Vol. 37: 89–97, 1999 | DISEASES OF AQUATIC ORGANISMS Dis Aquat Org | Published July 30

Association of herpesvirus with fibropapillomatosis of the green turtle *Chelonia mydas* and the loggerhead turtle *Caretta caretta* in Florida

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ABSTRACT: Sea turtle fibropapillomatosis (FP) is a disease marked by proliferation of benign but debilitating cutaneous fibropapillomas and occasional visceral fibromas. Transmission experiments have implicated a chloroform-sensitive transforming agent present in filtered cell-free tumor homogenates in the etiology of FP. In this study, consensus primer PCR methodology was used to test the association of a chelonian herpesvirus with fibropapillomatosis. Fibropapilloma and skin samples were obtained from 17 green and 2 loggerhead turtles affected with FP stranded along the Florida coastline. Ninety-three cutaneous and visceral tumors from the 19 turtles, and 33 skin samples from 16 of the turtles, were tested. All turtles affected with FP had herpesvirus associated with their tumors as detected by PCR. Ninety-six percent (89/93) of the tumors, but only 9% (3/33) of the skin samples, from affected turtles contained detectable herpesvirus. The skin samples that contained herpesvirus were all within 2 cm of a fibropapilloma. Also, 1 of 11 scar tissue samples from sites where fibropapillomas had been removed 2 to 51 wk earlier from 5 green turtles contained detectable herpesvirus. None of 18 normal skin samples from 2 green and 2 loggerhead turtles stranded without FP contained herpesvirus. The data indicated that herpesvirus was detectable only within or close to tumors. To determine if the same virus infected both turtle species, partial nucleotide sequences of the herpesvirus DNA polymerase gene were determined from 6 loggerhead and 2 green turtle samples. The sequences predicted that herpesvirus of loggerhead turtles differed from those of green turtles by only 1 of 60 amino acids in the sequence examined, indicating that a chelonian herpesvirus exhibiting minor intratypic variation was the only herpesvirus present in tumors of both green and loggerhead turtles. The FP-associated herpesvirus resisted cultivation on chelonian cell lines which support the replication of other chelonian herpesviruses. These results lead to the conclusion that a chelonian herpesvirus is regularly associated with fibropapillomatosis and is not merely an incidental finding in affected turtles.

KEY WORDS: Sea turtles · *Chelonia mydas* · *Caretta caretta* · Fibropapillomatosis · Chelonian herpesvirus

INTRODUCTION

Fibropapillomatosis (FP) of sea turtles is a debilitating disease characterized by multiple benign cutaneous fibropapillomas and occasional visceral fibromas (Herbst 1994). Fibroepithelial tumors commonly found on the turtle's conjunctivae and skin obstruct vision, and interfere with feeding and locomotion, while visceral nodules can fatally disrupt normal organ function (Herbst 1994, Herbst et al. 1999). Monitoring of sea turtle populations prior to 1982 found little or no

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disease, but prevalences rose rapidly in the 1980s and have remained high (Herbst 1994). FP has seriously affected green turtle *Chelonia mydas* populations in Florida and Hawaii and has recently emerged as a threat to the loggerhead *Caretta caretta* in Florida Bay (B. Schroeder pers. comm.). In 1998, a survey of green turtles in the Indian River Lagoon of Florida showed a FP prevalence of 72.5% (121/167 turtles) (L. Ehrhart pers. comm.). In addition, the disease has been reported in olive ridley *Lepidochelys olivacea* populations in the Pacific (Herbst 1994), has reached epizootic proportions in the Caribbean (Herbst 1994, Williams et al. 1994), and has been reported as high as 92% in some sea turtle populations in Hawaii (Balazs et al. 1991).

The etiology of FP is unproven, but the disease has been experimentally induced in tumor-free turtles in captivity by inoculation of cell-free filtrates of tumor homogenates, consistent with a viral etiology (Herbst et al. 1995). The transforming agent is chloroform-sensitive, suggesting that, if it is a virus it is most likely enveloped (Herbst et al. 1996). Although papillomaviruses and retroviruses have been considered as possible etiologic agents of FP, a chelonian herpesvirus present in transforming tumor filtrates continues to be a leading candidate (Herbst et al. 1995, Quackenbush et al. 1998, Klein 1999). Papillomaviruses were absent from normal and affected tissues examined by electron microscopy, immunohistochemistry, nucleic acid probes, and polymerase chain reaction (PCR) (Jacobson et al. 1989, Lackovich et al. 1998a). Retroviral elements, in contrast, were nearly ubiquitous in both normal and affected tissues (Casey et al. 1997, Herniou et al. 1998). The presence of herpesvirus in natural and experimentally induced fibropapillomas has been well documented by using electron microscopy and immunohistochemistry (Jacobson et al. 1991, Herbst et al. 1995, 1998c), and PCR (Herbst et al. 1998a, Quackenbush et al. 1998, Garber et al. unpubl.). Consistent with a causal hypothesis, Quackenbush et al. (1998) detected herpesvirus in tumors of 18 FP-affected marine turtles, but not in 16 unaffected turtles. Fourteen of the turtles examined were from Hawaii and Costa Rica while only 4 were from Florida.

Koch's postulates for evaluating the causal relationship between an infectious agent and a clinical disease remain unfulfilled for herpesvirus and FP (Evans 1976). This is due to the fact that FP-associated herpesvirus has been refractory to passage in cultured cells, and because of difficulties in conducting experimental infection studies of fatal diseases in a legally protected endangered species. However, epidemiologic data may provide alternative evidence of causation. In this study, consensus primer PCR amplification (VanDevanter et al. 1996) was used to detect herpesvirus in fibropapillomas, FP-associated visceral fibromas, skin near tumors, and in scar tissue from tumor removal surgeries, and also to demonstrate the absence of herpesvirus from normal skin of unaffected green and loggerhead turtles. The amplified conserved region within the herpesviral DNA polymerase gene was molecularly cloned and sequenced to determine the identity of herpesvirus present in both turtle species. Propagation of the FP-associated herpesvirus was attempted by using chelonian cell lines proven competent to support the replication of other chelonian herpesviruses. The current study extends earlier observations (Herbst et al. 1998a, Lackovich et al. 1998b, Quackenbush et al. 1998) by applying PCR testing to tumor and tissue samples from a large number of Florida sea turtles. The results lead to the conclusion that the same chelonian herpesvirus is regularly associated with fibropapillomatosis in green and loggerhead turtles in Florida and is not merely an incidental finding in affected turtles (Rivers 1937).

MATERIALS AND METHODS

Tissue collection. Turtles stranded at different locations along the Florida coast were transferred to The Turtle Hospital, Marathon, Florida; Mote Marine Laboratory, Sarasota, Florida; or the University of Florida Wildlife and Zoological Medicine Service, Gainesville, Florida. Samples were collected from 19 green and 4 loggerhead male and female turtles that varied in straight carapace length (Table 1). Two green and 2 loggerhead turtles did not have FP. The other 19 turtles all varied in disease severity, with a wide range in tumor number, size, and location.

Sterile instruments were used to collect individual samples during fibropapilloma removal surgeries or necropsies. Necropsies were performed following procedures previously described (Campbell 1996). Each sample was placed in a separate sterile sampling bag on ice immediately after removal. All samples were frozen and stored at −80°C. Eighty-nine fibropapilloma samples were collected from 19 turtles with FP. Fibropapilloma samples were collected from the conjunctivae, axillary, dorsal and ventral body surfaces. These varied in gross appearance as described by Herbst (1994) and ranged in diameter from 0.5 cm (0.1 g) to 30 cm (1410 g). Four fibromas were collected from the ventral surface of the lungs and the mucosal surface of the esophagus of 1 green turtle. The lung fibromas were 1 cm (1.1 g) and 3 cm (2.9 g) in diameter. One tracheal fibroma was 1 cm in diameter and a smaller nodule measured 0.4 × 0.2 cm. Thirty-three skin samples were obtained from the ventral and dorsal surfaces of the flippers and necks of 16 turtles

Table 1. Stranded sea turtles tested for chelonian herpesvirus

Identity	Species	Stranding date	Stranding location in Florida (County/Habitat)	Straight length (cm)
W94-19	Loggerhead	22 Jul 1993	Monroe Co./Florida Bay	72.9
SSL-406	Green	29 Sep 1996	Monroe Co./Florida Bay	49.2
SSL-438	Green	12 Oct 1996	Martin Co./Indian River Lagoon	40.3
SSL-408	Green	17 Oct 1996	Palm Beach Co./Atlantic Coast	40.0
SSL-414	Green	15 Nov 1996	Monroe Co./Florida Bay	44.0
SSL-471	Green	30 Nov 1996	St. Lucie Co./Atlantic Coast	44.1
97-11	Loggerhead	23 Mar 1997	Monroe Co./Florida Bay	69.9
SSL-398	Green	12 Apr 1997	Brevard Co./Indian River Lagoon	49.8
ST#11	Green	15 Jun 1997	Monroe Co./Atlantic Coast	40.0
ST#13	Loggerhead	22 Jun 1997	Volusia Co./Atlantic Coast	55.4
97-44	Green	8 Aug 1997	Brevard Co./Indian River Lagoon	44.2
SSL-442	Green	14 Aug 1997	Monroe Co./Atlantic Coast	55.8
SSL-445	Green	15 Aug 1997	Dade Co./Atlantic Coast	43.8
SSL-446	Green	16 Aug 1997	Indian River Co./Indian River Lagoon	33.2
SSL-448	Green	1 Sep 1997	Monroe Co./Florida Bay	38.1
Skinny	Green	12 Sep 1997	Monroe Co./Florida Bay	38.3
Corgey	Green	14 Sep 1997	St. Lucie Co./Atlantic Coast	66.7
SSL-468	Green	5 Oct 1997	Monroe Co./Florida Bay	43.3
SSL-473	Green	29 Oct 1997	St. Lucie Co./Atlantic Coast	40.3
3376	Green	26 Nov 1997	Monroe Co./Florida Bay	41.8
NLP971230	Green	30 Dec 1997	Sarasota Co./Gulf coast	36.4
SSL-486	Green	15 Mar 1998	St.Lucie Co./Atlantic Coast	50.2
ST#17	Loggerhead	9 Apr 1998	Dade Co./Atlantic Coast	81.9

with FP. Skin was sampled at 1 cm intervals away from selected fibropapillomas on the dorsal-proximal surface of the left and right rear flippers of 2 turtles. Thirteen tumors from 5 green and 2 loggerhead turtles were dissected into surface and interior regions using individual sterile instruments. Each region was tested for herpesvirus by PCR.

Also, 11 scar tissue samples from sites where fibropapillomas had been removed from 5 green turtles on the ventral and dorsal surfaces of the left and right flippers and the neck were collected. Scar tissue sampling occurred from 14 to 359 d after surgery. Eighteen normal skin samples, which showed no evidence of pathology by gross or subsequent microscopic examinations, were obtained from the ventral and dorsal surfaces of the flippers and necks of 4 turtles without FP.

Histopathology. Individual fibropapillomas, visceral fibromas, scar tissue, and normal skin biopsies were fixed in neutral buffered 10% formalin, embedded in paraffin, sectioned at 5 µm and stained with hematoxylin and eosin. Selected samples were also stained with Giemsa and Grocott stains. Stained sections of tissue samples were examined by using light microscopy.

DNA isolation. Frozen tissue samples were minced individually on ice in a biosafety containment cabinet by using sterile instruments. Selected punch biopsies of non-verrucous fibropapillomas, which had an epithelium clearly demarcated from the fibroblasts that composed the homogenous interior of the tumors, were first dissected into separate surface and interior portions. Approximately 0.5 g of each minced sample was digested with 50 mg of trypsin in 500 µl of digest buffer (10 mM Tris-HCl pH 8.0, 0.4 M NaCl, 0.6% SDS, 50 mM EDTA) at 37°C until liquified. Nucleic acids were purified by using standard phenol:chloroform extraction and ethanol precipitation methods and resuspended in 200 µl of nucleic acid-free water. The total nucleic acid concentration was determined for each sample by using UV spectrophotometry. One to two million cells were extracted from cell cultures for testing.

Detection of herpesvirus by consensus primer PCR. Herpesvirus was detected by using sequential nested consensus-primer PCR (VanDevanter et al. 1996) to amplify an internal region of the herpesvirus DNA polymerase gene. The PCR was performed by using a Perkin Elmer Gene Amp 2400 thermal cycler. The first round of amplification included 100 ng sample template nucleic acid or 10 ng positive control template varicella-zoster virus DNA (VZV; Varivax, Merck). In some experiments, DNA from another green turtle herpesvirus, lung-eye-trachea (LET) disease-associated virus (Jacobson et al. 1986), was used as an additional control template. Nucleic acid-free water was the negative control. A 2 µl aliquot of that amplification mixture was subsequently used as template for the second round of amplification. The products were electrophoresed through a 1.5% agarose gel in 1× TBE buffer, stained with ethidium bromide, and photographed under shortwave UV illumination. Samples containing herpesvirus yielded a 224 bp amplified fragment, which matched the size of the product ob-

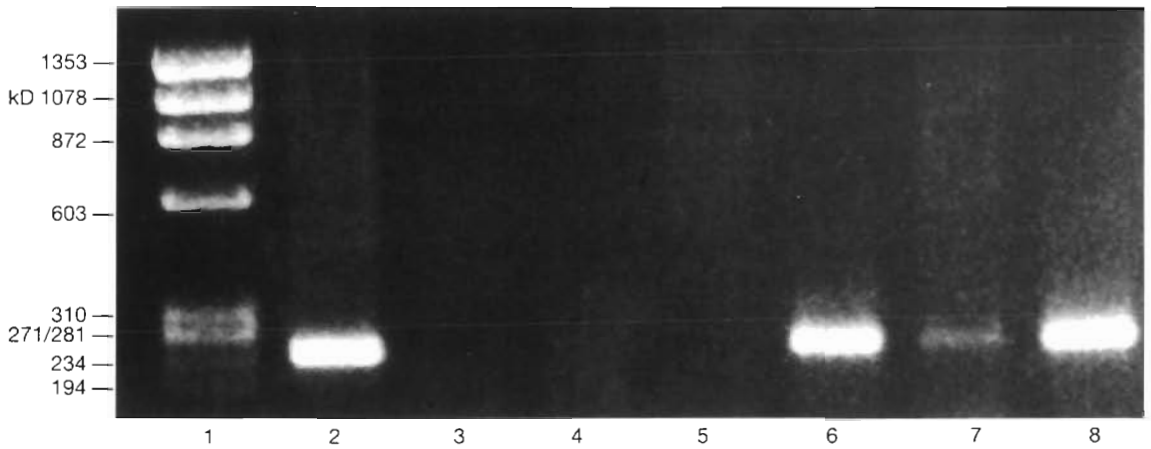

Fig. 1. Comparison of green and loggerhead turtle fibropapilloma, fibroma, and normal skin samples by PCR. Lane 1: PhiX174/Hae III DNA size markers. Lane 2: varicella-zoster virus (positive control). Lane 3: sterile water (negative control). Lane 4: ST#13 (loggerhead turtle). Normal skin from turtle without fibropapillomatosis (FP). Lane 5: SSL-414 (green turtle). Normal skin from turtle with FP. Lane 6: SSL-414 (green turtle). Fibropapilloma. Lane 7: SSL-406 (green turtle). Lung fibroma. Lane 8: 97-11 (loggerhead turtle). Fibropapilloma

tained by using the positive control template (Fig. 1). Serial dilution of VZV positive control template showed that under those conditions the assay had a limit of detection of about 1.1 herpesvirus genomic equivalents per 100 ng of sample nucleic acid.

Purification, cloning, and sequencing. The amplified segment of the herpesvirus DNA polymerase gene was analyzed to determine the identity of the FP-associated herpesvirus from both turtle species. The PCR amplification products from 6 tumors of a loggerhead turtle and 2 tumors from 2 green turtles were purified on QIAQUICK columns (Qiagen Inc., Chatsworth, CA, USA) and ligated to plasmid vector pCR2.1 (Invitrogen, Carlsbad, CA, USA) by using standard methods. Competent *E. coli* JM109 cells were transformed with recombinant plasmids by using standard heat-shock methods. Recombinant plasmid DNA from one clone of transformed cells from each of the 8 samples was prepared by standard methods (Promega Inc., Madison, WI, USA). The nucleotide sequences of both strands of each insert were determined by using standard M13 forward and reverse primers and automated dideoxy methods. The 8 sequences obtained were compared to each other and to herpesvirus sequences released to GenBank by using the Basic Local Alignment Search Tool of the National Center for Biotechnology Information.

Cultivation of the FP-associated herpesvirus. To propagate the FP-associated herpesvirus, unfiltered homogenates and cell-free filtrates (0.45 µm filter) were freshly prepared (Herbst et al. 1995) from PCR-positive fibropapillomas as a source of virus for inoculation of target cells. Twenty-five fibropapillomas and fibromas from green and loggerhead turtles were sampled. Homogenates and filtrates previously demonstrated competent to induce FP when inoculated into green turtles (Herbst et al. 1995, 1996) also were tested. In some experiments, cells freshly isolated from fibropapillomas, or passaged once *in vitro*, were co-cultivated with target cells. Target cells included a whole 14 d green turtle embryo line, a 33 d whole loggerhead turtle embryo line, a green turtle embryo kidney line (Moore et al. 1998), a whole 10 d gopher tortoise *Gopherus polyphemus* embryo line, and a terrapene heart (TH-1; ATCC No. CCL 50) line. Positive controls were 2 chelonian herpesviruses which replicated in all target cell lines: LET disease-associated herpesvirus (Jacobson et al. 1986) isolated from a green turtle, and HV4295 isolated from a Hermann's tortoise *Testudo hermanii* with vesicular stomatitis (Marschang et al. 1997). The culture medium was DME/F12 supplemented with 5% fetal bovine serum and antibiotics. Cells were cultured in standard T-25 plastic flasks with filter caps in a 5% CO_2 atmosphere at 20, 24, 28, or 32°C. Dilutions of homogenates and filtrates ranging from 1:5 to 1:20 were added to cell cultures which were 85 to 100% confluent. After 1 to 2 h, 9 ml of fresh medium was added and incubation continued for up to 21 d. For co-cultivation, 10^4 to 10^5 fibropapilloma cells were allowed to contact target cell monolayers by settling, and were not further disturbed by medium additions. Cultures were observed for cytopathic effects (CPE) which became evident in the LET and HV4295 infected controls 3 to 6 d after inoculation. Supernatants from inoculated cultures which did not show CPE were blind passaged 2 to 5 more times on fresh cells. Presence of herpesvirus was tracked by using PCR as described. Selected cultures were examined by electron microscopy.

RESULTS

General and histopathologic description of turtles and tissues

Eighteen of the 19 turtles with FP stranded in poor body condition, with fibropapillomas on both eyes and in the axillary and inguinal soft tissue adjacent to front and rear flippers. Their carapaces were covered with barnacles, leech eggs, and leeches. Randomly sampled barnacles and leeches tested by PCR were negative for herpesvirus. The 4 turtles without FP stranded with a similarly emaciated body condition.

The clinical diagnoses were confirmed histologically to meet the case definition of FP. All fibropapillomas showed papillary projections characterized by moderate to marked orthokeratotic hyperkeratosis, and mild hyperplasia of the stratified squamous epithelium. The stroma was composed of well-differentiated fibroblasts with compact bundles of collagen fibers. No inclusion bodies were seen in any tumor sections. Trematode eggs associated with inflammatory multinucleated giant cells were seen in less than 10% of the tumors tested.

The white nodules in the lungs of 1 turtle were not pediculated. The nodules were well-demarcated from adjacent normal tissue. The nodules were covered by a pseudostratified respiratory epithelium showing various degrees of epithelial hyperplasia. The nodules were composed of spindle-shaped fibroblast-like cells with abundant amounts of intervening collagen. Several trematode eggs were observed in the stroma of those fibromas. Two white oval-round nodules were detected and removed from the mucosal surface of the esophagus. The larger nodule was pediculated. The smaller nodule was not pediculated and infiltrated the mucosal surface.

Normal skin from both affected and unaffected turtles showed no histological evidence of papillary growth. Samples were marked by a dense stroma with numerous fibroblasts. No inclusion bodies were detected in normal skin.

Herpesvirus prevalence in fibropapillomas, fibromas, skin and scar tissue

All turtles affected with FP were positive for herpesvirus associated with their tumors as detected by PCR (Table 2). Herpesvirus was detected in 96% (89/93) of tumors tested. In cases where tumors were dissected into surface and interior regions, herpesvirus was detected in all regions in 12/13 tumors. However, in 1 tumor from loggerhead turtle W94-19 (Table 1), virus was detected only in the outermost (surface) region of the tumor. Four fibromas from the lungs and trachea of turtle SSL-406 were positive for herpesvirus. Only 3 of 33 skin samples from affected turtles contained detectable herpesvirus. The positive skin samples came from 3 different turtles, and all 3 samples were within 2 cm of a fibropapilloma. Only 1 of 11 scar tissue samples, obtained from sites where fibropapillomas had been removed surgically from 5 green turtles, was positive for herpesvirus (Table 3). Tumor re-growth was observed at multiple sites in 2 turtles (SSL-406 and SSL-471) while 3 turtles have remained tumor-free. Herpesvirus was absent from 18 normal skin samples from 2 green and 2 loggerhead turtles without FP. The findings lead to the conclusions that herpesvirus is regularly associated with fibropapillomatosis and is not merely an incidental finding in affected turtles.

Similarity of the FP-associated herpesvirus in tumors of green and loggerhead turtles from Florida

A 224 bp interior region of the herpesvirus DNA polymerase gene amplified from 6 tumors of a logger-

Table 2. Frequency of association of chelonian herpesvirus with individual fibropapillomas, fibromas, and normal skin samples of green and loggerhead sea turtles as tested by PCR

Turtle ID	Fibropapillomas (no. positive/total)	Fibromas (no. positive/total)	Normal skin (no. positive/total)
W94-19	(1/1)		No skin
SSL-406	(5/5)	(4/4)	(1/5)
SSL-438	(13/15)		(0/4)
SSL-408	(5/5)		(0/1)
SSL-414	(4/4)		(0/1)
SSL-471	(1/1)		(0/1)
97-11	(10/10)		(1/1)
SSL-398	(4/4)		(0/1)
ST#11	No tumor		(0/2)
ST#13	No tumor		(0/7)
97-44	(1/1)		(0/2)
SSL-442	(7/7)		(0/2)
SSL-445	(4/4)		(0/1)
SSL-446	(5/5)		(0/2)
SSL-448	(7/7)		(0/1)
Skinny	(3/5)		(1/5)
Corgey	No tumor		(0/4)
SSL-468	(5/5)		(0/2)
SSL-473	(7/7)		(0/3)
3376	(3/3)		No skin
NLP971230	(2/2)		(0/1)
SSL-486	(2/2)		No skin
ST#17	No tumor		(0/5)
Total positive	89/93	4/4	3/51
% positive	96%	100%	6%

Table 3. Detection by PCR of chelonian herpesvirus in scar tissue from sea turtle fibropapilloma surgical removal sites. +: chelonian herpesvirus detected in scar tissue; –: no chelonian herpesvirus detected in scar tissue

Identity	Fibropapilloma location	FP removal date/ scar sampling date	Time (d)	PCR result	Tumor re-growth (at scar site/other sites)
SSL-406	Front flipper	24 Oct 1996 / 8 Aug 1997	288	+	+/+
	Rear flipper	15 Mar 1997 / 8 Aug 1997	145	–	–/+
SSL-471	Rear flipper	19 Dec 1996 / 13 Dec 1997	359	–	–/+
	Rear flipper	19 Dec 1996 / 13 Dec 1997	359	–	–/+
SSL-398	Rear flipper	5 Jun 1997 / 13 Dec 1997	190	–	–/–
	Front flipper	31 Jul 1997 / 13 Dec 1997	135	–	–/–
SSL-442	Neck (top)	16 Oct 1997 / 30 Oct 1997	14	–	–/–
	Front flipper	16 Oct 1997 / 30 Oct 1997	14	–	–/–
	Front flipper	16 Oct 1997 / 30 Oct 1997	14	–	–/–
SSL-446	Neck (top)	30 Oct 1997 / 13 Dec 1997	14	–	–/–
	Neck (bottom)	30 Oct 1997 / 13 Dec 1997	14	–	–/–

head turtle and 2 tumors from 2 green turtles was sequenced to determine the similarity of herpesvirus present in both turtle species (Table 4). Alignments of the sequences revealed 95 to 99% nucleotide sequence similarities among the sequences derived from loggerhead and green turtles examined in this study and homologous herpesvirus sequences derived from FP-affected olive ridley, Hawaiian green, and Floridian green and loggerhead turtles by Quackenbush et al. (1998). The nucleotide sequences examined had only short motifs similar to herpesviruses from other hosts. Comparisons to all nucleotide sequences in GenBank showed that the closest overall similarities (51 to 52%) were to homologous sequences of bovine herpesviruses type 1 and 2.

The sequences obtained predicted that herpesvirus of loggerhead turtles differed from those of green turtles by only 1 of 60 amino acids in the sequence examined (Fig. 2), suggesting that the chelonian herpesvirus was the only herpesvirus present in the tissues examined. The predicted sequences also differed by only 1 of 60 amino acids at various positions in the homologous sequences derived from Hawaiian green turtles and Florida green and loggerhead turtles by Quackenbush et al. (1998), and only 2 of 60 amino acids in the homologous sequence Quackenbush et al. (1998) derived from olive ridley turtles. The conservative amino acid substitutions predicted suggested that the minor variation observed among the sequences simply reflected intratypic variants of a single FP-associated chelonian herpesvirus.

Table 4. Similarity (%) among herpesvirus DNA polymerase gene partial nucleotide sequences derived from green, loggerhead, and olive ridley turtles affected with fibropapillomatosis

	Sequences from this study	
	Loggerhead[a]	Green[b]
Florida loggerhead[a]	100	95
Florida green[b]	95	100
Olive ridley[c]	97	97
Hawaiian green[d]	95	99
Florida green and loggerhead[e]	95	99

GenBank Accession Numbers:
[a]Florida loggerhead turtle (this study) AF120208
[b]Florida green turtles (this study) AF120209
[c]Olive ridley turtle (Quackenbush et al. 1998) AF049904
[d]Hawaiian green turtle (Quackenbush et al. 1998) AF035003
[e]Floridian green and loggerhead turtles (Quackenbush et al. 1998) AF035004

Cultivation of FP-associated herpesvirus *in vitro*

More than 300 attempts to propagate the FP-associated herpesvirus were carried out under various conditions described. The typical CPE caused consistently by chelonian herpesviruses LET and HV4295 was never observed in cultures inoculated with tumor homogenates, tumor filtrates, or tumor cells containing the FP-associated herpesvirus. Tracking of infectivity by PCR showed that the FP-associated herpesvirus could not be detected for more than 1 to 2 passages in inoculated cultures. PCR confirmed that positive control cultures which exhibited CPE did contain herpesvirus. Electron microscopy did not detect virus in 24 cultures inoculated with FP-associated herpesvirus.

DISCUSSION

This study has demonstrated a high frequency of association of a chelonian herpesvirus with tumors of Florida green and loggerhead sea turtles afflicted with fibropapillomatosis. Herpesvirus could be detected in both surface and inner regions of individual tumors.

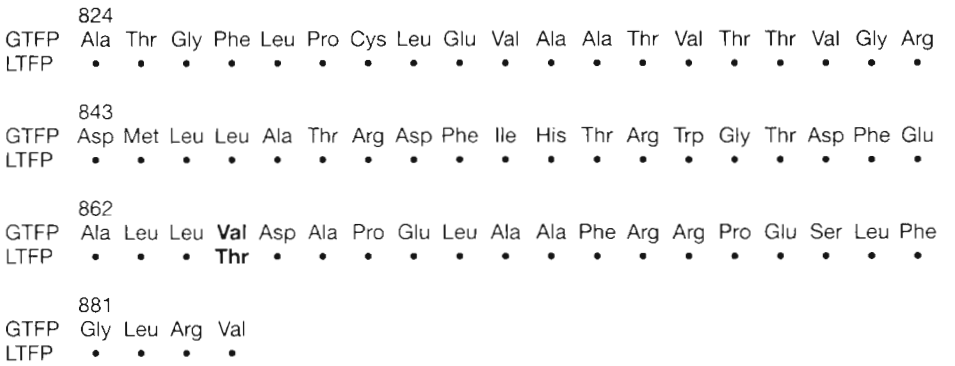

Fig. 2. Predicted partial herpesvirus DNA polymerase sequences from individual fibropapillomas of green and loggerhead turtles. Dots indicate identical amino acids

Normal skin samples from green and loggerhead turtles stranded without FP did not contain herpesvirus, but the virus could be detected in apparently normal skin within 2 cm of tumors in FP-affected turtles. These results confirm and extend earlier observations of the association of herpesvirus with this disease (Jacobson et al. 1991, Herbst et al. 1995, 1998a, 1999, Quackenbush et al. 1998), which supports a possible etiological role for this virus (Rivers 1937). However, while the presence of herpesvirus within or close to tumors suggests a potential role for this virus in the development of FP, the evidence presented to date is insufficient to prove causation.

The minor variation observed among chelonian herpesvirus polymerase gene partial sequences obtained in this study likely reflected intratypic variants of a single chelonian herpesvirus. Quackenbush et al. (1998) inferred the existence of multiple chelonian herpesvirus from variation among similar sequences amplified from green, loggerhead, and olive ridley turtles with FP. However, because the sources of the samples which exhibited any sequence divergence were geographically very distant, the 1 to 2% amino acid sequence divergence predicted from the short gene segment examined in that study may simply reflect intratypic variants of a single FP-associated chelonian herpesvirus. This chelonian virus has been characterized as a member of the alphaherpesvirinae subfamily (Herbst et al. 1998a, Quackenbush et al. 1998, Garber unpubl. 1999). Other chelonian herpesviruses which are presumptive agents of marine turtle diseases include gray patch disease (GPD) and LET disease viruses (Rebell et al. 1975, Jacobson et al. 1982, 1986). The relationship of these herpesviruses to the FP-associated herpesvirus is under investigation (Garber unpubl. 1999).

The persistence of this chelonian herpesvirus in scar tissue from FP-removal sites has not been previously investigated. The 11 scar tissue samples from the 5 green turtles were collected to learn whether virus could be detected in healed sites after fibropapilloma removal and whether tumor recurred at those sites. All individual fibropapillomas removed tested positive for herpesvirus while the resultant scar tissue tested 14 to 359 d after tumor removal surgery was virus negative in 10 of 11 samples from 5 turtles (Table 3). Three turtles, SSL-398, SSL-442, and SSL-446, have remained entirely tumor free for more than a year after FP-removal surgery (through December 1998) and one (SSL-446) has been recently released. However, despite numerous surgeries, tumors continued to recur in 2 turtles, SSL-406 and SSL-471, at external and internal sites resulting in these turtles having to be euthanatized. In turtle SSL-406, tumor recurred post surgery in the PCR positive scar tissue site as well as at numerous new sites both externally and internally. In turtle SSL-471, tumor did not recur at the PCR-negative scar tissue but did reappear at numerous new sites. This limited data shows that in some turtles surgical removal of fibropapillomas may potentially be effective in removing both virus and cutaneous tumor thus facilitating recovery from the disease. However, other turtles may not benefit from such treatment and these eventually succumb to recurring progressive FP. Turtles may not benefit if infected margins are left following surgery, if the virus has already spread systemically, or if new tissues have been independently infected. Understanding the basis for these different clinical outcomes would facilitate our understanding of FP pathogenesis and host resistance to FP (Herbst 1994, Herbst & Klein 1995, Herbst et al. 1998c, 1999).

Koch's postulates for evaluating the causal relationship between chelonian herpesvirus and FP remain unfulfilled due to the fact that FP-associated herpesvirus has been refractory to propagation in cultured cells, and because of difficulties in conducting experimental infection studies in a legally protected endangered species. However, epidemiologic data may provide some alternative evidence of causation. There are some parallels between FP and its associated herpesvirus and Kaposi's sarcoma (KS) in humans and its associated herpesvirus (KSHV, also called HHV-8). In both FP and KS, herpesvirus is not an incidental finding in affected individuals; the herpesvirus is rare in

unaffected individuals, but rarely absent from tumor tissues. Seroepidemiological studies have demonstrated a strong association of HHV-8 seropositivity and the development of KS (Kedes et al. 1996). Similarly, a strong association has been demonstrated between the antibody response to the FP-associated herpesvirus in green turtles and the development of clinical FP (Herbst et al. 1998c). In both diseases it is unclear whether tumor growth results entirely from the proliferation of virally transformed cells or results in part from the stimulation of cell growth by signals from nearby virus-infected cells (Herbst 1994, Gallo 1998, Herbst et al. 1999). Gallo (1998) has proposed that HHV-8 promotes hyperplasia by paracrine action of infected cells; many tumor cells are never infected with virus and true neoplastic cells are a minority in tumors. Cell lines derived from both KS and FP will grow into tumors in immunodeficient mice suggesting that some transformed cells exist in the original tumors (Gallo 1998, Herbst et al. 1998b).

FP may have a complex pathogenesis involving interactions among several viral and non-viral agents including environmental co-factors. (Herbst & Klein 1995, Herbst et al. 1999). Evaluation of the strength of this chelonian herpesvirus as a sole risk factor for FP awaits propagation of the virus, experimental inoculations with pure virus, and additional seroepidemiological studies. The results of this study and a similar (Quackenbush et al. 1998) case-control study establishes the rationale for such efforts. However, in this and other (D. Docherty, National Wildlife Health Center, pers. comm. 1998) studies to date, the virus has resisted propagation. Additional efforts are ongoing in different cell culture systems and may yet prove successful.

Acknowledgements. This research was supported by contract RWO 161 from NMFS, NOAA, U.S. Department of Commerce and RWO 180 from the U.S. Fish and Wildlife Service, Department of the Interior administered by the Cooperative Fish and Wildlife Unit, University of Florida, Gainesville, Florida.

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Editorial responsibility: Peernel Zwaart, Utrecht, The Netherlands

Submitted: February 2, 1999; Accepted: April 28, 1999
Proofs received from author(s): July 27, 1999