	B	0.0149	0
32	B	0.00487	14753.6
33	B	0.0187	2134.1
34	B	0.0219	0
35	B	0.003	8345.6
36	B	0.0109	1573.6
37	C	0.0224	0
38	C	0.0202	0
39	C	0.006	5968.2
40	C	0.0057	0
41	C	0.0121	10785.4
42	C	0.0204	3654.6
43	C	0.0388	0
44	C	0.038	0
45	C	0.0488	0
46	C	0.0528	326.47
47	C	0.0812	0
48	C	0.0272	0
49	C	0.0502	0
50	C	0.043	0
51	C	0.042	0
52	C	0.054	0
53	C	0.0365	0
54	C	0.0454	0
55	C	0.0269	0
56	C	0.01719	607.1
57	C	0.04695	0
58	C	0.015	1938
59	C	0.0559	0
60	C	0.0184	0
61	C	0.0217	0
62	C	0.0479	0
63	C	0.0469	0
64	C	0.0233	0
65	C	0.0337	28382.3
66	C	0.0183	874.6
67	C	0.0432	0

		n	65
Sample Type: Plasma			
Turtle ID	Study Group	[DNA] ug/ul	CFPHV copy#
1	A		
2	A		
3	A	0.092	0
4	A	0.053	0
5	A	0.033	0
6	A	0.075	424.9
7	A	0.0271	938
8	A	0.0217	0
9	A	0.0273	0
10	A	0.0259	0
11	A	0.018	0
12	A	0.0162	0
13	A	0.0434	0
14	A	0.02756	0
15	A	0.0643	0
16	A	0.05548	0
17	A	0.05256	0
18	A	0.0709	0
19	A	0.0689	0
20	A	0.03097	0
21	A	0.0522	0
22	A	0.0209	0
23	A	0.0285	0
24	B	0.05426	0
25	B	0.0401	0
26	B	0.0643	0
27	B	0.0658	0
28	B	0.0508	0
29	B	0.05329	0
30	B	0.05305	0
31	B	0.0334	0
32	B	0.07597	206.6
33	B	0.0489	0
34	B	0.03109	0
35	B	0.032	0
36	B	0.0179	0
37	C	0.0307	0
38	C	0.0259	0

39	C	0.0418	1306.1
40	C	0.08195	0
41	C	0.0324	0
42	C	0.0306	305.7
43	C	0.0583	0
44	C	0.094	0
45	C	0.0938	0
46	C	0.0384	0
47	C	0.0323	0
48	C	0.0561	0
49	C	0.067	0
50	C	0.0502	0
51	C	0.0515	0
52	C	0.018	0
53	C	0.027	0
54	C	0.017	0
55	C	0.0649	0
56	C	0.0754	0
57	C	0.0538	0
58	C	0.0539	0
59	C	0.0638	0
60	C	0.0643	0
61	C	0.0313	0
62	C	0.052	0
63	C	0.0591	0
64	C	0.0179	0
65	C	0.0159	0
66	C	0.0163	0
67	C	0.0159	0
		n	65

Sample Type: Urine			
Turtle ID	Study Group	[DNA] ug/ul	CFPHV copy#
1	A	0.1702	7511.8
2	A	0.0893	480
3	A		
4	A		
5	A		
6	A		
7	A		
8	A		
9	A	0.1415	279.7

10	A		
11	A		
12	A		
13	A		
14	A		
15	A		
16	A	0.0937	0
17	A		
18	A		
19	A	0.1202	0
20	A		
21	A		
22	A		
23	A	0.1341	5656.9
24	B	0.1134	361.9
25	B		
26	B		
27	B		
28	B	0.1232	0
29	B		
30	B		
31	B		
32	B	0.116	0
33	B		
34	B		
35	B	0.1354	220.2
36	B	0.0865	1513.5
37	C		
38	C	0.1526	0
39	C		
40	C	0.1317	0
41	C		
42	C		
43	C	0.0818	0
44	C	0.1305	0
45	C		
46	C	0.1378	448.8
47	C	0.1201	0
48	C		
49	C		
50	C		
51	C	0.1121	0
52	C	0.0934	0

53	C		
54	C		
55	C		
56	C		
57	C		
58	C		
59	C		
60	C		
61	C		
62	C		
63	C		
64	C		
65	C		
66	C		
67	C	0.1341	0
		n	20

Sample Type: Cloacal swabs			
Turtle ID	Study Group	[DNA] ug/ul	CFPHV copy#
1	A		
2	A	0.202	1311.6
3	A	0.0276	12864.3
4	A	0.1354	0
5	A		
6	A	0.0248	0
7	A	0.0191	3536.4
8	A		
9	A	0.028	0
10	A		
11	A	0.0466	0
12	A		
13	A		
14	A	0.0547	149.4
15	A		
16	A	0.0254	0
17	A	0.0367	0
18	A	0.0734	0
19	A	0.0309	0
20	A	0.0479	0
21	A	0.019	142.69
22	A	0.0239	497
23	A	0.0241	0

24	B	0.0182	0
25	B	0.0418	0
26	B	0.0434	0
27	B	0.0418	0
28	B	0.0441	0
29	B	0.043	0
30	B	0.1137	0
31	B	0.1317	62.53
32	B	0.0968	65.51
33	B	0.0194	0
34	B	0.023	0
35	B	0.038	109
36	B	0.0175	0
37	C	0.0491	0
38	C	0.0564	0
39	C	0.0491	4439.4
40	C	0.0604	0
41	C	0.0364	0
42	C	0.0293	0
43	C	0.0318	0
44	C	0.128	89.78
45	C	0.0258	0
46	C	0.0282	0
47	C	0.0426	0
48	C	0.0427	0
49	C	0.0427	0
50	C	0.043	0
51	C	0.0434	0
52	C	0.0458	0
53	C	0.0467	0
54	C	0.028	3536.4
55	C	0.0187	0
56	C	0.0212	0
57	C	0.0224	0
58	C	0.0249	157.31
59	C	0.0239	0
60	C	0.064	0
61	C	0.0333	0
62	C	0.027	0
63	C	0.0322	0
64	C	0.0302	0
65	C	0.0339	0
66	C	0.0356	0

67	C	0.0751	0
		n	60

Sample Type: Oral swabs			
Turtle ID	Study Group	[DNA] ug/ul	CFPHV copy#
1	A	0.039	0
2	A	0.0579	0
3	A	0.0331	0
4	A	0.0205	0
5	A	0.0734	0
6	A	0.0235	0
7	A	0.0751	0
8	A		
9	A	0.0487	0
10	A		
11	A	0.0653	0
12	A		
13	A		
14	A	0.0344	0
15	A		
16	A	0.0503	0
17	A	0.0466	0
18	A	0.0546	0
19	A	0.0466	0
20	A	0.0527	0
21	A	0.0548	0
22	A		
23	A	0.0528	0
24	B		
25	B	0.0591	0
26	B		
27	B		
28	B		
29	B		
30	B		
31	B	0.0605	0
32	B		
33	B	0.0229	0
34	B	0.0223	0
35	B	0.0194	0
36	B	0.0236	0
37	C	0.0192	0

38	C	0.0195	0
39	C	0.0179	0
40	C	0.023	0
41	C	0.0201	0
42	C	0.03	0
43	C	0.0259	0
44	C	0.0278	0
45	C	0.0288	0
46	C	0.0353	0
47	C		
48	C	0.0366	0
49	C		
50	C		
51	C		
52	C		
53	C		
54	C	0.0293	0
55	C	0.0368	0
56	C	0.0282	0
57	C	0.0423	0
58	C	0.0282	0
59	C	0.0492	0
60	C	0.0319	0
61	C	0.0496	0
62	C	0.051	0
63	C	0.0377	0
64	C	0.0175	0
65	C	0.0244	0
66	C	0.0225	0
67	C	0.0318	0
		n	48

Sample Type: Feces			
Turtle ID	Study Group	[DNA] ug/ul	CFPHV copy#
1	A		
2	A		
3	A		
4	A		
5	A		
6	A		
7	A		
8	A		

9	A	0.018	0
10	A		
11	A	0.0555	0
12	A		
13	A		
14	A	0.0337	0
15	A		
16	A	0.01	0
17	A	0.0097	0
18	A	0.071	0
19	A	0.0187	0
20	A	0.0241	0
21	A	0.0191	0
22	A	0.068	0
23	A		
24	B		
25	B	0.0102	0
26	B		
27	B		
28	B		
29	B		
30	B		
31	B		
32	B	0.0223	0
33	B		
34	B		
35	B	0.0137	0
36	B		
37	C		
38	C		
39	C		
40	C		
41	C	0.0535	0
42	C	0.075	0
43	C	0.0137	0
44	C	0.019	0
45	C	0.0216	0
46	C		
47	C		
48	C	0.0109	0
49	C	0.0298	0
50	C		
51	C	0.0159	0

52	C	0.0122	0
53	C	0.0137	0
54	C	0.026	0
55	C	0.0126	0
56	C	0.066	0
57	C	0.06	0
58	C		
59	C	0.0398	0
60	C	0.0963	0
61	C	0.027	0
62	C	0.0222	0
63	C	0.0103	0
64	C	0.0152	0
65	C	0.06	0
66	C	0.0297	0
67	C		
		n	35

Table S2. qPCR data (viral DNA copy number per μg DNA) for *C. mydas* biological samples taken during necropsy from tumored turtles

Samples	Turtles				
	FP-1	FP-2	FP-3	FP-4	FP-5
Cutaneous tumor	7,859	84,873	47,403,325	3,868,368	74,472,352
Internal tumor	--	--	360	--	--
Non-tumored skin	0	864	0	0	0
Skeletal muscle	0	0	0	0	0
Heart	0	0	0	0	0
Liver	0	0	0	0	0
Lung	0	0	0	0	0
Kidney	4,181	782	18,080	855	0
Thymus	0	0	0	0	0
Adrenal gland	0	0	0	0	--
Small intestine	--	0	0	0	0
Urinary bladder	0	0	0	0	0
Brain	--	0	0	0	--
Brachial plexus	9,597	0	0	0	0
Sacral plexus	0	0	2,456	0	0
Spinal nerve(s)	--	0	0	0	--
Optic nerve(s)	3,666	0	0	0	--
Blood	0	0	5,533	0	--
Urine	124	--	13,481	--	--

Table S3. qPCR data (viral DNA copy number per µg DNA) for *C. mydas* biological samples taken during necropsy from non-tumored turtles

Samples	Turtles				
	Non-FP-1	Non-FP-2	Non-FP-3	Non-FP-4	Non-FP-5
Cutaneous tumor					
Internal tumor					
Non-tumored skin	0	0	0	0	0
Skeletal muscle	0	0	0	0	0
Heart	0	1,717	0	1,455	0
Liver	0	0	0	0	0
Lung	0	0	0	0	0
Kidney	0	0	9,645	0	0
Thymus	0	0	0	0	0
Adrenal gland	--	--	0	0	0
Small intestine	0	--	0	0	0
Urinary bladder	--	0	0	0	0
Brain	0	0	0	0	0
Brachial plexus	0	0	1,564	0	0
Sacral plexus	0	0	0	0	0
Spinal nerve(s)	--	0	0	0	0
Optic nerve(s)	0	60,495	0	0	0
Blood	0	0	0	1,071	0
Urine	--	--	--	--	--

Table S4. qPCR data (CFPHV UL30 copy number per cell) for *C. mydas* biological samples taken during necropsy from tumored turtles

Samples	Turtles				
	FP-1	FP-2	FP-3	FP-4	FP-5
Cutaneous tumor	0.039	0.424	237.017	19.342	372.362
Internal tumor	--	--	0.002	--	--
Non-tumored skin	0	0.004	0	0	0
Skeletal muscle	0	0	0	0	0
Heart	0	0	0	0	0
Liver	0	0	0	0	0
Lung	0	0	0	0	0
Kidney	0.021	0.004	0.09	0.004	0
Thymus	0	0	0	0	0
Adrenal gland	0	0	0	0	--
Small intestine	--	0	0	0	0
Urinary bladder	0	0	0	0	0
Brain	--	0	0	0	--
Brachial plexus	0.048	0	0	0	0
Sacral plexus	0	0	0.012	0	0
Spinal nerve(s)	--	0	0	0	--
Optic nerve(s)	0.018	0	0	0	--
Blood	0	0	0.028	0	--
Urine	0.001	--	0.007	--	--

Table S5. qPCR data (CFPHV UL30 copy number per cell) for *C. mydas* biological samples taken during necropsy from non-tumored turtles

Samples	Turtles				
	Non-FP-1	Non-FP-2	Non-FP-3	Non-FP-4	Non-FP-5
Cutaneous tumor					
Internal tumor					
Non-tumored skin	0	0	0	0	0
Skeletal muscle	0	0	0	0	0
Heart	0	0.009	0	0.007	0
Liver	0	0	0	0	0
Lung	0	0	0	0	0
Kidney	0	0	0.048	0	0
Thymus	0	0	0	0	0
Adrenal gland	--	--	0	0	0
Small intestine	0	--	0	0	0
Urinary bladder	--	0	0	0	0
Brain	0	0	0	0	0
Brachial plexus	0	0	0.008	0	0
Sacral plexus	0	0	0	0	0
Spinal nerve(s)	--	0	0	0	0
Optic nerve(s)	0	0.302	0	0	0
Blood	0	0	0	0.005	0
Urine	--	--	--	--	--

Figure S3. Example capillary (Sanger) sequencing chromatogram for a CFPHV UL30-positive amplicon isolated from a green turtle (FinchTV DNA trace viewer software, Geospiza, Inc.)