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Molecular evolution of fibropapilloma-associated herpesviruses infecting juvenile green and loggerhead sea turtles

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ABSTRACT

Chelonid Alphaherpesvirus 5 (ChHV5) has long been associated with fibropapillomatosis (FP) tumor disease in marine turtles. Presenting primarily in juvenile animals, FP results in fibromas of the skin, connective tissue, and internal organs, which may indirectly affect fitness by obstructing normal turtle processes. ChHV5 is near-universally present in tumorous tissues taken from affected animals, often at very high concentrations. However, there is also considerable asymptomatic carriage amongst healthy marine turtles, suggesting that asymptomatic hosts play an important role in disease ecology. Currently, there is a paucity of studies investigating variation in viral genetics between diseased and asymptomatic hosts, which could potentially explain why only some ChHV5 infections lead to tumor formation. Here, we generated a database containing DNA from over 400 tissue samples taken from green and loggerhead marine turtles, including multiple tissue types, a twenty year time span, and both diseased and asymptomatic animals. We used two molecular detection techniques, quantitative (q)PCR and nested PCR, to characterize the presence and genetic lineage of ChHV5 in each sample. We found that nested PCR across multiple loci out-performed qPCR and is a more powerful technique for determining infection status. Phylogenetic reconstruction of three viral loci from all ChHV5-positive samples indicated widespread panmixia of viral lineages, with samples taken across decades, species, disease states, and tissues all falling within the same evolutionary lineages. Haplotype networks produced similar results in that viral haplotypes were shared across species, tissue types and disease states with no evidence that viral lineages associated significantly with disease dynamics. Additionally, tests of selection on viral gene trees indicated signals of selection dividing major clades, though this selection did not divide sample categories. Based on these data, neither the presence of ChHV5 infection nor neutral genetic divergence between viral lineages infecting a juvenile marine turtle is sufficient to explain the development of FP within an individual.

1. Introduction

Family Herpesviridae represents a diverse group of large-DNA icosahedral viruses with widespread host distribution throughout animal lineages, including avian, mammalian, and reptilian hosts (Davison et al., 2009). Extensive efforts to classify associations between herpesvirus and host have produced three broad groupings: alpha-, beta- and gamma-herpesvirus. Of herpesviruses, Alphaherpesviruses are the most specieous, widely distributed amongst host species (Davison, 2000), and tend to coevolve with their hosts (Severini et al., 2013; McGeoch et al., 2000). This coevolution consequently leads to specialization on hosts, resulting in lethal spillover events (Huff and Barry, 2003; Huemer et al., 2002), host-specific transmission routes (including sexual, mechanical, and vectored), and system-specific host symptoms (Whitley and Roizman, 2001a; Gershon et al., 2015). Of note, Alphaherpesviruses are frequently implicated in tumorigenesis of host tissues (Goldberg, 1981; Whitley and Roizman, 2001b). Herpesviruses, such as Epstein-Barr, frequently cause large chromosomal rearrangements when inserting into host genomes (Gerber et al., 1969), and other cellular mechanisms induced by herpesvirus infection can result in tumor formation (Cavallin et al., 2014). Herpesvirus-associated tumor formation is known in many disease systems, including reptilian hosts. Of the reptilian herpesviruses, Alphaherpesviruses in testudinids are phylogenetically basal (McGeoch et al., 2006). This old lineage of herpesvirus is diverse, with novel divergent strains found across numerous host species (Sim et al., 2015; Bicknese et al., 2010; Ossiboff et al., 2015): one of which is strongly associated with tumor formation.

Fibropapillomatosis (FP) is the only known example of a widespread herpsvirus-associated tumorigenic disease in a reptile. FP is a neoplastic tumor disease of marine turtles that presents as fibropapillomas of the

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epidermal tissues, and fibromas of the internal organs (Herbst, 1994). Most affected individuals are neritic (coastal) juveniles and subadults, with FP prevalence declining prior to sexual maturity (National Research Council, 1990). Tumors are benign, though biological processes such as digestion, locomotion, and vision may be seriously impaired via obstruction (Herbst, 1994; Brooks et al., 1994a). Severe cases of FP have additionally been linked to increased risk of bacteremia (Brooks et al., 1994b). While green sea turtles (*Chelonia mydas*) are most commonly affected by FP, all marine turtles are capable of contracting the disease (Herbst, 1994). Despite the conservation implications this disease might have for these endangered and threatened species, there are still many unresolved questions pertaining to the disease ecology of FP and its marine turtle hosts.

The putative etiological agent of FP is Chelonid Alphaherpesvirus 5 (ChHV5) (Quackenbush et al., 1998). A genome of 132,233 bp was sequenced and assembled, identifying ChHV5 as a novel genus within Alphaherpesvirus (Herbst et al., 2004), and a suite of molecular markers facilitated numerous phylogenetic analyses (Ene et al., 2005; Greenblatt et al., 2005; Quackenbush et al., 2001). Recently, efforts to culture ChHV5 were successful (Work et al., 2009). Though viral evolution rates, ancient origins of the virus, and co-divergence with host species were demonstrated (Gerber et al., 1969), ecological and genetic variables important for disease progression are still being studied. ChHV5 was initially implicated in FP via histological investigations (Jacobson et al., 1991). These results were further confirmed with the advent of sequencing technology, as fibromas are consistently found to harbor ChHV5 viral DNA (Quackenbush et al., 1998). However, transmission pathways for this virus are still largely unknown, with hypotheses ranging from mechanical, vertical, and leech vectored, though cell-free extract has proven infectious in cell cultures (Herbst et al., 1995).

Most investigations interested in sequence data from ChHV5 rely on samples sourced from tumors or the skin immediately adjacent to tumors (Ene et al., 2005; Patrício et al., 2012). The high concentration of viral DNA in these tissues simplifies the process of amplification, making them attractive targets. Conversely, investigations interested in presence or absence of ChHV5 in asymptomatic animals by necessity utilize non-tumorous tissues, where lower viral concentrations have required the use of more intensive amplification efforts via nested PCR to enhance the likelihood of detection (Herrera et al., 1998; Lu et al., 2000). Detection of ChHV5 in healthy turtles is often as low as 15%, though disentangling low asymptomatic carriage from low probability of detection (i.e., false negatives) remains challenging (Alfaro-Núñez et al., 2016). Due to the differing methods employed in ChHV5 sequencing and detection studies, there is a paucity of information pertaining to viral sequence variation in asymptomatic hosts, save for a few notable studies (Alfaro-Nunez et al., 2014; Page-Karjian et al., 2015). However, such investigations are necessary for understanding the relationship between viral infection and FP, as viral variation may explain why asymptomatic and tumorous hosts can both arise following ChHV5 infection.

Asymptomatic carriers of ChHV5, by definition, cannot be distinguished from non-carriers without molecular analyses and extensive sample collection, presenting a challenge for understanding viral dynamics outside of tumorous hosts. Additionally, asymptomatic tissue often produces inconsistent gene amplification results across viral loci, ostensibly due to low viral DNA concentrations (Page-Karjian et al., 2012). Intensive sampling efforts of many individual turtles are therefore necessary to ensure adequate numbers of sequences for downstream analyses. However, most ChHV5 sequencing studies sample only a few individuals due to challenges associated with sampling large numbers of sea turtles, and consequently do not obtain a large number of viral sequences (Stacy et al., 2008; Rodenbusch et al., 2012). Limitations on sample size is thus a major ongoing challenge for understanding ChHV5 epidemiology in juvenile sea turtles.

Here, we utilize a robust long-term dataset characterizing FP in juvenile marine turtles from 1983 to the present in a well-documented juvenile developmental habitat, the Indian River Lagoon adjacent to the central Atlantic coast of Florida, USA (Abreu-Grobois et al., 2000). This dataset, collected by the University of Central Florida Marine Turtle Research Group (UCF MTRG), represents one of the longest in-water studies for juvenile *C. mydas* and loggerhead (*Caretta caretta*) sea turtles in the US. An average of 145 *C. mydas* juveniles are captured annually, and approximately half of these turtles have visible tumors (Hirama and Ehrhart, 2007). We sampled normal skin, blood and tumors (when present) from asymptomatic and symptomatic *C. mydas* and *C. caretta* turtles over twenty years (1998–2017) to evaluate the relationship between FP dynamics and herpesvirus evolution within turtle tissues, including tissue from numerous asymptomatic individuals. Specifically, we sequenced a suite of three herpesvirus genes (Capsid Maturation Protease *cap*, DNA polymerase catalytic subunit *pol*, and glycoprotein b *glyb*) (Page-Karjian et al., 2012) and used phylogenetic and haplotype analysis to determine whether viral genetic lineages associate with species, tissue types, time periods, or development of FP. We quantified positive natural selection acting within each viral gene to determine if selection drives diversification of viral lineages across different classes of turtle hosts. Additionally, we used quantitative (q)PCR to compare herpesvirus infection intensity among tissues, species and asymptomatic compared to FP individuals. Finally, we compared the efficacy of PCR and qPCR approaches for detecting herpesviruses across marine turtle species and tissues, as well as among viral genetic lineages. Together these analyses provide comprehensive insight into ChHV5 strain relationships in marine turtles with and without FP, as well as a robust assessment of the efficiency of common surveillance and monitoring techniques.

2. Material and methods

2.1. Study site and sample collection

We collected tissue samples from all *C. mydas* and *C. caretta* captured during bi-monthly in-water sampling trips in the Indian River Lagoon (1998–2017). These species regularly occur sympatrically both in this site and throughout their respective ranges, and are known to hybridize (James et al., 2004). Our study site is located approximately 1–2 km south of the Sebastian Inlet within the Indian River Lagoon, Florida (27.8324°N; 80.4420°W). Turtles were captured by large-mesh tangle nets during bi-monthly sampling trips. Nets were set for approximately three hours per sampling session, and tended continuously by boat while soaking. Captured turtles were immediately brought onboard and transferred to a larger work-up boat. Turtles were tagged with flipper and PIT (Passive Integrated Transponder) tags, and standardized morphometrics and weights were collected from each turtle (Bjorndal et al., 1999). Any tumor-like growths indicative of FP were photographed and documented using a standardized scoring system (Balazs and Pooley, 1991): Category 0 – no symptoms, Category 1 – mildly afflicted, Category 2 – moderately afflicted, and Category 3 – severely afflicted. Category scores are subjectively determined by the size, number, and location of the tumors present on the turtles (Work and Balazs, 1999) and the majority of animals with FP in this study were Category 1. Blood was drawn from the dorsal cervical sinus of each turtle into evacuated blood collection tubes using antiseptic protocol and 20-gauge or 22-gauge needles depending on size of the turtle. We collected tissue biopsies following protected species permitted protocols. Each asymptomatic turtle (without FP) was sampled singularly. Any turtle with FP had an additional tissue biopsy taken directly from the most severe tumor observed in order to compare herpesvirus presence and quantity in tumorous versus non-tumorous tissue. The area biopsied was first scrubbed with an isopropyl alcohol swab. The skin biopsy was obtained using a 4-mm sterile biopsy punch. If needed, a coagulant powder was used to control bleeding after tissue sampling. Non-tumor skin samples were taken from the fleshy (non-scale) portion