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GEOGRAPHIC VARIATION IN MARINE TURTLE FIBROPAPILLOMATOSIS

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Abstract: We document three examples of fibropapillomatosis by histology, quantitative polymerase chain reaction (qPCR), and sequence analysis from three different geographic areas. Tumors compatible in morphology with fibropapillomatosis were seen in green turtles from Puerto Rico and San Diego (California) and in a hybrid loggerhead/hawksbill turtle from Florida Bay (Florida). Tumors were confirmed as fibropapillomas on histology, although severity of disease varied between cases. Polymerase chain reaction (PCR) analyses revealed infection with the fibropapilloma-associated turtle herpesvirus (FPTHV) in all cases, albeit at highly variable copy numbers per cell. Alignment of a portion of the polymerase gene from each fibropapilloma-associated turtle herpesvirus isolate demonstrated geographic variation in sequence. These cases illustrate geographic variation in both the pathology and the virology of fibropapillomatosis.

Key words: *Chelonia mydas*, fibropapillomatosis, green turtle, herpesvirus.

BRIEF COMMUNICATION

Fibropapillomatosis (FP) is a neoplastic disease of marine turtles characterized by the presence of external and internal tumors.[3] Because FP has a high prevalence in immature animals, it may impact the long-term survival of these threatened and endangered species. An alphaherpesvirus fibropapilloma–associated turtle herpesvirus (FPTHV) has consistently been associated with tumors in three different species of marine turtles.[4,6,7] However, most cases of FP have historically been diagnosed based on gross morphology, with less effort spent on microscopic examination of tissues or confirming the presence of FPTHV using molecular assays. Here we apply gross examination, histology, and quantitative molecular techniques to cases of FP from three different geographic areas that illustrate the complex nature of the disease.

Turtles with FP from Puerto Rico came from Puerto Manglar, Culebra Island, and included one case sampled in October 2000 and five cases (including three that had been free of visible fibropapillomas 18 months previously) sampled in December 2001. One green turtle was captured in San Diego Bay in January 2001 with approximately 80 small tumors on its limbs, neck, and axial areas. A hybrid turtle with FP, suspected of being a cross between a hawksbill and loggerhead based on morphologic criteria, came from the Turtle Hospital (Marathon, Florida, USA). Tumors were sampled using standard 6 mm biopsy punches after surgical scrubbing of the biopsy site. For histology, tumors were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E). For polymerase chain reaction (PCR) analyses, tumors were stored frozen at −80°C and DNA prepared as described.[7] Briefly, tissues were homogenized in lysis buffer and DNA extracted according to manufacturer instructions (Qiagen Inc., Valencia, California 91355, USA). Samples were assayed for FPTHV polymerase (*pol*) DNA using PCR in the presence of each primer: GTHV1 (5′TGTCTGGAGGTGGCGGCC ACG3′) and GTHV2 (5′GACACGCAGGCCAAA AAGCGA3′) which generated a 163 bp amplicon. Quantitative PCR (qPCR) was employed to measure the copies of viral genome per cells.[6]

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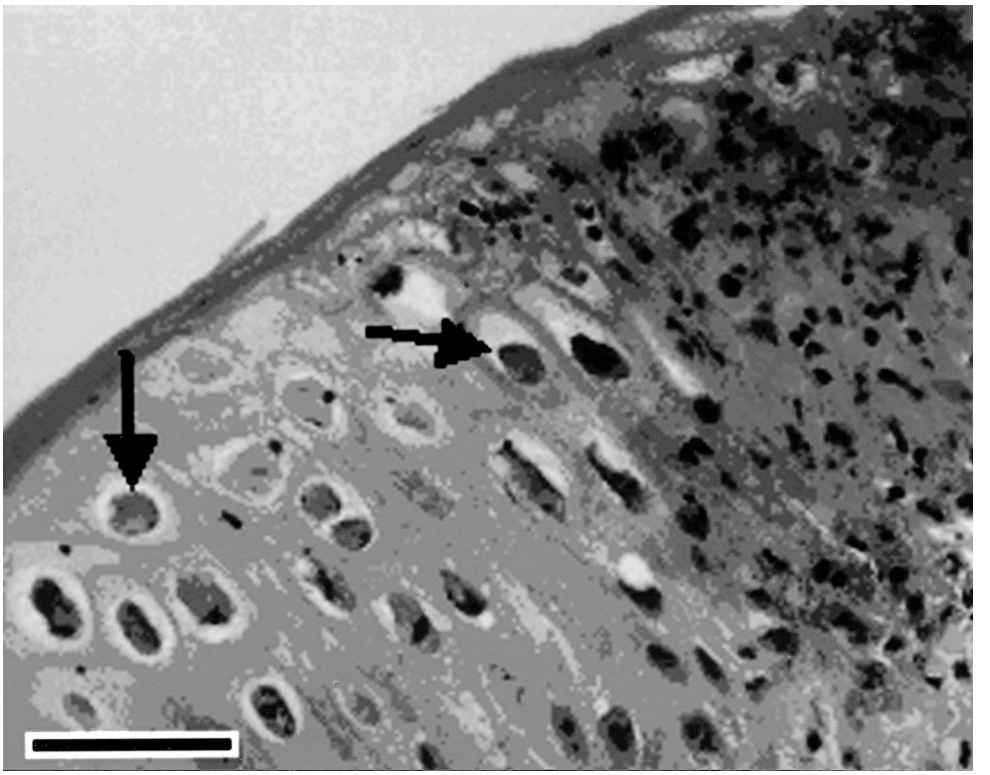

Figure 1. Microscopic appearance of a fibropapillomatosis (FP)–affected hybrid marine turtle captured in Florida. Note ballooning degeneration of the epidermis, nuclear swelling, intranuclear inclusions (arrow), and superficial to full thickness necrosis of the epidermis (right). Bar = 50 μm.

A portion of the FPTHV DNA polymerase gene (UL30, *pol*) from each FPTHV(+) tissue sample was cloned, sequenced, and compared with the homologous sequences prepared from the tumors of other FPTHV(+) turtles. The 482 bp PCR amplicon for sequencing was obtained with previously published primers DFA and GTHV2 using the original reaction conditions.[6] The partial *pol* sequence of the hybrid FPTHV has been submitted to GenBank (accession number AY395516). Previously submitted sequences used here for comparison are Australian green *pol* (AF299108), Australian loggerhead *pol* (AF299107), Barbados green *pol* (AF299110), Costa Rica olive ridley *pol* (AF049904), Florida green *pol* (AF035004), and Hawaii 7 green 2 *pol* (AF035003, bases 15840–16322), as well as Hawaii green 1 *pol* (AY390420), Puerto Rico green *pol* (AY390421), and San Diego green *pol* (AY390422).

Grossly, tumors in turtles from Puerto Rico were firm, rugose, pedunculated, and ranged in size from <1 cm to >5 cm diameter. Tumors from the San Diego turtle were numerous (>80), small (<3 cm), and sessile with a papillary to rugose surface. The hybrid turtle presented with multiple large (>5 cm) tumors on the shoulders, flippers, and neck. Microscopically, the tumors were characterized by prominent connective tissue matrix with proliferating fibroblasts overlaid by acanthotic orthokeratotic epidermis. Intracellular edema and focal necrosis were also observed. The San Diego turtle had prominent dermal perivascular mononuclear infiltrates, and there was poor distinction between the fibroblastic matrix and underlying collagen. Tumors from the hybrid turtle had ballooning degeneration of epidermis, nuclear swelling, and intranuclear inclusions (Fig. 1).

All six tumors from the Puerto Rico turtles were positive for FPTHV; however, tumor samples 2, 4, and 5 had very low viral DNA loads, and normal tissue was negative (Table 1). Tumors and normal skin from the San Diego turtles were positive for FPTHV with normal tissue having <1 copies per cell and tumors having 7–10 copies per cell (8 ± 2). All tumors from the hybrid turtle were FPTHV-positive with viral loads ranging from 40 to 713 copies per cell, the highest yet reported (178 ± 298; Table 1).

Table 1. Copies of fibropapilloma-associated turtle herpesvirus (FPTHV) *pol* DNA sequence in unaffected skin and tumors of a Florida Bay hybrid turtle, San Diego (SD) green turtle 990A, and Puerto Rico (PR) turtle ppm209/ppm211, as measured by qualitative polymerase chain reaction (qPCR). Copy numbers provided are the averages of two to four replicate samples that differed by <5%. For comparison, average numbers of copies in unaffected skin and fibropapillomas of turtles from various geographic regions are provided (asterisk: from [6]). TaqMan PCR performed as in [2].

Sample	Average copies (per 1,000 cells)
SD unaffected skin 1 of 2	5
SD unaffected skin 2 of 2	4
SD tumor 1 of 3	7,500
SD tumor 2 of 3	10,000
SD tumor 3 of 3	6,900
PR turtle 1, eye tumor	5,900
PR turtle 1, shoulder tumor	4,700
PR turtle 2, normal skin	0
PR turtle 2, tumor	26
PR turtle 3, tumor	12,000
PR turtle 4, tumor	30
PR turtle 5, tumor	60
Hybrid tumor 1 (right front flipper)	52,000
Hybrid tumor 2 (right shoulder)	44,000
Hybrid tumor 3 (right rear flipper base)	43,000
Hybrid tumor 4 (neck)	40,000
Hybrid tumor 5 (left shoulder)	713,000
Average reported skin*	30
Average reported tumor*	13,900

The predicted translation of partial *pol* sequences from the Puerto Rico, San Diego, and hybrid FPTHVs were aligned with homologous sequences from FPTHVs of eight other genetically and geographically diverse marine turtles (data not shown).[1] The alignment, taken in combination with more extensive surveys of FPTHV sequence diversity published elsewhere, underscores the idea that FPTHV sequences separate into geographic clades.[1]

FP has been documented in marine turtles from San Diego, Puerto Rico, and Florida; however, here we quantitate FPTHV infection in turtles from Puerto Rico and San Diego and in a hybrid loggerhead/hawksbill turtle.[3,5,9] FP in San Diego turtles was the mildest as exemplified by the small size of tumors and the fact that tumor size and number of tumors in these turtles has not changed appreciably for the past 10 years. In contrast, FP in the hybrid turtle followed the other extreme and was more similar to tumor score 3 (severe disease) turtles in Hawaii.[10] FP severity in turtles from Puerto Rico appeared to fall in between on severity with some turtles having large tumors and other small tumors. The pathology was reflected in viral loads per cell in tissues that were most variable in the hybrid and Puerto Rico turtles and highest in the hybrid turtle, perhaps suggesting active viral replication (Table 1). The latter was also suggested by the presence of intranuclear inclusions on histology (Fig. 1).

The variable manifestation of FP in these geographic areas could be due to environmental cofactors or differences in strains of virus.[2] Supporting the former hypothesis are reports that FP is more prevalent in habitats that are proximal to agricultural and urban development. Examples include the high prevalence of FP on the island of Oahu, Hawaii, and its absence on the west coast of the island of Hawaii.[11] Puerto Manglar (Culebra Island), the only site in Puerto Rico where FP has emerged, is near urban development, and the first cases of FP in San Diego Bay were among a group of turtles that customarily aggregated in warm effluent water from a power plant. Alternatively, differences in FP pathology may follow from variations among different FPTHV strains. For example, the tumorigenicity of Marek's disease herpesviruses of chickens depends upon the relative virulence of the infecting strain.[8] Separating the effects of environmental cofactors and FPTHV strain variation on FP severity will require extensive serologic monitoring and sequence characterization of newly isolated FPTHV strains.

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