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Histologic and Immunohistochemical Characterization of Pheochromocytoma in Six Cotton-Top Tamarins (*Saguinus oedipus*)

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Abstract

Pheochromocytomas are uncommon neoplasms of the adrenal medulla that are most frequently reported in rats and select mouse strains. In the many cases, especially those in man, pheochromocytoma is associated with familial tumor syndromes due to inherited mutations in a variety of proto-oncogenes and tumor suppressor genes. Non-human primates are valuable animal models for a variety of human diseases due to their similar anatomy and physiology; however, cases of pheochromocytomas have only rarely been reported in non-human primates. Herein we characterize the gross, histologic, and immunohistochemical features of pheochromocytoma in 6 cotton-top tamarins (*Saguinus oedipus*). Pheochromocytomas represented 6/114 (5.3%) of the total causes of death in the studied population and corresponded to 16% of the total number of neoplasms. The average age of affected animals was 17.9 years. Histologically, all cases were defined by tight bundles, nests, and cords of neoplastic chromaffin cells. All cases had concurrent myocardial degeneration and fibrosis of varying severity and chronicity. Three (50%) of the cases also had hyalinization and medial thickening of coronary arteries consistent with hypertension. Immunohistochemically, 6/6 (100%) of the cases stained positively for chromogranin A, synaptophysin, N-CAM (or CD56), and protein gene product 9.5. None of the cases stained for glial fibrillary acidic protein. Pedigree analysis revealed inter-relatedness in 4/6 animals with progenitor animals also affected with pheochromocytomas. The tumors in this population illustrate comparable histologic and immunohistochemical staining patterns to cases in other laboratory animals and humans, and, therefore, may indicate common underlying genetic alterations that precipitate tumor development.

The adrenal gland is an anatomically simple organ divided into a cortex and medulla with the latter populated predominately by specialized chromaffin cells that are derived from the neural crest and a small population of support (sustentacular) cells.¹⁰ The chromaffin cells synthesize and secrete various catecholamines, including epinephrine and norepinephrine, which lead to a variety of physiologic effects including increased cardiac contraction and vasoconstriction. 10 The most common pathology associated with the adrenal medulla is the genesis of tumors including pheochromocytomas and neuroblastomas.⁵ Pheochromocytomas are variably functional and can secrete epinephrine leading to a variety of hypertensive and myocardial degenerative effects.¹⁷ Microscopic findings typically include an alveolar or trabecular pattern and, in general, cells resemble normal medullary chromaffin cells. Tumors can contain small

numbers of ganglion cells, neuroblasts, and melanin-containing cells; however, these cell types not predominate in cases of pheochromocytoma.¹⁵

Although pheochromocytomas are considered rare in humans (estimated incidence of 1/300,000), considerable interest and research has been focused on developing a suitable animal model to study pheochromocytomas.^{11,16} Both knockout mice and rats have been extensively used in mechanistic studies of the genesis of spontaneous and genotoxic pheochromocytoma.^{14,16} Non-human primates serve as an excellent model for many human diseases due to their similar physiology and pathology; however the incidence of pheochromocytomas in non-human primates is not currently known. A solitary case of an angiomatous pheochromocytoma has been reported in a Rhesus macaque (*Macaca mulatta*).⁹ Endocrine neoplasia has been reported slightly more frequently in new world primates with tumors arising within the adrenal gland reported most commonly. These include pheochromocytomas in three golden lion tamarins (*Leontopithecus rosalia rosalia*), two mantled howler monkeys (*Alouatta villosa*), one cotton-top tamarin (*Saguinus oedipus*), and one brown spider monkey (*Ateles fusciceps*).^{3,4} Only the brown spider monkey and the cotton-top tamarin were reported to have concurrent myocardial degeneration, presumably from epinephrine excess. Cases of solitary adrenal cortical adenoma have been reported in a single black-tailed marmoset and a single cotton-top tamarin.^{3,4}

Immunohistochemistry is commonly used to differentiate adrenal medullary tumors from cortical or extra-adrenal tumors.^{11,12,17,20} Markers commonly used to differentiate pheochromocytomas include chromogranin A (CGA), protein gene product (PGP) 9.5, synaptophysin (SYN), CD56 (also known as neural cell adhesion molecule or N-CAM) and glial fibrillary acidic protein (GFAP). CGA is one of the most specific neuroendocrine immunohistochemical markers currently available and is a part of catecholamine-containing secretory granules and reliably stains adrenal medullary cells.^{4,7,11,12,20} PGP9.5 forms a portion of the ubiquitin-proteasome system and is highly specific to neurons and cells of the neuroendocrine system.^{7,11,13,20} Synaptophysin is a glycoprotein expressed in the pre-synaptic vesicles of neurons as well as diffusely in the neuroendocrine system.^{11,12,20} N-CAMs are involved in the morphogenesis of neural epithelial cells and is a cohesive cell adhesion molecule found in peripheral and central nervous system cells.^{8,11} GFAP is a class III intermediate filament and although considered relatively specific for astrocytes and neural stem cells, has rarely been used as an immunomarker that stains a sub-population of sustentacular cells. It is not universally positive in cases of pheochromocytoma.^{1,11}

Herein we characterize a cohort of six cotton top tamarins that developed spontaneous pheochromocytomas and had histologic evidence of myocardial degeneration and hypertension. Additionally we use immunohistochemistry for CGA, SYN, PGP 9.5, N-CAM, and GFAP to compare these tumors to published human cases.

Materials and Methods

A relational database of necropsy records at the New England Primate Research Center (NEPRC) was searched from 2000-2008 for adrenal pathology in cotton-top tamarins. From the cases chosen, the case criteria was further refined to the diagnosis of pheochromocytoma resulting in six recognized cases. There were no tumors other than pheochromocytoma recorded. All animals were necropsied within 2 hours of death and representative sections of all major organs were collected, fixed in 10% neutral buffered formalin (NBF), embedded in paraffin, sectioned at 5µm, and stained using hematoxylin and eosin (HE). Additional sections were prepared for immunohistochemistry. A pedigree analysis to examine the relationship between affected animals was performed using Pedigraph, a software program developed by the Department of Animal Science, University of Minnesota.

To characterize the neoplasms using immunohistochemical methods we used standard immunoperoxidase staining for SYN, CGA, PGP 9.5, NCAM, and GFAP. Formalin fixed, paraffin embedded sections were deparaffinized, rehydrated and subsequently blocked with hydrogen peroxide. Pre-treatment for GFAP involved using proteinase K whereas pre-treatment for PGP 9.5, SYN, NCAM, and CGA involved microwaving for 20 minutes in 0.01M citrate buffer followed by 20 minutes of cooling. All steps were followed by a tris-buffered saline (TBS) wash. Prior to application of primary antibodies, all slides were treated with Dako protein block for 10 minutes. Sections were incubated with anti-human CGA (Dako [Carpinteria, CA, USA], polyclonal, 1:1000, 30' at room temperature), anti-mouse SYN (Dako, monoclonal, 1:50, overnight in refrigerator), anti-cow GFAP (Dako, polyclonal, 1:500, 30' at room temperature), anti-human NCAM (Zymed [San Francisco, CA, USA], monoclonal, 1:500, overnight in refrigerator), and anti-human PGP 9.5 (Biomedica [Foster City, CA, USA], monoclonal, 1:400, overnight in refrigerator). Slides were then incubated with secondary antibody biotinylated goat anti-rabbit (Vector laboratories, [Burlingame, CA, USA], 1:200, 30' at room temperature) for CGA and GFAP and biotinylated horse anti-mouse (Vector laboratories, 1:200, 30' at room temperature) for SYN, NCAM, and PGP9.5. This was followed by 30 minute incubation at room temperature with Vectastain ABC Elite [Vector laboratories] (CGA, GFAP, and SYN) or Vectastain ABC standard [Vector laboratories] (NCAM, PGP 9.5). All slides were developed with DAB chromagen (Dako) and counterstained with Mayer's hematoxylin. In all cases, step sections were incubated with isotype-specific irrelevant antibodies for negative controls. Positive controls consisted of sections of small intestine (PGP 9.5), brain (GFAP), and adrenal gland (SYN, CGA, NCAM) from age-matched cotton-top tamarins.

Results

Animals

From 2000 to 2008, 114 adult (older than 1 year old) cotton-top tamarins were necropsied at the New England Regional Primate Research Center (NEPRC). Of these, 37 had malignant neoplasms with pheochromocytomas corresponding to 6 (16%) of the total number of neoplasms recognized (Table 1). Pheochromocytomas represented 5.3% of the total number of deaths in this population during the defined time period. There were 4 male and 2 female animals affected. The average age at time of death was 17.9 years (ranging from 15.1 to 21.9). Following identification of affected animals, a pedigree map was developed to ascertain any possible relationships between the affected animals (Fig. 1). The pedigree analysis revealed that 4/6 (67%) of the affected animals were derived from offspring of one mating (442-1977 × 441-1977). Subsequently, all animals in the pedigree were analyzed for adrenal pathology and two additional animals 442-1977 (case no. 7) and animal 444-1977 (case no. 8) were found to have unilateral adrenal masses consistent with pheochromocytomas. The relevant clinical data for these animals are presented at the end of Table 1. These two affected progenitor animals were significantly younger compared to the study cohort; however these two animals were not included in the current study group due to time elapsed since necropsy and lack of appropriate tissues for immunohistochemistry.

Clinically, all animals had gradual weight loss and palpation of an intra-abdominal mass was recorded in 2 animals. One animal had an arrhythmia and systolic ejection murmur with skipped beats and an abnormal QRS complex on electrocardiogram examination. Complete blood count and serum biochemistry analysis were within normal limits in all animals.

Grossly, 5 of the 6 animals had variably sized, ovoid masses arising at the cranial aspect of the right kidney (case no. 1 and 5), left kidney (case no. 3), or involving both left and right kidneys (case no. 2 and 4). Case no. 6 had no noticeable mass detected. There was no vascular invasion noted grossly in any of the animals.

Histopathology

The adrenal neoplasms were expansile and partially to completely replaced the normal cortical architecture (Fig. 2). The neoplastic cells were arranged in tight bundles, cords, and occasional pseudorosettes that were oriented in close approximation to blood-filled capillaries (Fig. 3). Cells were embedded in a small amount of fibrous connective tissue stroma. Generally, the cells had abundant, finely granular, eosinophilic cytoplasm with large, basally situated nuclei, finely stippled to margined chromatin, and a single prominent nucleolus. Cellular and nuclear atypia was mild in all cases with an average of 1-2 mitotic figures per 10 hpf. 3 cases (case no. 2, 5, 6) had a small proportion (<5%) of neoplastic cells with megalokaryocytic nuclei or multinucleate cells. In three animals there were rafts of neoplastic cells located within extracapsular lymphatics (Fig. 4); however, no evidence of distant metastasis was noted in any of the animals. The neoplastic cells were interspersed with hemosiderin laden macrophages in 3 cases (case no. 1, 3, 6), extramedullary hematopoiesis in 4 cases (case no. 1, 2, 4, 6), and in 3 cases intratumoral sinusoids were dilated and partially to completely occluded by fibrin thrombi (case no. 1, 2, 6) (Fig. 5).

In the myocardium of all affected animals were multifocal regions of myofiber cytolysis characterized by sarcoplasmic hypereosinophilia, sarcoplasmic vacuolation, loss of cellular detail, and myofiber shrinkage (Fig. 6). All animals also had areas of interstitial fibrosis that entrapped and replaced variable numbers of myofibers; this fibrosis was extensive in two of the affected animals. Two of the animals also had marked interstitial fibrosis in the lung coupled with increased numbers of hemosiderin laden macrophages (heart-failure cells) (Fig. 7). Three of the cases (case no. 1, 2, and 4) had marked hyalinization of the coronary arteries with thickening of the tunica media and increased number and size of smooth muscle cells.

Two of the affected animals (case no. 3 and 4) had unilateral thyroid cystadenomas characterized as expansile, encapsulated cystic neoplasms that had projections of neoplastic follicular epithelium forming papillary fronds within the cystic cavities. Additional co-morbid histologic findings were chronic lymphoplasmacytic and fibrosing nephritis (6/6), vacuolar hepatopathy (5/6), multifocal hepatic necrosis (2/6), moderate lymphoplasmacytic enterocolitis (1/6), adrenal myelolipoma (1/6), and jejunal lymphangiectasia (1/6).

Immunohistochemistry

Immunohistochemical analysis of the 6 cases of pheochromocytomas was similar for all antibodies studied. SYN immunoreactivity was strong, diffusely intracytoplasmic, and confined solely to the neoplastic chromaffin cells (Fig. 8). Cortical tissue exhibited no immunoreactivity with scattered capsular nerves strongly positive. CGA exhibited diffuse, granular, cytoplasmic immunoreactivity within the neoplastic chromaffin cells, but spared the cortical cells (Fig 9). PGP 9.5 exhibited diffuse cytoplasmic immunoreactivity in the neoplastic cells as well as staining occasional interstitial cells in the adrenal cortex and the capsular nerves (Fig. 10). N-CAM immunoreactivity was strongly membranous in all of the neoplastic chromaffin cells (Fig. 11). Cells in the zona glomerulosa and the zona fasciculata had scattered, less intense membrane staining for N-CAM. GFAP was universally negative in all cases examined staining only occasional solitary interstitial cells in the cortex and capsular nerves (Fig. 12).

Discussion

Herein we report the clinical, histologic, and immunohistochemical properties of 6 cases of pheochromocytoma in cotton-top tamarins (*Saguinus oedipus*). Pheochromocytomas are neuroendocrine tumors derived from the chromaffin cells of the adrenal medulla. Complications typically associated with these tumors include myocardial degeneration

resulting from excess catecholamine release and systemic hypertension typified by vascular medial hypertrophy. In this study, all animals with pheochromocytomas had concurrent myocardial degeneration and fibrosis. Random myocardial degeneration and fibrosis is not a common age-associated lesion in cotton-top tamarins housed at the NEPRC and therefore an association with increased circulation catecholamines is plausible in this cohort of animals. In addition two of these animals also had alveolar septal fibrosis and hemosiderin laden macrophages within the alveoli indicative of long-standing cardiac dysfunction. Three animals had marked hyalinization and thickening of the tunica media of intramural coronary arteries, a common finding associated with hypertension. Although other organs (i.e. stomach, eye, kidney) did not have any histologic evidence of vascular disease, the lesions in the heart are highly suggestive of hypertension, a common side effect of pheochromocytomas.¹⁰

In humans, pheochromocytomas are considered an uncommon neoplasm with an average annual incidence of 1/300,000 individuals.¹¹ Although rare, considerable interest has been spent attempting to define an appropriate animal model for this tumor.¹⁶ Pheochromocytomas are uncommon in all domestic and laboratory animals except for some strains of rats and various transgenic mouse models.^{14,16} In the former, tumors typically arise either *de novo* in aged animals or are associated with exposure to a variety of genotoxic compounds.¹⁶ They are typically observed in males and can be either bilateral or unilateral.¹⁶ Similar murine neoplasms have been studied in a variety of transgenic models including mice expressing polyoma viral T-antigens and *c-mos* in addition to *Nf1* knockout mice.^{14,16} Although both mice and rats provide valuable information into the pathogenesis of pheochromocytomas, their different physiology make comparisons to human cases of pheochromocytoma problematic. Non-human primates are physiologically similar to humans and could potentially provide a more concise model to study the disease. Although there are several reports of pheochromocytomas in new world primates, the neoplasms appear to be rare or underreported.^{3,4} In the current study, 5.3% of the cotton-top tamarins that died were found to have pheochromocytomas and resultant cardiac pathology sufficient to have led to death. This is a significantly higher percentage than is indicated in the literature for other species and may indicate an increased propensity to develop these tumors in this population of cotton-top tamarins.

In humans, most cases are unilateral and solitary with fewer than 10% of the tumors considered malignant.^{11,16,17,18} In the present study two-thirds of the cases were unilateral and one-third bilateral. No prognostic significance exists for unilateral versus bilateral tumors; however, bilateral tumors are more commonly associated with inherited, familial tumor syndromes.¹⁷ Many cases of pheochromocytoma in humans are associated with multiple endocrine neoplasia (MEN) type 2, von Hippel-Lindau (VHL) syndrome, and neurofibromatosis (NF) type 1.^{6,9} Pheochromocytomas are present in roughly half of the cases of MEN2 and result from a mutation in the RET proto-oncogene.^{6,17,18} In fact, bilateral pheochromocytomas are a strong factor in screening for RET mutations in affected human patients.^{6,18} In cases of VHL syndrome, the absence of the VHL protein leads to stabilization of hypoxia inducible factor 1 α thereby potentiating cell growth and angiogenesis.⁹ In the current study the increased incidence of pheochromocytomas in this population suggests an effect beyond background tumor development and genetic studies into the population are being pursued. Additionally, the presence of concurrent thyroid cystadenomas in several of these tamarins raises the specter of a multiple endocrine neoplasia-like syndrome possibly occurring in several of the affected animals. Indeed, the pedigree map illustrates the inter-relatedness of the affected animals. Especially intriguing is that one of the original progenitor animals (442-1977) also died from a pheochromocytoma, albeit at a much younger age than the other animals. Although no distinct inheritance pattern can be discerned from the pedigree, appearance of the tumor occurs in both sexes and in most cases skips a generation.

Immunohistochemistry is considered an invaluable aid in diagnosing tumors of the adrenal medulla. Neuroendocrine markers like CGA, PGP 9.5, N-CAM, and SYN are typically positive in cases of pheochromocytoma, particularly with CGA which is reported to stain 100% of pheochromocytomas.^{1,8,11,12,20} Chromogranins are the major secreted protein of the adrenal medulla and play an important role in binding and aggregating intracellular calcium.⁴ In addition, there are defined roles for chromogranins in functioning as pro-hormones, molecular chaperones, and modulators of gene expression.^{4,20} PGP 9.5 is a part of the ubiquitin-proteasome system, and although originally isolated from the brain, expression has now been found in many human tissues including the diffuse neuroendocrine system, female and male reproductive organs, and hematopoietic cells.^{7,11,13,20} To our knowledge, this is the first study to utilize PGP9.5 in cotton-top tamarin tissue and further illustrates the utility of the marker in diagnosing neuroendocrine tumors. Synaptophysin is a membrane glycoprotein found in pre-junctional neuroendocrine granules and is used as a specific marker of neuroendocrine tumors.^{11,20} N-CAM (also known as CD56) is a common marker also used in diagnostic immunopathology and has specificity to cells of the nervous system, neuroendocrine system and skeletal muscle.^{8,11} In human adrenal glands, N-CAM immunoreactivity has been reported in both cortical and medullary cells thereby making it a poor marker to differentiate primary tumors arising in each site.⁸ Immunostaining in the present case series, however, showed much more intense staining in the neoplastic medullary chromaffin cells compared to the cortical cells, suggesting that NCAM is a better marker for chromaffin cells. GFAP is also occasionally used in the diagnosis of pheochromocytomas and is used to stain a subpopulation of the support cells of the adrenal medulla (sustentacular cells).¹

In conclusion, pheochromocytomas are uncommon tumors in all domestic and laboratory animals except for rats and select mice strains. Although there are scattered reports of these tumors developing in new-world primates, the incidence is not known, although is presumed to be sporadic. The 6 cotton-top tamarins described herein had uni- or bilateral pheochromocytomas. In addition all animals had acute, multifocal myocardial degeneration and fibrosis consistent with chronic, ongoing myocardial injury, presumably from released epinephrine. Immunohistochemically, chromogranin A, synaptophysin, neural cell adhesion molecule, and PGP9.5 strongly stained the neoplastic cells whereas GFAP was uniformly negative. Pheochromocytomas in this colony of cotton-top tamarins represented 5.3% of the total causes of death in the studied time frame and, due to genetic inter-relatedness of the affected animals and the identification of affected progenitor animals, genetic studies are ongoing in this population.

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Fig. 1. Pedigree for all affected tamarins. The present cohort is highlighted in grey boxes. Animal numbers 442-1977 and 444-1977 represent earlier progenitor animals that also had histologically confirmed pheochromocytomas.

Fig. 2. Adrenal gland, Cotton-top tamarin, case No. 2. A large, unencapsulated mass arising from the adrenal medulla compresses the adjacent adrenal cortex (asterisk). HE.

Fig. 3. Adrenal gland, Cotton-top tamarin, case No. 6. The neoplastic cells are arranged in tight clusters, nests, and pseudo-rosettes that are oriented around small blood vessels (arrows). HE.

Fig. 4. Adrenal gland, Cotton-top tamarin, case No. 2. Rafts of neoplastic cells partially occlude peri-adrenal lymphatics (arrows). HE.

Fig 5.
Adrenal gland, Cotton-top tamarin, case No. 1. Within the neoplasm there are several loose aggregates of extramedullary hematopoiesis (arrow) and a single large fibrin thrombus (asterisk) partially fills a small blood vessel. HE.

Fig. 6. Heart, Cotton-top tamarin, case No. 3. Multifocally, the myocytes are swollen, vacuolated, and degenerate with loss of striations (arrows). HE.

Fig. 7.
Lung, Cotton-top tamarin, case No. 4. The alveoli contain large numbers of macrophages that are filled with hemosiderin (arrow). HE.

Fig. 8. Adrenal gland, Cotton-top tamarin, case No. 2. The neoplastic cells are exhibit strong cytoplasmic immunoreactivity for synaptophysin. Intratumoral blood vessels (asterisk) and compressed cortical cells (arrow) have no immunoreactivity. Immunoperoxidase staining, DAB chromagen, Mayer's hematoxylin counterstain.

Fig. 9. Adrenal gland, Cotton-top tamarin, case No. 6. The neoplastic cells exhibit moderate cytoplasmic immunoreactivity for chromogranin A. Intratumoral blood vessels (asterisk) have no immunoreactivity. Immunoperoxidase staining, DAB chromagen, Mayer's hematoxylin counterstain.

Fig. 10. Adrenal gland, Cotton-top tamarin, case No. 5. The neoplastic cells exhibit strong cytoplasmic immunoreactivity for PGP 9.5. Rare interstitial cells in the adjacent cortex (arrow) are also positive. Immunoperoxidase staining, DAB chromagen, Mayer's hematoxylin counterstain.

Fig. 11. Adrenal gland, Cotton-top tamarin, case No. 5. The neoplastic cells exhibit strong membranous immunoreactivity for N-CAM. All intratumoral blood vessels (arrows) have no immunoreactivity. Immunoperoxidase staining, DAB chromagen, Mayer's hematoxylin counterstain.

Fig. 12. Adrenal gland, Cotton-top tamarin, case No. 3. The neoplastic cells exhibit no immunoreactivity for GFAP. A single nerve (arrow) is strongly positive and there are scattered hemosiderin laden macrophages (asterisk). Immunoperoxidase staining, DAB chromagen, Mayer's hematoxylin counterstain.

Table 1

Case summaries

Case Number	Sex	Age (years)	Site of mass
Case No. 1 (341-1985)	M	15.1	Right adrenal
Case No. 2 (250-1986)	M	16.8	Right and left adrenal
Case No. 3 (211-1983)	F	20.7	Left adrenal
Case No. 4 (317-1983)	M	21.9	Right and left adrenal
Case No. 5 (294-1990)	M	16.8	Right adrenal
Case No. 6 (219-1992)	F	16.4	Not recorded
Case No. 7 (442-1977)	F	9.3	Not recorded
Case No. 8 (444-1977)	M	12.9	Not recorded