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Orogenital Neoplasia in Atlantic Bottlenose Dolphins (*Tursiops truncatus*)

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Abstract

This study describes lingual papillomas and squamous cell carcinomas (n = 11) and genital papillomas (n = 4) in Atlantic bottlenose dolphins (*Tursiops truncatus*) evaluated from January 2000 to January 2005. Tumors were found primarily in adult dolphins of both sexes living in free-ranging and captive conditions. Three dolphins had multiple lingual tumors of mixed histological type, consisting of papillomas and squamous cell carcinomas, suggesting malignant transformation of the benign papillomatous lesions. To our knowledge, this is the first report of oral papillomas in bottlenose dolphins and concurrent oral neoplasia that included both sessile papilloma and squamous cell carcinoma in the same dolphin. Additionally, it is the first known report of genital papillomas in free-ranging bottlenose dolphins from Atlantic coastal waters. The unusually high occurrence of related benign and malignant orogenital epithelial neoplastic lesions in a short period suggests that the lesions may represent one or more emerging diseases. Preliminary evidence suggests that these tumors may be of infectious etiology, possibly having an orogenital route of transmission.

Key Words: Atlantic bottlenose dolphin, *Tursiops truncatus*, pathology, neoplasia, papilloma, squamous cell carcinoma, emerging disease, virus

Introduction

In 1987, a comprehensive review reported only 41 confirmed tumors in cetaceans (Geraci et al., 1987). The majority of tumors represented benign mesenchymal and epithelial neoplasms; the organ systems most commonly involved were the gastrointestinal tract, skin, and internal female reproductive tract. Since 1987, reports of neoplasia, particularly malignant neoplasia, have increased in certain marine mammal species (Gulland et al., 2001). This increasing occurrence may represent an emerging disease phenomenon or be the result of increased surveillance by veterinarians, pathologists, and others who deal with marine mammal strandings, free-ranging animal health assessment studies, and captive animal health care.

In this report, we describe lingual papillomas and squamous cell carcinomas in 11 Atlantic bottlenose dolphins (*Tursiops truncatus*) in addition to genital papillomas in four bottlenose dolphins evaluated from January 2000 to January 2005. Tumors were present in both free-ranging and captive animals. Three dolphins had multiple tumors of mixed histological type, consisting of papillomas and squamous cell carcinomas, suggesting malignant transformation of the benign papillomatous lesions. Pathological evidence suggests that these tumors may be of viral etiology, possibly transmitted by the orogenital route.

Materials and Methods

Biopsies of oral and genital lesions from adult dolphins were obtained from captive ($n = 9$) and free-ranging Atlantic bottlenose dolphins ($n = 6$), the latter as part of a dolphin health assessment study conducted in the Indian River Lagoon, Florida, USA, and the coastal waters of Charleston, South Carolina, USA (Table 1). For this study, adults were defined as dolphins ≥ 6 years. Two dolphins < 6 years of age were designated as subadults. Ages were estimated in captive dolphins by length of time in captivity, and in free-ranging dolphins by examination of dental enamel layers after cross sectioning (Hohn et al., 1989).

Incisional biopsies were obtained aseptically from all oral and genital lesions following local anesthesia by infiltration of 2% lidocaine hydrochloride. Biopsies were from the tongue, frenulum, or penile/vulvar mucosa in 12 dolphins. Three dolphins (Cases 9, 10, and 13) had two grossly distinct lesions from the tongue (see Table 1). Cases 5, 6, and 14 had lung, kidney, liver, heart, spleen, gastrointestinal tract, brain, diaphragm, urinary bladder, reproductive tract, pleura, adrenal gland, and multiple lymph nodes sampled at necropsy. Tissues were placed in 10% neutral buffered formalin, routinely processed, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin for examination by light microscopy. Special stains (Gomori's methenamine silver, Brown and Brenn) were used as indicated.

Formalin-fixed portions of several lesions (from Cases 1-6, 9-11, and 13-14) were examined by transmission electron microscopy (TEM) using standard embedding procedures. One-micron sections, stained with toluidine blue, were examined by light microscopy to select sites for electron microscopy evaluation. Ultrathin sections were obtained with an ultramicrotome, stained with uranyl acetate and lead citrate, and subsequently examined as previously described (Bossart et al., 1996). Cases 7, 8, 12, and 15 were not evaluated by TEM due to inadequate sample size.

For immunohistochemistry, additional 4-mm paraffin sections (from Cases 1-7, 9-11, and 13-14) were stained with a commercially available polyclonal rabbit antibody developed against denatured bovine papillomavirus type 1 (BPV-1), bovine herpes virus-1 (BHV-1), and human herpesvirus simplex-1 (HSV-1) (DAKO Corp., Carpenteria, CA) using a standard avidin-biotin-peroxidase staining technique as previously described (Bossart et al., 2002). Positive controls included bovine and human tissues containing specific BPV-1, BHV-1, and HSV-1 antigens. Negative controls consisted of omitting the primary antibody and substituting it with an unrelated primary antibody. BPV-1 is the prototype papillomavirus (PV) and has at least 17 distinct linear epitopes that are conserved to various degrees among mammalian and avian papillomaviruses (Jenson et al., 1991). The BPV-1 immunohistochemical technique is used routinely to screen animal tissues for evidence of productive PV infections (Jenson & Lancaster, 1991). Immunological cross-reactivity among cetacean herpesviruses, BHV-1, and HSV-1 has been reported and used as an immunohistochemical screening technique for the presence of herpesviruses in cetaceans (Kennedy et al., 1992).

Table 1. Cases of orogenital neoplasia in 15 bottlenose dolphins

Case	Age	Sex	Status	Site	Diagnoses
1	Adult	F	FR	Tongue	Sessile papilloma
2	Subadult	F	FR	Vulva	Sessile papilloma
3	Adult	M	FR	Tongue	Sessile papilloma
4	Adult	M	FR	Genital mucosa	Sessile papilloma
5	Adult	F	C	Tongue	Squamous cell carcinoma
6	Adult	F	C	Tongue	Squamous cell carcinoma
7	Adult	F	FR	Vulva	Sessile papilloma
8	Adult	M	C	Penis	Sessile papilloma
9	Adult	M	C	Tongue	Squamous cell carcinoma, Sessile papilloma
10	Adult	M	C	Tongue	Squamous cell carcinoma, Sessile papilloma
11	Adult	M	C	Tongue	Sessile papilloma
12	Adult	F	C	Tongue	Squamous cell carcinoma
13	Adult	F	C	Tongue	Squamous cell carcinoma, Sessile papilloma
14	Adult	F	C	Tongue	Squamous cell carcinoma
15	Subadult	M	FR	Tongue	Sessile papilloma

FR = free-ranging; C = captive

Results

The age, sex, vital status, tumor site, and histopathologic diagnosis of the dolphins are summarized in Table 1. Tumors were found primarily in male and female adults living in free-ranging and captive conditions. Oral tumors occurred as both singular (Cases 1, 3, 14, and 15) and multiple (Cases 5, 6, 9, and 10-13) lesions located on the anteriodorsal aspect of the tongue (Cases 1, 3, 5, 6, 9-12, 13, and 15) and/or under the tongue near the base of the frenulum (Cases 5, 6, 9, 10, and 14). Genital lesions were found on the penis or external male genital mucosa (Cases 4 and 8) or on the external female genital mucosa (vulva) beneath the genital slit (Cases 2 and 7).

Figure 1. Oral lesion from the anteriodorsal tongue of a dolphin (Case 3); the lesion is multifocal, raised, and white, with a fissured, non-ulcerative mucosa (Type 1 lesion).

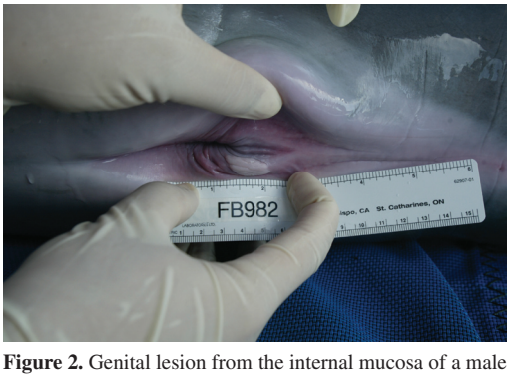

Figure 2. Genital lesion from the internal mucosa of a male dolphin (Case 4); the lesion is focal, irregular, slightly raised, and white, with a mildly fissured, non-ulcerative mucosa (Type 1 lesion). Note the similar appearance to Figure 1.

Grossly, orogenital lesions were composed of two distinct types. Type 1 anteriodorsal tongue (Figure 1) and genital (Figure 2) lesions were focal to multifocal, irregular to circular, raised, soft, light pink to white, and sessile, ranging from 0.5 to 2 cm in diameter (Cases 1-4, 7-8, 11, and

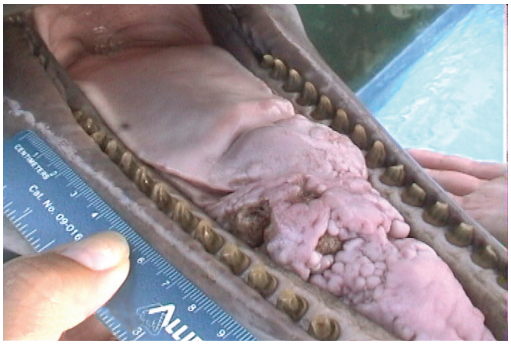

Figure 3. Tongue lesions from a dolphin (Case 5); lesions are multifocal, raised, pink, and coalescing nodules, which are sometimes ulcerated (Type 2 lesion).

15). The surface of these lesions appeared fissured or velvety and non-ulcerative. Type 2 lesions were found on the surface or beneath the tongue (Figure 3) and were focal to multifocal, raised, firm, pink, sometimes ulcerated and coalescing nodules, with irregular and thickened erythematous borders measuring from 3 to 7 cm in diameter (Cases 5-6, 12, and 14). Three dolphins (Cases 9, 10, and 13) had multiple lingual lesions involving both Type 1 and Type 2 forms.

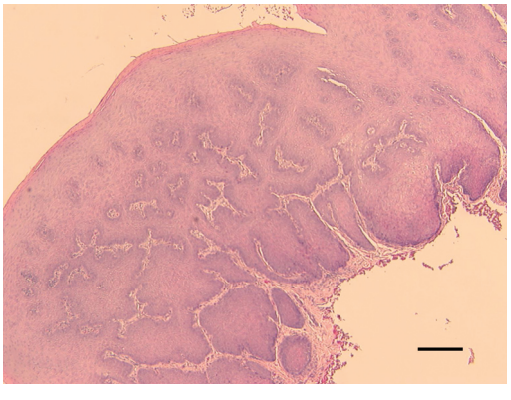

Figure 4. Low power photomicrograph of the center of the oral lesion from Figure 1; sessile plaques composed of proliferating keratinocytes with elongation of dermal papillae characterize the lesion, which is consistent with a sessile papilloma (Type 1 lesion). H&E stain; bar = 200 microns.

Microscopically, Type 1 orogenital lesions were diagnosed as sessile papillomas characterized by focal sessile plaques composed of uniformly proliferating keratinocytes and occasionally dysplastic keratinocytes with elongation of the dermal papillae (Figures 4 & 5). Rarely, keratinocytes contained vacuolated cytoplasm and pleomorphic, round, vesicular nuclei that were centrally or eccentrically located. These cells had features

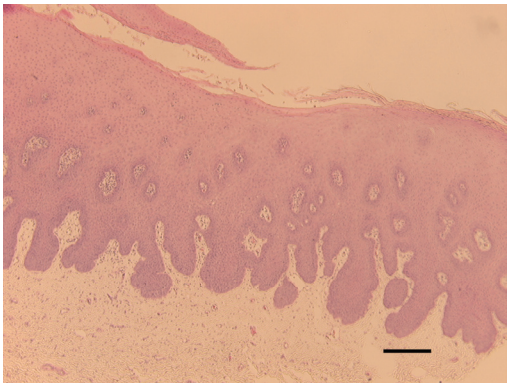

Figure 5. Low power photomicrograph of the center of the genital lesion from Figure 2; lesion is composed of proliferating keratinocytes with microscopic similarities to Figure 4 (Type 1 lesion). H&E stain; bar = 200 microns.

similar to koilocytes described in other species that are exhibiting the cytopathic effects of papillomavirus infection (Bossart et al., 1996, 2002). Inclusion bodies were not observed. Special stains to exhibit bacteria and fungi were negative.

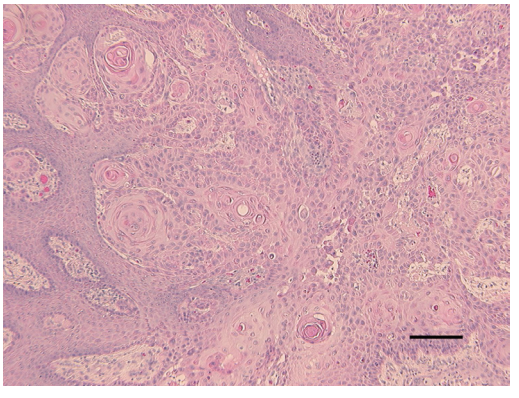

Figure 6. Photomicrograph of a tongue lesion from a dolphin (Case 10); the lesion is composed of deep nests and cords of atypical squamous cells with keratin pearl formation, which is consistent with a squamous cell carcinoma (Type 2 lesion). H&E stain; bar = 100 microns.

Type 2 tongue lesions were distinctly different and were diagnosed as squamous cell carcinomas. The mucosa and submucosa of these lesions contained a poorly circumscribed invasive malignant neoplasm characterized by nests and cords of atypical squamous cells (Figure 6). Squamous cells had prominent intercellular bridges; scant eosinophilic to lightly basophilic cytoplasm; pleomorphic round vesicular nuclei; prominent basophilic to magenta nucleoli; and abundant, often bizarre, mitoses (Figure 7). Additionally,

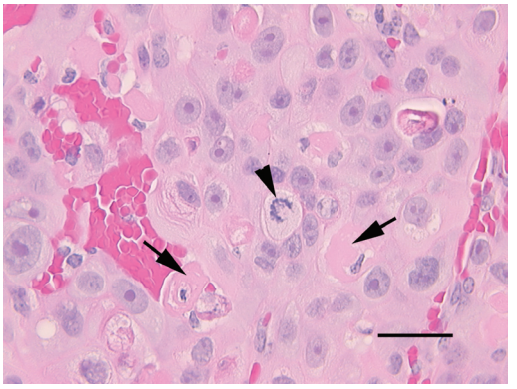

Figure 7. Higher power photomicrograph of Figure 6; note pleomorphic round to polygonal neoplastic cells with round vesicular nuclei, large prominent nucleoli, and a bizarre mitotic figure (arrow head). Cells often are keratinized (arrows). H&E stain; bar = 120 microns.

occasional nests of cells displayed central accumulations of compact, partially keratinized cells or keratin pearl formation (Figure 6). Tumor ulceration with associated multifocal infiltrates of neutrophils, lymphocytes, plasma cells, and/or histiocytes was present in Cases 5, 6, 9, and 10. In a few cases (Cases 6 and 10), degenerate and nondegenerate neutrophils infiltrated the stratum externum and were occasionally associated with keratinocyte necrosis and superficial colonies of a heterogeneous, gram-negative bacterial population. Fungal stains were negative, and inclusion bodies were not observed. Cases 9, 10, and 13, which had multiple lingual lesions consisting of both Type 1 and Type 2 lesions, were diagnosed with distinct sessile papillomas and squamous cell carcinomas, respectively.

At necropsy, three dolphins with oral squamous cell carcinoma (Cases 5, 6, and 14) had widespread metastatic squamous cell carcinomas that were histologically similar to the oral tumors. Case 5 had metastases in the lung, pleura, diaphragm, and lymph nodes (pre-scapular, mediastinal, and pulmonary-associated). Case 6 had metastases in the lung, kidney, adrenal gland, pericardial sac, retropharyngeal lymph nodes, and lymphatics of the urinary bladder and oviduct. In Case 14, metastases were present in the liver, kidneys, and lungs.

Transmission electron microscopy revealed that the nucleoplasm and cytoplasm of moderate numbers of epithelial cells of sessile oral and genital papillomas (Cases 1 and 2) contained encapsidated intranuclear particles and enveloped cytoplasmic virions, measuring approximately 120 to 160 nm in diameter (Figure 8). Enveloped virions were observed just outside the nuclear membrane, and occasionally, virions obtained an outer envelope

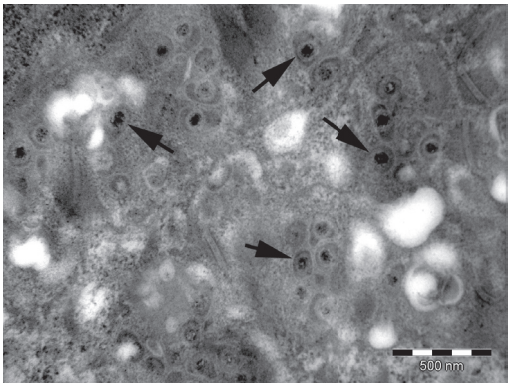

Figure 8. Transmission electron photomicrograph of a keratinocyte from a genital sessile papilloma from a dolphin (Case 2); enveloped cytoplasmic virions (arrows), measuring approximately 120 to 160 nm in diameter are present. Bar = 500 nm.

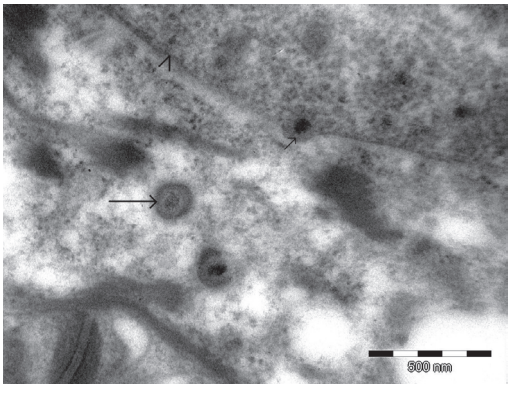

Figure 9. Transmission electron micrograph of a keratinocyte from a genital sessile papilloma from a dolphin (Case 2), which demonstrates enveloped virions within the cytoplasm (large arrow); a virion (small arrow) obtains an outer envelope in passage through the host nuclear membrane (arrowhead). Bar = 500 nm.

in passage through the nuclear membrane (Figure 9). The size and morphology of these virions were suggestive of herpesviruses (Cheville, 1983).

Immunohistochemically, all lesions were negative for BPV-1, BHV-1, and HSV-1. Control tissues were examined to confirm these negative results.

Discussion

Reports of neoplasia in marine mammals, particularly California sea lions (*Zalophus californianus*) and beluga whales (*Delphinapterus leucas*), have increased dramatically in the last two decades (Gulland et al., 2001); however, reports of neoplasia are relatively uncommon in bottlenose dolphins. In bottlenose dolphins, single case reports describe renal adenoma (Migaki et al., 1978), metastatic uterine adenocarcinoma (Sanchez et al., 2002), pulmonary reticulendotheliosis (Geraci et al., 1987), pulmonary squamous cell carcinoma (Ewing & Mignucci-Giannoni, 2003), or splenic lymphosarcoma and pancreatic carcinoma (Landy, 1980). Disseminated immunoblastic malignant lymphoma has been reported in three bottlenose dolphins from the southeastern United States (Bossart et al., 1997).

To our knowledge, this is the first report of oral papillomas in bottlenose dolphins and concurrent oral neoplasia that describes both sessile papilloma and squamous cell carcinoma in the same dolphins, suggesting malignant transformation of the papilloma to squamous cell carcinoma. Additionally, it is the first report of genital papillomas in free-ranging bottlenose dolphins from Atlantic coastal waters. The unusually high occurrence of related benign and malignant epithelial orogenital neoplastic lesions detected in a short time period suggests that the lesions may represent one or more progressive emerging diseases of infectious etiology. Support for this speculation derives from the novel nature of the disease, histopathologic similarities of the orogenital lesions, evidence of viral particles by electron microscopy, and pathologic data from other species that have similar tumors (Bossart et al., 1996; Cotran et al., 1999; Sundberg et al., 2000; Bossart et al., 2002; Goldschmidt, 2002).

Additionally, histopathologic similarities of the benign papillomatous lesions at both oral and genital sites could suggest a common etiology with an orogenital route of transmission. Herpesviruses and papillomaviruses may be associated with similar orogenital tumors in humans, domestic animals, and other marine mammal species (Bossart et al., 1996; Van Bressem et al., 1996; Cotran et al., 1999; Van Bressem et al., 1999; Sundberg et al., 2000; Bossart et al., 2002; Goldschmidt, 2002; Renner et al., 2004). In two dolphins (Cases 1 and 2), herpesvirus virions were observed by electron microscopy in a sessile papilloma from the tongue and vulva, respectively. While the role of herpesvirus in lesion pathogenesis is unknown, it is interesting to note that the orogenital lesions have gross and histologic features similar to oral hairy leukoplakia in humans. Hairy leukoplakia is associated with Epstein Barr virus (EBV) infection, a human gamma herpesvirus, which is generally restricted to immunosuppressed patients, particularly those with human immunodeficiency virus infection (Cotran et al., 1999). In humans, dual infection with EBV and human papillomavirus may play a role in oral and

nasopharyngeal carcinogenesis (Rassekh et al., 1998; Tsuhako et al., 2000; Higa et al., 2003); however, the specific contribution of EBV to oral and nasopharyngeal squamous cell carcinoma remains controversial due to conflicting data on the varied presence of EBV within the lesion (Rassekh et al., 1998; Cruz et al., 2000; Tsuhako et al., 2000; Higa et al., 2002).

The histological patterns of the benign orogenital tumors described in this report consist of uniform epithelial hyperplasia with attenuated submucosal papillae and rare koilocytosis. These features are uncommon for PV-associated tumors in other species, but recently were described as an emerging disease in Florida manatees with PV-associated cutaneous tumors (Bossart et al., 2002). While genus-specific PV or herpesvirus antigens were not detected immunohistochemically in the dolphin cases, these viruses, either singly or in combination, cannot be ruled out as components in lesion pathogenesis. Absence of herpesvirus or PV immunohistochemical reactivity may reflect a lack of antibody cross-reactivity or an intermittently productive viral infection. Absence of virions from other lesions could also be a result of a nonproductive viral infection, a condition well-described in other PV-induced lesions in other species (Lack et al., 1980; Sundberg et al., 1985; Bossart et al., 1996, 2002). Also, support for a possible PV etiology comes from our recent findings of a novel virus representing the first North American dolphin papillomavirus identified (Rehtanz et al., 2005), along with two other abstract reports of PV, which have been detected in genital papillomas from captive bottlenose dolphins using polymerase chain reaction techniques (Chiers et al., 2004; Renner et al., 2004).

Genital papillomas in three free-ranging bottlenose dolphins and lingual papillomas from a single long-snouted common dolphin (*Delphinus capensis*) from coastal Peru have been reported previously (Van Bressem et al., 1996). Grossly, the lesions from both species of Peruvian dolphins were sessile, similar to those observed in the present study; however, the histological pattern of the Peruvian dolphin's genital lesions was a sharply limited epithelial hyperplasia involving the intermediate or outer mucosal layers with elongated submucosal papillae and frequent koilocytosis, which are features typical of PV cutaneous lesions in other species (Bossart et al., 2002). The lingual papilloma from the common dolphin was described as uniformly hyperplastic with no koilocytosis observed. Genus-specific PV antigens were not detected immunohistochemically, and virions were not observed by electron microscopy from the Peruvian bottlenose and common dolphins; however, the authors suggested that PV may have been the etiological agent of the genital tumors based on the histopathological findings and positive PV immunohistochemical findings found in genital tumors from dusky dolphins (*Lagenorhynchus obscurus*) and Burmeister's porpoise (*Phocoena spinipinnis*) (Van Bressem et al., 1996).

Solitary, sublingual squamous cell carcinoma was described previously in a single bottlenose dolphin (Renner et al., 1999); however, the presence of concurrent benign (papilloma) and malignant (squamous cell carcinoma) oral lesions in three of the dolphins in our study was novel and suggests a progressive pathologic process involving malignant transformation. Malignant transformation or conversion of papillomas to squamous cell carcinoma occurs in sheep, goats, cats, and humans (Moulton, 1954; Jablonska & Majewski, 1994; Sundberg et al., 2000). Interestingly, the flat or sessile papillomas described in this study are similar in gross and histopathological appearance to those in humans and cats that typically undergo malignant conversion. In cats, this phenomenon often is associated with immunosuppression (Jablonska & Majewski, 1994; Sundberg et al., 2000). The widespread metastases of squamous cell carcinoma observed in three of this study's dolphins was an unusual finding. In domestic animals and pinnipeds with oral squamous cell carcinoma, such lesions are typically locally aggressive and invasive with occasional regional lymph node metastases. Widespread metastases such as those we have reported are uncommon (Bossart, 1990; Goldschmidt, 2002).

In summary, the occurrence of a cluster of orogenital neoplasms among free-ranging and captive dolphins suggests that an emerging disease, presumably of infectious etiology, may be present. Further research to define the extent of the condition, isolate and characterize the causal agent(s), and search for factors that may be responsible for the recent apparent increase in incidence of these tumors is warranted.

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