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Histopathological and DNA Content Analysis of a Dermal Sarcoma in the Soft-shelled Turtle *Pelodiscus sinensis*

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A dermal sarcoma was found in a freshwater, soft-shelled turtle *Pelodiscus sinensis*. The neoplasm consisted of proliferating fibrous tissue and extended from the dermis. The overlying epidermis was hyperplastic and partially folded. The deeper dermis and hypodermis contained three large, discrete necrotic foci of ~10 mm diameter. Numerous eosinophilic granule cells and macrophages surrounded the necrotic areas. A mixed population of cells with nuclear pleomorphism was observed between the papillary layers of vessels. This area also had regions of different histological structures: (1) regularly arranged, spindle-shaped cells with compact nuclei in a fine-fibrillar matrix; (2) haphazardly arranged cells (≤ 23 μm diameter) with ovoid, highly hypertrophic, faintly stained nuclei; and (3) cells (3.6-5.8 μm diameter) with irregularly shaped nuclei and marginal condensed chromatin in a myxomatous matrix. Some mitotic figures, binucleate cells, and multinucleate giant cells of up to 50 μm in length were also found. Flow cytometry of propidium iodide-stained cells yielded different histograms for the normal skin and the skin (primarily epidermis) and fibrous dermis of the tumor, indicating DNA heterogeneity in the dermal portion of the tumor. The ploidy indices for the dermal cells were 1.91 and 0.78, as compared to normal cells.

Key words: Dermal sarcoma, *Pelodiscus sinensis*, Aneuploidy, Eosinophilic granule cells, Giant cells, Inflammation, Tetraploidy

Introduction

Two types of neoplastic skin disease have been described in turtles: squamous cell carcinomas and fibropapillomatosis (FP). Squamous cell carcinomas, which seldom occur, are characterized by the proliferation of abnormal keratinocytes and metastases to the muscle tissue, liver, lungs, and kidneys (Billups and Harshbarger, 1976; Orós et al., 2004). FP is characterized by multiple cutaneous papillomas, fibromas, and fibropapillomas, as well as internal fibromas. FP was first described in 1938 in the green turtle *Chelonia mydas*, in a captive specimen that had been previously captured near Key West, Florida (Smith and Coates, 1938). It has subsequently been found in olive Ridley (*Lepidochelys olivacea*), loggerhead (*Caretta caretta*), flatback (*Natator depressus*) (Herbst, 1994), and hawksbill (*Eretmochelys imbricate*) (D'Amato and Moraes-Neto, 2000) marine turtles. The pathogenesis of FP is not well understood and whether FP is a hyperplastic or neoplastic process is an unresolved (Herbst, 1994). FP-derived fibro-blasts have recently been found to exhibit properties characteristic of neoplastic cells; a green turtle FP-derived fibroblast line was tumorigenic in immu-nodeficient mice (Herbst et al., 1998), and neoplastic transformation of fibroblasts was associated with the overexpression of four genes and underexpression of two genes (Herbst et al., 2001).

One of the consequences of failed cell cycle regulation in tumors is that tumor cells vary in DNA content (i.e., aneuploidy, polyploidy), increasing the genetic heterogeneity of the cell population. Tumor ploidy has been previously described as an important factor in prognosis and in malignancy estimation of human carcinomas and soft tissue neoplasms (Collin et al., 1997; Seoane et al., 1999; Okita et al., 2003). Flow cytometry is an accurate and rapid technique for measuring the DNA content of cells, and it is

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routinely used for quantitative analysis of the DNA content of mammalian tumors. It has broad applications in studies of fish and reptilian physiology, genetics, and toxicology. In particular, this technique is very useful in environmental toxicology studies because a number of environmental contaminants can affect cellular DNA content (Bickham et al., 1988; Lamb et al., 1991). However, this method has seldom been used in the analysis of tumors from lower vertebrates; only one study of DNA content in turtle neoplastic cells has been reported (Papadi et al., 1995). In the present study, we used flow cytometry to analyze the nuclear DNA content of a tumor in the freshwater, soft-shelled turtle *Pelodiscus* (=*Trionyx*) *sinensis*, Crother, 2000. We also examined the histological structure of the tumor using light microscopy.

Meterials and Methods

Histological analysis

Healthy juvenile soft-shelled turtles, with a mean body weight of 182.6±23.7 g were obtained from a commercial soft-shelled turtle farm in Daegu, South Korea. The turtles were reared and maintained at the Marine Aquarium of Fishery Genetics and Breeding Laboratory of Korea Maritime University, Busan, South Korea, in accordance with the guidelines set forth by the South Korean National Institute of Safety Research. A cutaneous growth was found in one of the 27 soft-shelled turtles held in the laboratory (Fig. 1). The turtle was anesthetized and sacrificed before examination (Park et al., 2006), and tissue samples were fixed in 10% formalin solution, processed, and embedded in paraffin. Sections (5 μm thick) were stained with hematoxylin and eosin and examined under a light microscope equipped with an AxioVision digital camera (Carl Zeiss, Germany). Institutional abbreviations are as listed in Leviton et al. (Leviton et al., 1985).

Tissue preparation and staining for flow cytometry

We studied three formalin-fixed samples obtained from the tumor-bearing turtle: normal skin, skin (primarily epidermis) of the tumor, and fibrous dermis of the tumor. Samples fixed in 10% formalin were washed under running water and placed in 70% ethanol. Small pieces of tissue were mechanically disaggregated and filtered through a 30-μm filter tube (Partec, Germany) to trap large particles of cellular debris. The cell suspension was then centrifuged at 200×*g* for 5 min and decanted. The cell pellet was resuspended in 1 mL of propidium iodide staining solution I and incubated for 30 min at room temperature before analysis. The nuclear DNA content was measured using a FACSCAN flow cytometer (Partec, Germany), and DNA histograms were generated using Partec FloMax Software. Blood cells of the mud loach (*Misgurnus mizolepis*) with 2.80 pg DNA/nucleus were used as an internal standard.

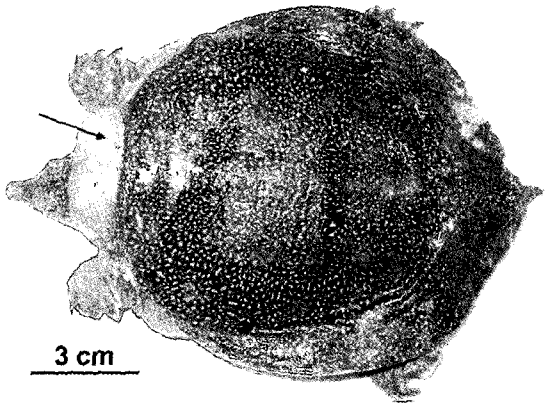

Fig. 1. A soft-shelled turtle, *Pelodiscus sinensis*, with cutaneous growth on the neck (arrow).

DNA histogram analysis

The DNA index was calculated as the ratio of the mean values of the aneuploid/polyploid and diploid G_0/G_1 peaks. By definition, normal diploid cells have a DNA index of 1.0.

Results

Histology of the tumor

The tumor was a single mass of about 20×40×20 mm that was removed from the neck of the soft-shelled turtle specimen. Visceral nodules were not found after postmortem examination of internal organs. The mass consisted of proliferating fibrous tissue and extended from the dermis. The overlying epidermis was hyperplastic and folded in some areas, forming structures similar to papillomas (Fig. 2a); in other areas, no folds were observed, and cell numbers were increased only in the stratum basale and stratum spinosum. The degree of hyperplasia in different parts of the tumor epidermis varied from mild (7-15 cells thick) to moderate (up to 20 cells thick), whereas the normal skin was only 3-5 cells thick. The tumor epidermis contained vacuolated epithelial cells (Fig. 2b) and was covered by a keratin layer of normal thickness. Under a light microscope, numerous parasites (eggs or single-cell protozoa) were detected on the surface of, but not inside, the tumor.

The prominent histological feature of the tumor

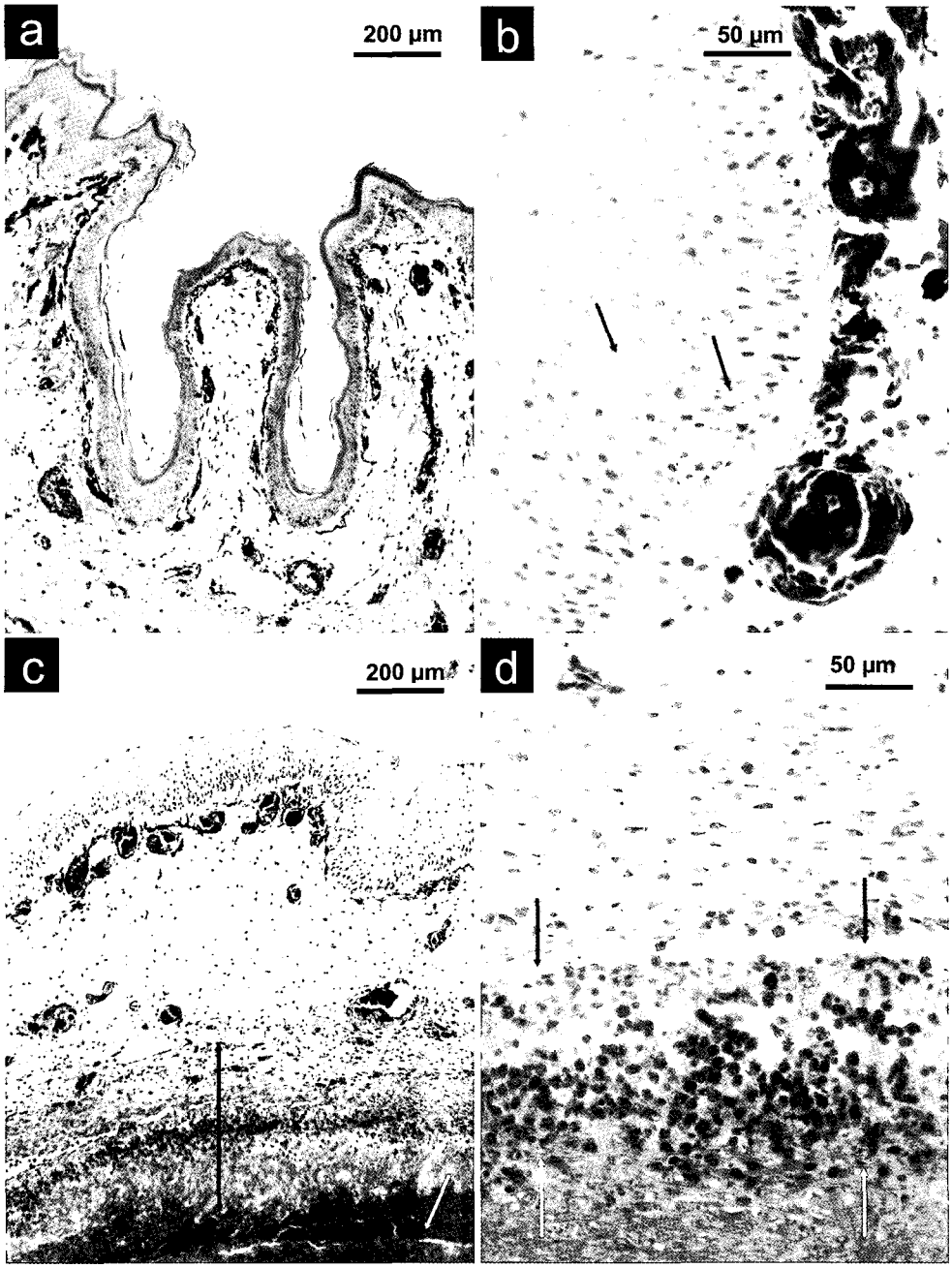

Fig. 2. Dermal tumor of soft-shelled turtle. (a) Folded hyperplastic epidermis overlying the dermal tumor of soft-shelled turtle; (b) Moderate hyperplasia of epidermis. The vacuolization of epithelial cells within the stratum basale and stratum spinosum (arrows) and congestion are seen in small blood vessels in dermis; (c) the peripheral zone (double edge arrow) of the necrotic focus (arrow) located in tumorous mass. The fibrous area is located between the papillary layer of epidermis and the surface of necrotic focus. The overlying epidermis is hyperplastic; (d) numerous eosinophilic granule cells are observed in the peripheral zone of necrotic focus (area between arrows); (e) atypical cells with pleomorphic nuclei located in myxomatous matrix. Arrows point to irregularly shaped moderately hyperchromic nuclei, whereas short arrows show ovoid, highly hypertrophied pale-staining nuclei with prominent nucleolei. In the centre of the picture there is an eosinophilic granule cell; (f) loosely arranged atypical cells with small nuclei (arrows) measured from 3.6 to 5.8 μm maximum diameter. The nuclei are characterized by the irregular shape and the marginal condensed chromatin. The short arrow point to a mitosis; (g) multinucleate giant cells (arrows) with nuclei occupying a central part of the cell. The area is characterized by low cellularity and myxomatous matrix; and (h) a giant cell with multiple nuclei arranged around the periphery (arrow). Numerous eosinophilic granule cells are seen in the vicinity.

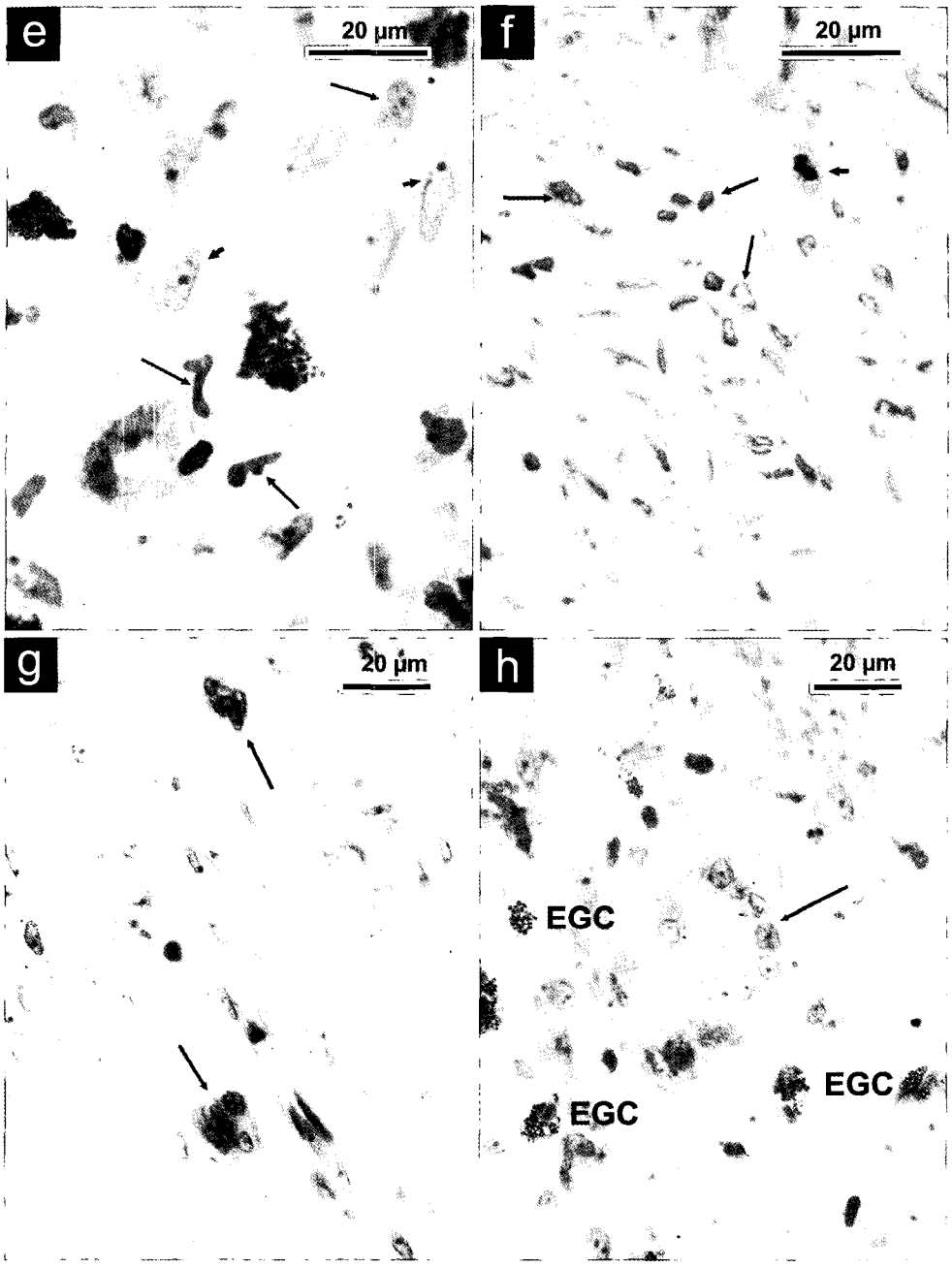

Fig. 2. Continued.

dermis. As a few layers of necrotic tissue were present, these foci may have arisen via progressive central necrosis. Where the necrotic focus had spread close to the epidermis, the epidermis was also necrotic. Numerous eosinophilic granule cells (EGCs) and macrophages surrounded the necrotic areas (Fig. 2c). The EGC density in the peripheral zone of the necrotic foci was very high (Fig. 2d), but they were less numerous in the fibrous part of the dermis, the epidermis, and on the surface of the keratin layer. Most of the EGCs located close to necrotic foci appeared to be in the process of degranulation, and extracellular granules were observed in close proximity to these cells.

The portion of the tumor located between the epidermal papillary layer and the necrotic foci was occupied by fibrous tissue with numerous small blood vessels. Flow cytometry revealed that this tissue contained a mixed population of cells with nuclear pleomorphism. Microscopic observation revealed

areas of three different histological types: (1) regularly arranged, spindle-shaped cells with compact nuclei in a fine-fibrillar matrix; (2) haphazardly arranged, atypical cells of up to 23 μm in length with ovoid, highly hypertrophic, faintly stained nuclei (Fig. 2e); and (3) cells of 3.6-5.8 μm in diameter with irregularly shaped nuclei containing marginal condensed chromatin in a myxomatous matrix (Fig. 2f). Some mitotic figures and binucleate cells were also present. These cells and nuclei were small, suggesting that they were formed as the result of mitotic failure rather than as the result of fusion of two existing cells. Numerous small blood vessels were present within the stroma.

Multinucleate giant cells with different nuclear arrangements were also present in the tumor. The nuclei were present either at the center of reshaped cells (Fig. 2g) or around the periphery (Fig. 2h). The appearance and nuclear localization of some of these cells were similar to those of floret-type, multinucleate giant cells found in mammalian tumors. The diameters of the multinucleate cells ranged from 13 to 50 μm. The smaller cells (13-25 μm in diameter) were found throughout the fibrous tissue, and the larger ones (-50 μm in diameter) were located in close proximity to the periphery of the necrotic foci.

DNA content of tumor cells

Flow cytometry analysis of the normal and tumor (epidermis predominantly) skin cells yielded histograms indicative of diploid profiles. The average nuclear DNA contents of these cells were 3.48 and 3.24 pg/nucleus, respectively (Table 1). Histograms of fibrous dermis from the tumor showed two distinct peaks. The average DNA contents of the first peak (69.07% of total area) and second peak (30.93% of total area) were 2.70 and 6.63 pg/nucleus, respectively. When compared to normal skin, the first and second fibrous dermis peaks yielded ploidy indices of 0.78 and 1.91, respectively, indicating that cells with abnormal karyotypes were present within the fibrous part of the dermis.

Coefficient of variation

Statistical analyses were performed on the coefficient of variation (CV) of the G_1 and other peaks for the three types of tissue analyzed. The frequency distributions of the CV is a sensitive indicator of within-sample variability and instrumental variation. Assuming intrinsic variability, the observed CV for normal diploid nuclei should reflect only instrumental variation.

Examination of the CVs for the normal and tumor tissues revealed three major features. First, the mean CV of a nearly tetraploid peak (5.96%) was not larger than the mean CV for diploid distributions (6.20%). Second, the CV of the aneuploid peak for the fibrous dermis (19.84%) was greater than that of the diploid or tetraploid peaks. Third, the DNA content distributions of the aneuploid and diploid peaks of the fibrous dermis were qualitatively different, i.e., the base of the former was wider than that of the latter.

Discussion

Our examination of a tumor from a soft-shelled turtle revealed an expanded epidermis and dermis. Spontaneously induced fibropapillomas of the green turtle also exhibit proliferation of the epidermis and dermis, but as a whole they appear benign (Jacobson et al., 1989; Herbst et al., 1995). In contrast, the tumor described herein exhibited signs of malignancy: vast necrosis, intensive immunological response (neoplasia and necrosis), histologically different structures in different areas of the tumor and heterogeneity of nuclear DNA content. Consequently, the structure of this tumor is not compatible with published descriptions of sea turtle fibropapillomas.

A very important consideration in the classification of a tumor is its site of origin. We presume that the morphological alterations within one neoplasm will be more pronounced at its center than at the periphery, if the tumor mass is of recent origin. The centers of the necrotic foci were located deep within the dermis, and necrosis had spread into the hypodermis and superficial dermal layer. Alterations in the papillary layer of the dermis were not as pronounced, and massive structural alterations were observed only where the necrotic focus had spread close to the epidermis. Therefore, we suspect that this tumor arose either in the deeper dermis or at the dermis-hypodermis border, unlike the fibropapillomas of sea turtles. Fibropapillomas of green turtles induced by inoculation of the transmitted agent primarily occupy the epidermis and dermis, with more extensive proliferation of fibroblasts within the papillary layer of the dermis. In our study, the deeper part of the tumor dermis had been drastically altered and was necrotic.

We conclude that the morphological features of the tumor and the presence of proliferating areas composed of atypical, spindle-shaped cells within the tumor warrant a diagnosis of sarcoma. In the World Health Organization Classification of soft-tissue sarcomas published in 2002 (Fletcher et al., 2002), the importance of correct histotyping was acknow-

Table 1. DNA content (pg/nucleus) and ploidies of the different cell populations from dermal sarcoma of soft-shelled turtle

Tissue specimen	Mean DNA content	Area (%)	CV (%)	Ploidy index
Normal skin	3.48	100.00	6.20	1.00
Epidermis from tumor	3.24	100.00	18.80	0.93
Dermis from tumor*	2.70[1]	69.07[1]	19.84[1]	0.78[1]
	6.63[2]	30.93[2]	5.96[2]	1.91[2]

*Dermis from the tumor showed two distinct peaks, [1]The first peak, [2]The second peak.

ledged by the incorporation of immunohistochemical criteria into the diagnostic guidelines for human tumors. Precise diagnosis of sarcomas in lower vertebrates is also difficult without immunohistochemical data. The technique has yet to be used successfully in analysis of growths in turtles. One attempt to characterize turtle tumor cells by immunohistochemistry using several monoclonal and polyclonal antisera against mammalian intermediate filaments was unsuccessful (Orós et al., 2004). Although we have not identified the type of sarcoma in the present case, it has histological similarities to benign and malignant fibrous histiocytomas of mammals. Its microscopic features, such as nuclear pleomorphism and vast necrosis, point to a malignancy.

Our analysis of the tumor revealed inflammation with massive aggregation of eosinophilic granule-containing cells (EGCs). EGCs in teleost fishes have been proposed to be the functional equivalent of mast cells in mammals (Reite, 1998). High densities of EGCs have been demonstrated in neurofibromas and malignant peripheral nerve sheath tumors in the bicolor damselfish *Stegastes partitus*, but the role of these cells in tumor development remains uncertain (Vicha and Schmale, 1994). EGC granules in several species have been shown to contain antimicrobial peptides, and EGCs can participate in anti-parasitic as well as cytotoxic responses in fishes (Reimschuessel et al., 1987; Sharp et al., 1989; Cammarata et al., 2000; Silphaduang and Noga, 2001). In our study, signs of degranulation were seen in the EGCs located near the necrotic foci. We speculate that the large number of EGCs present in the tumor tissue is a consequence of the inflammatory response, rather than a characteristic of this type of tumor.

In many human tumors, cells with tetraploid DNA content arise as an early step in tumorigenesis and precede the formation of aneuploid cells (Andreassen et al., 1996; Shackney et al., 1989). Tetraploidy also occurs as an intermediate step prior to aneuploidy and tumor formation in certain rodent model systems (Cross et al., 1995), further suggesting a role for tetraploidy in tumor development. Aneuploidy is linked to the progressive development of higher-grade, invasive tumors (Rabinovitch et al., 1989), and the emergence of aneuploid clones is a representative marker of malignancy progression in cutaneous and cutaneous fibrohistiocytic tumors (Seoane et al., 1999; Okita et al., 2003).

In our study, heterogeneity of DNA ploidy was found in the dermis. We previously defined heterogeneity as the presence of areas differing in DNA index within a single tumor (Oriyama et al., 1998). In recent years, heterogeneity of DNA ploidy within a single tumor has been reported in a hepatocellular carcinoma and in malignant soft tissue sarcomas (Collin et al., 1997; Oriyama et al., 1998).

DNA histograms with a G_0/G_1 coefficient of variation (CV) of greater than 10% are generally classified as unsuitable for tumor analysis (Coon and Weinstein, 1991). In our study, a fibrous area between the papillary layer of the dermis and the margin of necrotic foci was used for flow cytometry analysis. The G_0/G_1 CV of the data was 6.20%, indicating that the data were suitable for analysis. Histological study showed that this area consisted of cells similar to normal fibroblasts, atypical cells of up to 23 μm in length with highly hypertrophic nuclei, which may be tetraploid, and atypical cells with diameters ranging from 3.6 to 5.8 μm with small, irregularly shaped nuclei and condensed chromatin. The heterogeneity of nuclear DNA content may be connected with the presence of pleomorphic atypical nuclei in the tumor. Aneuploid peaks should include the sum of instrumental and within-sample variation.

The data for the tumor skin cells also exhibited a high CV. This phenomenon has two possible explanations. First, the tumor skin peak may actually be a composite of two distinct distributions: that of normal cells and that of abnormal cells of the superficial layer of dermis. Since we analyzed tissues, not cell lines, and dissection of the skin to subdivide epithelial cells of the epidermis from fibroblasts of the papillary layer of skin was not possible, we cannot directly confirm or disprove this conjecture. Our analysis did show a mixed population containing epithelial cells, fibroblasts, and possibly some in-

flammatory cells, which is consistent with this conjecture. We assume that some abnormal aneuploid tumor cells were present in this sample.

The second possible explanation for the high CV for the tumor skin cells may be the presence of dying cells. Hyperplasia and vacuolization of the epithelial cells were observed. In tissues with pathology, cell death usually occurs through necrosis or apoptosis. When apoptosis is triggered, cells undergo a distinct set of structural changes; endonucleases break down chromatin, and the nuclei become shrunken with condensation of chromatin beneath the nuclear membrane. Later in the process, the cells are fragmented into apoptotic bodies, each one being membrane-bound and many containing nuclear remnants. Fragments of destroyed nuclei and debris might increase the CV.

To our knowledge, this report represents the first description of a soft tissue sarcoma in the freshwater soft-shelled turtle. As for other turtle species, one case of myxofibrosarcoma in a tortoise is described in the Registry of Tumors in Lower Animals (number 5774), but the species of turtle is not mentioned (presumably, Testudinidae).

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