Prevalence and Phylogeny of Herpesvirus Sequences from Normal and Fibropapilloma Tissues of Green and Loggerhead Turtles Sampled at Moreton Bay, Australia

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Fibropapillomatosis of sea turtles is a recently emerging disease and likely represents a new viral epizootic (Balazs, 1991; Casey *et al.* 1997; Herbst, 1994; Quackenbush *et al.*, 1998). We have identified turtle herpesvirus (THV) DNA sequences that are associated with fibropapillomas from green (*Chelonia mydas*), loggerhead (*Caretta caretta*) and olive ridley (*Lepidochelys olivacea*) turtles from Hawaii, Florida, and Costa Rica, respectively (Quackenbush *et al.*, 1998). We now report the nucleic acid analysis of fibropapillomas and normal tissues from green and loggerhead turtles sampled in June 1998 at Moreton Bay, Australia. There is a strong correlation (93%) of the turtle herpesvirus DNA polymerase gene with fibropapillomatosis as measured by PCR. DNA sequence analysis has allowed for the phylogenetic positioning of these geographically distinct viral isolates and further confirms herpesviruses as etiologic agents in the genesis of fibropapillomatosis.

Methods

Consensus sequence PCR amplification and cloning of amplicons: Biopsies of tumors and normal skin were collected from green and loggerhead turtles captured from Moreton Bay, Australia (Aguirre *et al.*, this volume). Tissues were homogenized and DNA prepared as previously described. One mg of DNA isolated from various tissues was subjected to PCR amplification with turtle-specific herpesvirus primers (GTHV1: 5' TGTCTGGAGGTGGCGGCCACG 3', GTHV2: 5' GACACGCAGGCC AAAAAGCGA 3', for cloning GTHV2 and GTHV3: 5' AGCATCATCCAGGCCCACAA 3' were used). The PCR mixture consisted of 20 mM Tris-HCl (pH 8.3), 2 mM MgCl2, 50 mM KCl, 2.5% DMSO, 200 mM of each dNTP, 10 pmol of each primer, and 2.5 units of Taq DNA polymerase (Gibco BRL, Gaithersburg, Maryland). All samples were denatured at 94°C for 5 min and then amplified for 30 s at 94°C, 30 s at 62°C, 30 s at 72°C for 35 cycles followed by 5 min at 72°C for one cycle. Fifteen ml of each PCR amplification reaction was separated on a 2% agarose gel. PCR products were gel purified with a Qiaex II gel extraction kit (Qiagen, Inc., Chatsworth, Calif.) according to the manufacturer's instructions and cloned into pCR2.1 TOPO (Invitrogen, Carlsbad, Calif.). Automated sequencing was done with an ABI 373A automated sequencer (Applied Biosystems, Inc. Foster City, Calif.) at the Biotechnology Resource Center at Cornell University.

Results

Detection of herpesvirus pol sequences in green and loggerhead turtles: To determine if fibropapillomas from Australian green and loggerhead turtles contain a herpesvirus, DNA was extracted and subjected to PCR using the specific GTHV primers. A total of fifteen tumors were assayed from 13 green and 2 loggerhead turtles. Similarly, uninvolved skin from 13 tumor bearing and 27 non-tumor bearing turtles were also assayed. The results are presented in **Table 1**.

Table 1. Detection of turtle herpesvirus sequences in Australian green and loggerhead turtles.

Green turtles	N	No. positive (%)
Fibropapillomas	13	12 (92)
Skin from tumor positive turtles	11	5 (45)
Skin from tumor free turtles	14	3 (21)
Loggerhead turtles		
Fibropapillomas	2	2 (100)
Skin from tumor positive turtles	2	0 (0)
Skin from tumor free turtles	13	2 (15)

There is a high prevalence of herpesvirus associated with fibropapillomatosis in both the Australian green (92%) and the loggerhead (100%) turtles. Histologically normal skin samples from fibropapillomatosis positive green turtles also shows a significant level of herpesvirus positivity (45%). There were not enough loggerhead skin samples from fibropapillomatosis positive turtles for a valid comparison in this category. However, some skin samples from fibropapillomatosis free green and loggerhead turtles at Moreton Bay were also herpesvirus infected, 21% and 15% respectively.

Cloning and sequence analysis of green and loggerhead pol amplicons: To further evaluate the specificity of the PCR positive signals, especially with fibropapilloma samples, amplicons from both green and loggerhead tumors were cloned and sequenced. The amino acid sequence derived from the 483 bp fragments of the two herpesvirus DNA polymerase genes was aligned with the turtle herpesvirus sequences from the Hawaiian green, Florida green, Florida loggerhead, and Costa Rican olive ridley samples. The Australian green has a unique amino acid substitution of a (L) leucine to (S) serine but is

otherwise identical to the Hawaiian green. Similarly, the Australian loggerhead is identical within this region to the Hawaiian green. Interestingly, as previously reported the Florida green and Florida loggerhead turtles were also shown to be identical within this region (Quackenbush *et al.*, 1998).

Conclusion

In this study we used a PCR assay with turtle specific primers, designed to amplify a region of the DNA polymerase gene of herpesviruses that contains highly conserved amino acid motifs, to identify herpesvirus sequences that are etiologically associated with fibropapillomatosis of Australian green and loggerhead turtles. To date, turtle herpesvirus sequences are detected in green, loggerhead and olive ridley fibropapillomas, suggesting the presence of newly identified turtle herpesviruses as the causative agents of disease. We now extend these data to include a geographically unique population of turtles.

We conclude that:

Turtle herpesviruses are etiologically associated with fibropapillomatosis of green and loggerhead turtles at Moreton Bay, Australia.

PCR amplification, cloning and sequence analysis show that the Australian turtle herpesvirus are most similar to the Hawaiian turtle herpesvirus.

Literature Cited

Aguirre, A.A., C.J. Limpus, T.R. Spraker, and G.H. Balazs. This volume, oral presention. Survey of fibropapillomatosis and other potential diseases in marine turtles from Moreton Bay, Queensland, Australia.

Balazs, G.H. 1991. Current status of fibropapillomas in the Hawaiian green turtle, *Chelonia mydas*. pp. 47-57. *In* G. H. Balazs and S. G. Pooley (eds.), *Research plan for marine turtle fibropapilloma*. NOAA Tech. Memo. NMFS-SWFSC-156.

Casey, R.N., S.L. Quackenbush, T.M. Work, G.H. Balazs, P.R. Bowser, and J.W. Casey. 1997. Evidence for retrovirus infections in green turtles *Chelonia mydas* from the Hawaiian Islands. Dis. Aquat. Org. 31:1-7.

Herbst, L.H. 1994. Fibropapillomatosis of marine turtles. Ann. Rev. Fish Dis. 4:389-425.

Quackenbush, S.L., T.M. Work, G.H. Balazs, R.N. Casey, J. Rovnak, A. Chaves, L. duToit, J.D. Baines, C.R. Parrish, P.R. Bowser, and J.W. Casey. 1998. Three closely related herpesviruses are associated with fibropapillomatosis in marine turtles. Virology 246: 392-399.

Quantification of Tumor Severity and Hematology in Green Turtles Afflicted with Fibropapillomatosis in the Hawaiian Islands

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In this study hematologic status was related to the severity of tumor affliction in green turtles (*Chelonia mydas*) with fibropapillomatosis (FP). In July 1997 and July 1998, 108 free-ranging green turtles were captured and bled at Palaau, a coastal foraging area on the island of Molokai where FP is endemic (Balazs *et al.*, 1998). Blood was analyzed for hematocrit, estimated total solids, total white count and differential (Work *et al.*, 1998). Based on earlier work (Balazs, 1991), each turtle was assigned a subjective tumor score indicating the severity of FP as follows: TS0 - no visible external tumors, TS1 - lightly tumored, TS2 - moderately tumored, and TS3 - heavily tumored. Individual tumors were graded into four size classes (A=<1cm, B=1-4cm, C=>4-10cm, D=>10cm) and tumor scores were assigned based on the number of tumors in each class.

There was a progressive increase in monocytes and a decrease in all other hematologic parameters except heterophils and total white counts as tumor score increased. These data indicate that tumor score can relate to physiologic status of green turtles afflicted with FP and is a useful field tool for monitoring and reporting the severity of the disease in this species.

Literature Cited

Balazs, G. H. 1991. Current status of fibropapillomatosis in the Hawaiian green turtle, *Chelonia mydas*. *In* Balazs, G.H. and S. Pooley (eds.), *Research plan for marine turtle fibropapilloma*, p. 47-57. Tech. Memo. NMFS-SWFC-156.

Balazs, G.H., W. Puleloa, E. Medeiros, S.K.K. Murakawa and D.M. Ellis. 1998. Growth rates and incidence of fibropapillomatosis in Hawaiian green turtles utilizing coastal foraging pastures at Palaau, Molokai. *In* Proc. 17th Ann. Symp. Sea Turtle Biol. Conserv. NOAA Tech. Memo. NMFS-SEFSC-415:130-132.

Work, T.M., R.E. Raskin, G.H. Balazs, and S. Whittaker. 1998. Morphological and cytochemical characteristics of blood cells from the green turtle, *Chelonia mydas*, in the Hawaiian Islands. Amer. J. Vet. Research 59:1252-1257.

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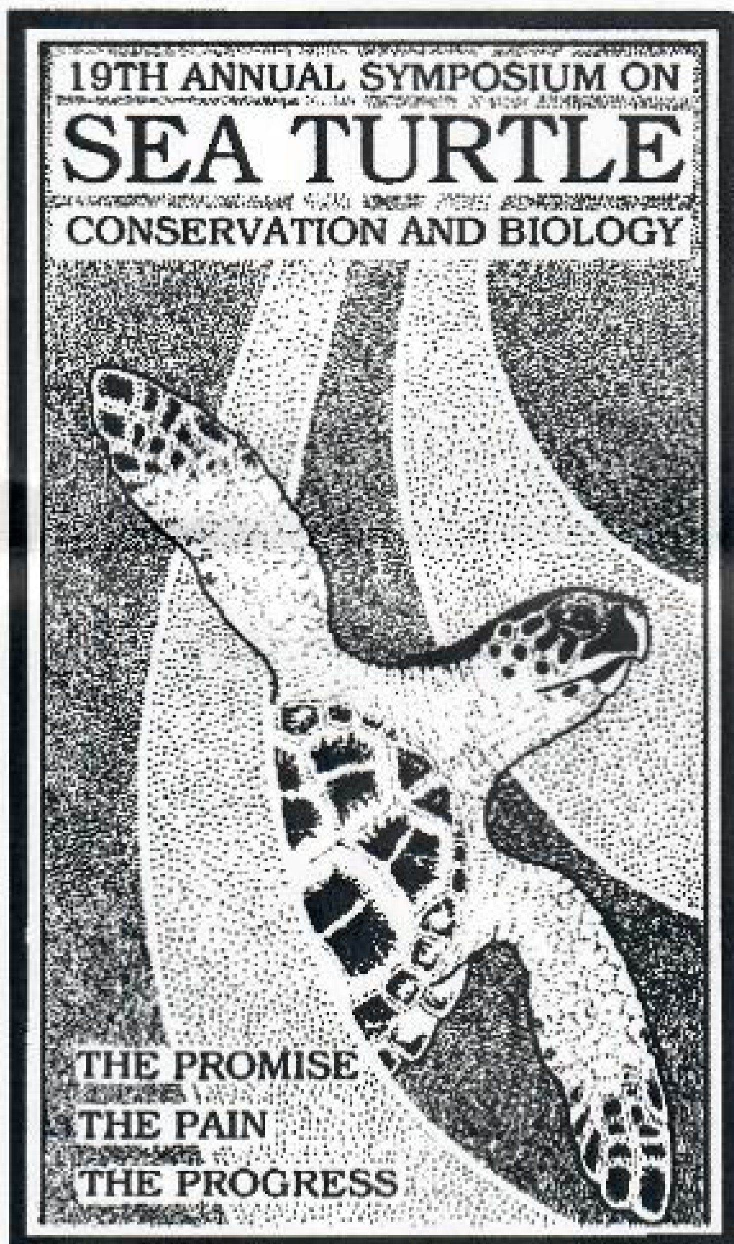

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