1   *Pathology*

2   *Note*

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4   **Pulmonary neuroendocrine tumor in a female wolf (*Canis lupus lupus*)**

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14   **SHORT RUNNING TITLE**: PULMONARY NEUROENDOCRINE TUMOR IN WOLF

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20   ABSTRACT. A 17-year-old female wolf (*Canis lupus lupus*) had a right lung mass that

21   was adhered to the thoracic cavity. Histopathological examination revealed that the mass

22   consisted of sheets, cord or ribbon-like structures of monotonous, small, cuboidal cells

23   with round, oval or short-spindle nuclei and scant clear cytoplasm, demarcated by a fine

24   fibrovascular stroma. Focal necrosis, congestion and thrombi were observed.

25   Immunohistochemically, the tumor cells diffusely expressed cytokeratin AE1/AE3, and

26   some expressed chromogranin A, neural cell adhesion molecule (CD56) and thyroid

27   transcription factor-1. The number of proliferating cell nuclear antigen-positive tumor cells

28   was low. A diagnosis of pulmonary neuroendocrine tumor was based on the resemblance to

29   carcinoids.

30   **KEYWORDS**: chromogranin A, lung; NCAM (CD56), neuroendocrine tumor, wolf

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32   Pulmonary neuroendocrine tumors are considered to be derived primarily from pulmonary

33   neuroendocrine cells, known classically as bronchial Kulchitsky-type cells [12]. In

34   humans, these tumors are associated with a wide prognostic spectrum, ranging from low-

35   and intermediate-grade carcinoid tumors (typical carcinoma [TC] and atypical carcinoma

36   [AC], respectively) to high-grade, large-cell neuroendocrine carcinomas (LCNECs) and

37   small-cell carcinomas (SCLCs) [12]. The incidence of neuroendocrine lung tumors is

38   extremely low in animals, including dogs [3-5] and cats [11]. We reported the case of a 17-

39   year-old female wolf (*Canis lupus lupus*) that had pathological features of pulmonary

40   neuroendocrine tumor that appeared to be both TC and AC.

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42   The female wolf (*Canis lupus lupus*) was born at a zoo in Russia in 1997 and was moved

43   to the first zoo in Japan in 1998. She was moved in 2006 to a second zoo, Hirakawa

44   Zoological Park, where she lived for the rest of her life. She received good nutrition, and

45   before her death, she showed no clinical signs, including respiratory abnormalities. In

46   2014, she was found dead, with foamy vomit on the floor.

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48   A necropsy revealed that the anterior and middle lobes of the right lung were occupied

49   with a large, solid, irregular-shaped mass. On cut sections, the mass had pale pink or white

50   laminar structures, approximately 20 cm in diameter that had adhered to the thoracic wall

51   (Fig. 1). The posterior lobe of the right lung was collapsed. The right lung mass was fixed

52   in 10% neutral-buffered formalin, routinely processed and embedded in paraffin. Paraffin

53   sections (5 μm) were stained with hematoxylin and eosin (H&E). The sections were also

54   stained with Masson trichrome and Grimelius. For immunohistochemistry, sections were

55   incubated with the following primary antibodies against cytokeratin AE1/AE3 (1:50;

56   Dako, Glostrup, Denmark), chromogranin A (CGA, 1:3,000; Yanaihara Institute Inc.,

57   Fujinomiya, Japan), neuron-specific enolase (NSE, 1:200; Calbiochem, San Diego, CA,

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58   USA), synaptophysin (SYP, 1:500; Chemicon International Inc., Temecula, CA, USA),

59   neural cell adhesion molecule (NCAM, CD56, 1:200; Sigma-Aldrich, St. Louis, MO,

60   USA), thyroid transcription factor-1 (TTF-1, 1:200; Dako), proliferating cell nuclear

61   antigen (PCNA, 1:200; Dako) and factor VIII-related antigen (1:1,000; Dako). For antigen

62   retrieval, deparaffinized sections were heated in 10 mM citrate buffer (pH 6.0) by

63   autoclaving at 121°C for 10 min for the SYP and TTF-1, in Dako target retrieval solution

64   (pH 9.0, Dako Denmark A/S) by autoclaving at 121°C for 10 min for NCAM or in 10 mM

65   citrate buffer (pH 6.0) by microwaving at 90°C for 10 min for cytokeratin AE1/AE3.

66   Expression was detected using a VECTASTAIN® Elite ABC kit (Vector Laboratories, Inc.,

67   Burlingame, CA, USA) with 3,3′-diaminobenzidine/hydrogen peroxide as the chromogen.

68   The sections were then counterstained with hematoxylin. For negative controls, the

69   primary antibodies were replaced with non-immunized sera, and for positive controls,

70   these were applied to the lung, adrenal and small intestine tissues.

71

72   Histopathological examination revealed that the mass was not encapsulated, but

73   demarcated by a fine to thick fibrovascular stroma (Fig. 2A), which was positive for

74   Masson trichrome. Focal necrosis and congestion were common, sometimes together with

75   the formation of thrombi. The tumor cells were arranged in sheets (Fig. 2B), cord (Fig. 2C)

76   or ribbon-like (Fig. 2D) structures of monotonous small columnar tumor cells, relatively

77   small but a little larger than that of a resting lymphocyte. The tumor cells had round, oval

78   or short-spindle nuclei with finely granular chromatin and inconspicuous nucleoli. A

79   ribbon-like growth pattern of the tumor cells was the most common arrangement. Nuclear

80   palisading and moulding were common; however, no mitotic figures were found in our

81   observation. Pale eosinophilic or clear cytoplasm was very scant and showed little positive

82   reaction for Grimelius stain, despite adequate controls. Pseudofollicular formation of tumor

83   cells was observed; however, no typical tubular or acinar growth pattern of the tumor cells

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84   were observed in any part of the tumor mass. Immunohistochemically, the strong

85   expression of cytokeratin AE1/AE3 was observed diffusely in the tumor mass (Fig. 3A). A

86   patchy or focal strong expression of NSE was identified (Fig. 3B), and the weak expression

87   was noted in the periphery of the strongly reactive areas. A similar patchy strong

88   expression of CGA was scattered (Fig. 3C); however, the areas with CGA expression were

89   limited, compared with the NSE expression. Nuclear expression of TTF-1 was seen in a

90   small population of tumor cells. Membranous expression of NCAM was also observed in a

91   very small population of tumor cells (Fig. 3D). No expression of synaptophysin and

92   vimentin was confirmed. The number of PCNA-positive tumor cells was very low: 1.3% in

93   10-high-power field ($\times$400); this was consistent with the absence of mitotic figures. Small

94   to large vasculatures labeled with factor VIII-related antigen were observed in the

95   interstitium of the tumor mass. Reliable immunoreactions of all the markers were

96   confirmed in internal control tissues.

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98   The present case was an extremely large intrathoracic mass and was characterized by

99   histopathologic features, such as proliferation of relatively small, monotonous, round cells

100   that were arranged primarily in a ribbon-like growth pattern. There was evidence of a

101   pseudofollicular formation of tumor cells; however, these structures appeared to be simply

102   derived from disassociation between tumor cells with sheet or cord-like structures. The

103   microscopic features suggested the tumor was a neuroendocrine tumor; however, evidence

104   of immunohistochemical neuroendocrine differentiation was limited. Non-small cell lung

105   carcinoma, especially adenocarcinoma with neuroendocrine differentiation, has been

106   identified in humans; a population of the tumors reacted positively with neuroendocrine

107   markers, such as chromogranin A or synaptophysin [1, 10]. The tumor is considered a

108   transitional form of neuroendocrine carcinoma, characterized by increasing neuroendocrine

109   differentiation that parallels the worsening of the prognosis compared with ordinary non-

110   small cell lung carcinoma [10]. This category might not be suitable to the present case,

111   because our case was composed of tumor cells with only neuroendocrine morphology, but

112   lacking morphological features of bronchiolar/alveolar adenocarcinoma.

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114   To further characterize the tumor cells, we compared the present case with dogs and other

115   species with pulmonary neuroendocrine/carcinoid tumors. In many cases of tumors in

116   dogs, neuroendocrine tumor cells with relatively abundant cytoplasm are arranged in an

117   endocrine (organoid nesting) growth pattern, such as packets, lobules or sheets

118   separated by a fibrovascular stroma [3-6, 8]. As in our case, strands or ribbon-like

119   arrangements of the tumor cells were reported less often in the lung of a cat [11], but

120   were observed in other organs, such as cutaneous [2] and hepatobiliary systems of cats

121   [8, 9]. In humans, carcinoid tumors are characterized by cells with uniform cytological

122   features and several growth patterns (i.e. organoid, trabecular, insular, palisading, ribbon

123   and rosette-like arrangements) and highly vascularized fibrovascular stroma that suggest

124   neuroendocrine differentiation. Such tumors are subdivided into low-grade TC and

125   intermediate-grade AC based on the appearance of mitosis and necrosis [12]. The

126   present case had common tumor features that are shown by both types of carcinoids;

127   key features were the presence of focal necrosis, such as AC, and a low proliferative

128   activity of tumor cells, such as TC. Similar cases involving apparent TC [3] and AC [5]

129   are reported in dogs. Therefore, in this case, the diagnosis made was a neuroendocrine

130   tumor with intermediate characteristics of TC and AC, as reported in dogs.

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132   Use of immunohistochemistry for CGA, synaptophysin, NSE and/or NCAM may help

133   to diagnose neuroendocrine tumors [12]. The present case had a relatively limited

134   positive reaction to CGA and NCAM and a negative reaction to synaptophysin. The

135   data, however, were supported by previous findings of variable immunoreaction in each

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136   case of neuroendocrine tumors. For instance, a positive reaction to both CGA and

137   synaptophysin was shown in neuroendocrine tumors that occurred in the mediastinum

138   [7] and the carina [4] of dogs, and only a positive reaction to CGA, not synaptophysin,

139   was shown in a lung carcinoid case in a dog [3]. NCAM is known as one of the most

140   reliable markers for pulmonary neuroendocrine tumors in humans [12]; however, to the

141   best of our knowledge, no such data were reported in animal tumors. Of note, the

142   staining with TTF-1 was confirmed to be restricted in a small population of tumor cells

143   in a similar manner to that observed in human neuroendocrine tumors [12].

144   Furthermore, the unique nature of these tumor cells is demonstrated by

145   immunohistochemical responses for cytokeratin and vimentin: one case was positive for

146   cytokeratin and vimentin [6], and another was negative for both [3]. In contrast these

147   findings, our case was positive for cytokeratin and negative for vimentin. These data

148   suggest that the tumors might have a broad spectrum of cellular differentiation or are

149   derived from different cell lineages. Even though a limited population of positive cells

150   reacted to these selected neuroendocrine markers, the morphological features in our case

151   facilitate the diagnosis of neuroendocrine tumor.

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189 **Figure legends**

190 Fig. 1. Macroscopic findings of the lung tumor. The solid, irregular-shaped tumor mass

191 with pale pink or white laminar structures spread over the thoracic cavity. Heart and

192 lung lobes are observed close to the tumor mass in the upper left side.

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194 Fig. 2. Histopathological findings in the lung tumor. (A) The tumor was demarcated by

195 thick fibrous stroma. Thrombi and congestion are seen at the upper left site. (B-D)

196 Small monotonous tumor cells with ovoid nuclei and scant cytoplasm are arranged in a

197 sheet (B) and cord (C) or ribbon-like (D) growth pattern. H&E stain. Bar = 12.5 or 50

198 μm (B-D).

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200 Fig. 3. Immunhistochemistry of cytokeratin AE1/AE3, NSE, CGA and NCAM in the

201 lung tumor. (A) Strong expression of cytokeratin AE1/AE3 is observed in tumor cells.

202 (B, C) Strong to moderate expression of NSE (B) and CGA (C) is observed in some

203 populations of the tumor cells. (D) Strong to weak expression of NCAM (CD56) is

204 observed in the membranes of the restricted numbers of tumor cells. Bar = 50 (A-C) or

205 100 μm (D).

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207

Fig. 1. Macroscopic findings of the lung tumor. The solid, irregular-shaped tumor mass
with pale pink or white laminar structures spread over the thoracic cavity. Heart and
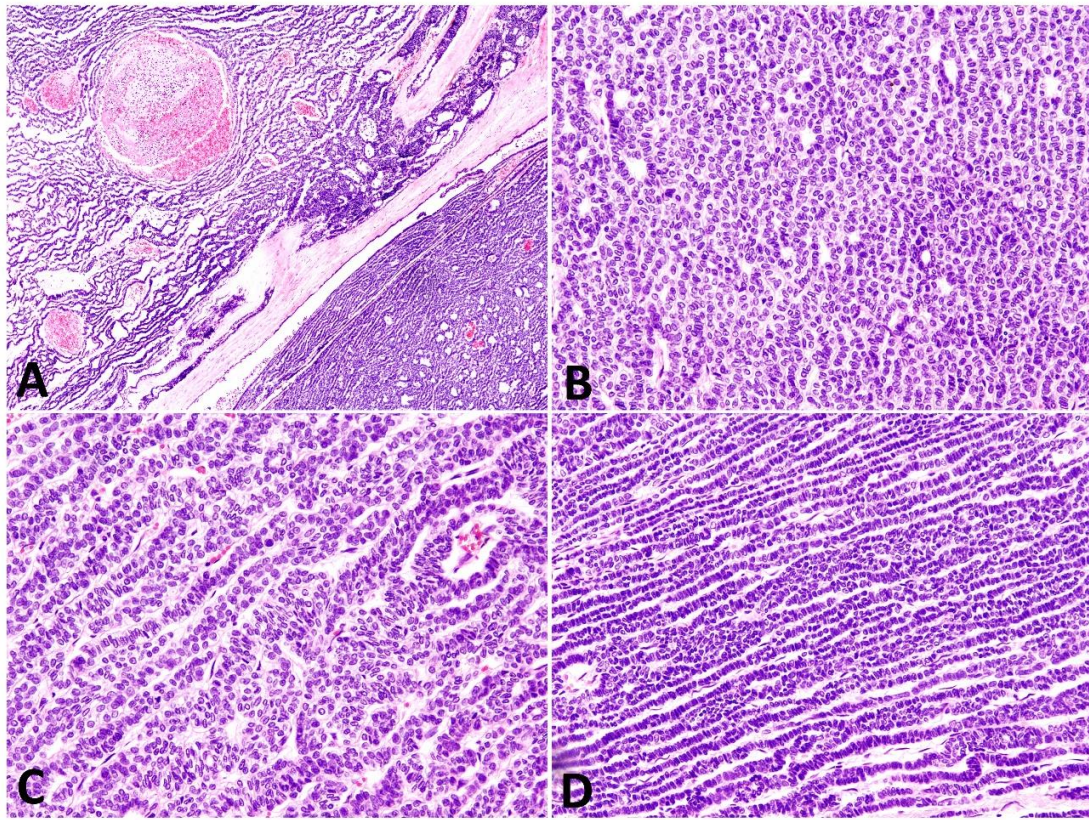lung lobes are observed close to the tumor mass in the upper left side.

Fig. 2. Histopathological findings in the lung tumor. (A) The tumor was demarcated by thick fibrous stroma. Thrombi and congestion are seen at the upper left site. (B-D) Small monotonous tumor cells with ovoid nuclei and scant cytoplasm are arranged in a sheet (B) and cord (C) or ribbon-like (D) growth pattern. H&E stain. Bar = 12.5 or 50 μm (B-D).
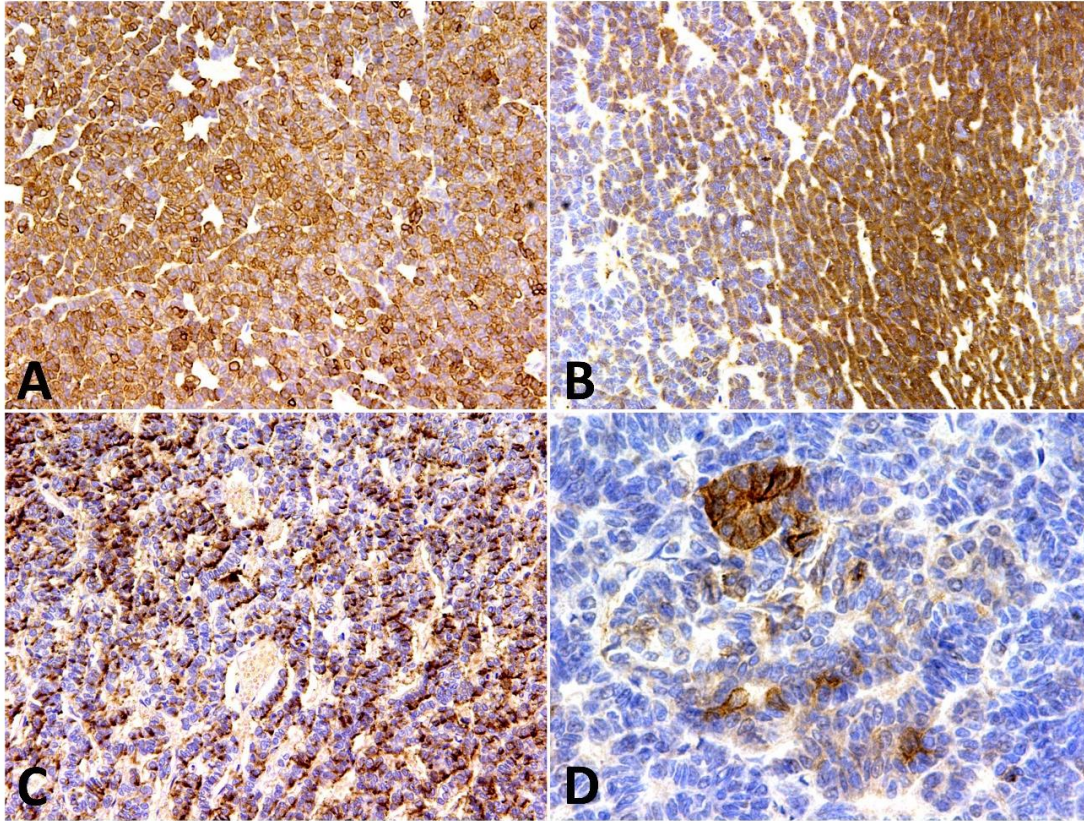
Fig. 3. Immunhistochemistry of cytokeratin AE1/AE3, NSE, CGA and NCAM in the lung tumor. (A) Strong expression of cytokeratin AE1/AE3 is observed in tumor cells. (B, C) Strong to moderate expression of NSE (B) and CGA (C) is observed in some populations of the tumor cells. (D) Strong to weak expression of NCAM (CD56) is observed in the membranes of the restricted numbers of tumor cells. Bar = 50 (A-C) or 100 μm (D).