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Spontaneously arising disease

Epidermal growth factor-containing fibulin-like extracellular matrix 1-derived amyloidosis with fatal gastric bleeding in a Tsushima leopard cat (*Prionailurus bengalensis euptilurus*)

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ABSTRACT

Epidermal growth factor-containing fibulin-like extracellular matrix 1 (EFEMP1)-derived amyloidosis causes gastrointestinal bleeding in humans. Amyloidosis has also been reported in the Tsushima leopard cat (*Prionailurus bengalensis euptilurus*) and rhesus macaque (*Macaca mulatta*). However, the clinical signs and lesions in humans and animals differ. A captive Tsushima leopard cat, aged 19 years, died of haemorrhage due to a gastric ulcer and a pathological analysis was performed. Amyloid was deposited in the venous walls and interstitium of the systemic organs, especially the stomach and intestines. The amyloid appeared as a weakly basophilic hyaline-like substance and stained with Congo red. Immunohistochemistry and liquid chromatography-tandem mass spectrometry revealed that the amyloid was composed of both N- and C-terminal EFEMP1 peptides. The amyloid fibrils were approximately 10 nm wide, unbranched, short and randomly arranged. The patient died from gastric bleeding caused by EFEMP1-derived amyloidosis. These findings suggest that EFEMP1-derived amyloidosis in Tsushima leopard cats has pathological features similar to those in affected humans, including amyloid deposition predominantly in the walls of veins and fatal gastrointestinal bleeding.

In animals, 22 types of amyloidosis have been identified, each differing in affected tissues, animal species and pathological features due to variations in their precursor proteins [1,2]. Epidermal growth factor-containing fibulin-like extracellular matrix protein 1 (EFEMP1)-derived amyloidosis was first reported as a cause of fatal gastrointestinal bleeding in older people [3]. EFEMP1, also known as fibulin-3, is an extracellular matrix glycoprotein belonging to the eight-membered fibulin protein family, and is expressed in systemic organs including the stomach, intestine, pancreas, lung, heart, skeletal muscle, liver, spleen, kidney, prostate gland, testis, ovary, placenta and brain [4]. EFEMP1-derived amyloid is deposited systemically in venous walls and the interstitium with age [3,5]. In animals, EFEMP1-derived amyloid deposition has been reported in the venous walls of an aged rhesus macaque (*Macaca mulatta*) and in the arterial walls of an aged Tsushima leopard cat (*Prionailurus bengalensis euptilurus*) [6,7]. However, the pathogenesis and distribution of amyloid deposition in these species is unclear because of a lack of information on clinical signs and a limited number of cases. The Tsushima leopard cat is an endangered wild cat inhabiting the Tsushima Islands, Nagasaki Prefecture, Japan, and is regarded as a subspecies of the leopard cat [8]. This species is classified as critically endangered by the Ministry of the Environment and designated as a national natural monument by the Government of Japan [8,9].

A 19-year-old male Tsushima leopard cat, weighing 3.3 kg, bred in a zoo in Japan, died after a 5-month deterioration in its general condition, which included anorexia, haematuria and difficulty standing. At necropsy, samples from the heart, lung, liver, kidney, spleen, pancreas, gastrointestinal tract, oesophagus, eye, bladder, ureter, thyroid, adrenal gland and brain were collected, fixed with 10 % phosphate-buffered formalin and embedded in paraffin. Sections were cut at 3 μm for haematoxylin and eosin (HE) staining and immunohistochemistry (IHC) and at 6 μm for Congo red staining. Staining was performed according to

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standard protocols. To identify the amyloid precursor protein, IHC was performed using the anti-N-terminal and anti-C-terminal region of the human EFEMP1 antibody (Cloud-Clone Corp., www.cloud-clone.com). The secondary antibody used was horseradish peroxidase-conjugated polymer anti-rabbit IgG antibody (Dako, www.agilent.com). Reactions were visualized by 3,3′-Diaminobenzidine tetrahydrochloride staining. As a negative control, the primary antibody was omitted. Tissues previously diagnosed with EFEMP1-derived amyloidosis were used as the positive control [6].

Transmission electron microscopy (TEM) examination was outsourced to PCL Japan (www.pcljapan.co.jp). Formalin-fixed materials from the stomach were post-fixed with 2 % osmium tetroxide solution and embedded in epoxy resin. Ultrathin sections were examined using a transmission electron microscope (HT7700; Hitachi High-Tech, www.hitachi-hightech.com).

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed as previously described [10,11]. Briefly, Congo red-positive amyloid deposits were dissected under a stereomicroscope from the stomach, digested with trypsin and subjected to LC-MS/MS. To elucidate the protein composition of the amyloid deposits, MS/MS spectra obtained from trypsin-digested samples were collated to theoretical fragment ion patterns of tryptic peptides derived from *Felis catus* proteins listed in the UniProt database, using the Mascot Server (Matrix Science Inc., www.matrixscience.com). To determine which region of the precursor protein was incorporated into the amyloid, the MS/MS data were additionally aligned with the EFEMP1 protein sequence of the Tsushima leopard cat available in the NCBI database (accession no: BEI31667.1). Peptides with statistically significant matches (pep_expect <0.05) were identified using Mascot's probability-based scoring algorithm.

A large blood clot was attached to the mucosa of the cardiac region of the stomach and removal of the clot revealed an underlying ulcer (1.0 × 1.5 cm) (Fig. 1). Cloudy brown ascites fluid with mixed gastric contents was seen due to a full-thickness rupture of part of the gastric wall. A small amount of cloudy, brown liquid was also present in the small and large intestines but no significant lesions were seen in the intestinal walls. The liver had a mosaic pattern due to regions of haemorrhage, light brown discolouration, dark red congestion and areas with normal colour. Multiple poorly circumscribed nodules with a maximum diameter of 2.8 cm were seen in each lobe. Bilaterally, the kidneys were small (right: 2.3 × 3.0 cm; left: 2.3 × 3.1 cm) and anaemic with rugged cortical surfaces. Pus accumulation was present in the subcutaneous tissue on the right side of the shoulder. The lungs were incompletely collapsed with mild oedema that was more pronounced on the right side. A corneal ulcer of the left eye and decubitus ulcer on the left side of the lumbar region were observed. There were multiple white nodules in the pancreas (1.0–6.0 mm) and adrenal glands (1.0–3.0 mm). No significant lesions were found in the heart or spleen.

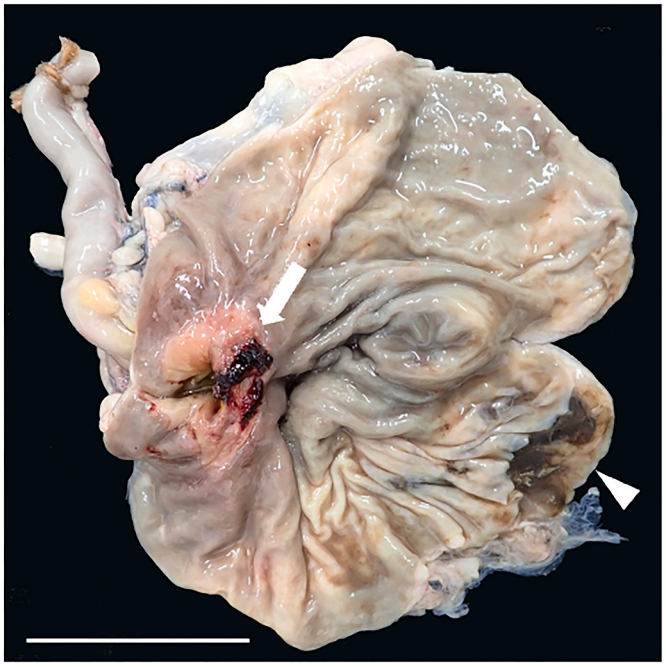

Fig. 1. EFEMP1-derived amyloidosis, stomach, Tsushima leopard cat. Ulcer with haemorrhage in cardia (arrow). Stomach wall ruptured (arrowhead). Bar, 5 cm.

Histologically, a weakly basophilic hyaline-like substance was seen in the venous walls and interstitium of the submucosa, serosa of the stomach and adipose tissue surrounding the stomach (Fig. 2a). The substance was also found in the wall of veins and interstitium of the intestine, lung, bronchial gland, muscular layer of the urinary bladder and oesophagus, pancreas, surrounding tissue of the adrenal gland and ureter, femoral muscle, thyroid gland and sclera. Additionally, it was detected in the small arteries of the thyroid, lung, pancreas and adrenal glands. In the gastrointestinal tract, although the substance was deposited widely, the ulcerated area was especially affected. In the vascular wall, the substance was seen in all layers of the venous wall and predominantly in the adventitia of the arteries. The hyaline-like substance stained with Congo red and had green birefringence under polarizing light, confirming it as amyloid (Fig. 2b and c). In the stomach, the ulcer consisted of a mucosal defect that extended into the submucosa with coagulative necrosis in the submucosa as well as haemorrhage. The lesion was accompanied by inflammatory cell infiltration, predominantly of neutrophils. In the muscular layer, mild neutrophilic inflammation was seen; necrosis was not seen in most regions except for the inner portion of the muscular layer adjacent to the submucosa. In addition, moderate to severe neutrophilic inflammation, mild necrosis and mild fibrin deposition were observed in the serosa and adipose tissue surrounding the stomach. These inflammatory changes were seen only at the site of the gastric ulcer. In a rupture of part of the gastric wall, there was no evidence of an ante-mortem reaction, such as infiltration of inflammatory cells, fibrin deposition or haemorrhage. Severe interstitial fibrosis of both the medulla and cortex of the kidneys, localized, mild papillary hyperplasia of the urinary bladder mucosa, bilateral parathyroid adenomas, multiple biliary duct hyperplasia and multiple nodular hyperplasia of hepatocytes, multifocal cortical nodular hyperplasia of both adrenal glands, multifocal nodular hyperplasia of the pancreatic exocrine tissue and localized neutrophilic inflammation in the subcutis of the right shoulder were also seen.

Immunohistochemically, the amyloid was positive with the antibodies against both the N- and C-terminal regions of EFEMP1 (Fig. 3). Ultrastructurally, the amyloid fibrils were approximately 10 nm wide, unbranched, short and randomly arranged (Fig. 4).

In the proteomic analysis, EFEMP1 was detected as a major component of the amyloid deposits using LC-MS/MS, with a high score on the exponentially modified protein abundance index in the Mascot analysis. N-terminal peptides of EFEMP1 were detected across a broader sequence range than C-terminal peptides (Supplementary data).

The patient was diagnosed with EFEMP1-derived amyloidosis and gastric bleeding. In this case, EFEMP1-derived amyloid deposition was particularly severe in the ulcerated region of the stomach, prominently affecting the venous wall and interstitium. IHC and proteomic analysis revealed that the amyloid consisted of both N- and C-terminal EFEMP1 peptides. The stomach had a large ulcer and haemorrhage associated with EFEMP1-derived amyloidosis. Commonly, amyloid deposition increases the functional and structural vulnerability of tissues, and venous EFEMP-derived amyloid deposits may affect venous function in older humans [3]. We reasoned that excessive EFEMP1-derived amyloidosis contributed to the development of the gastric ulcer and massive haemorrhage, which was considered the cause of death of this cat. Gastric rupture with amyloid deposition was observed but there was no evidence of any ante-mortem reaction at the site. Therefore, it was

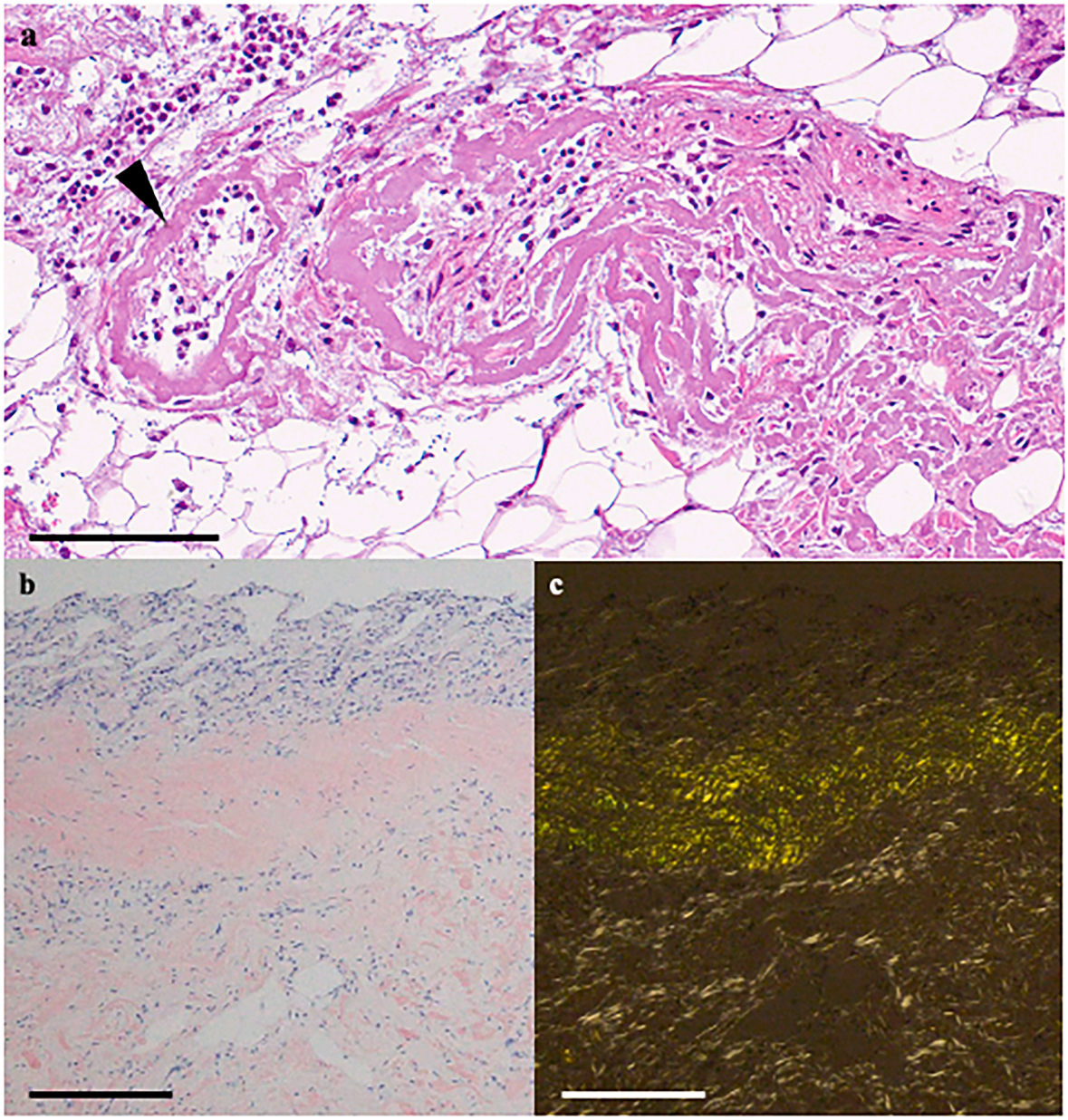

Fig. 2. EFEMP1-derived amyloidosis, Tsushima leopard cat. (**a**) Deposition of weakly basophilic amyloid in venous wall (arrowhead). HE. Bar, 100 μm. (**b**) Amyloid stained with Congo red and (**c**) had green birefringence under a polarizing microscope. Congo red. Bar, 200 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

considered that the gastric rupture was an agonal or post-mortem change, likely resulting from increased gastric wall vulnerability due to amyloid deposition.

EFEMP1-derived amyloid deposits were most frequently seen in the venous wall and interstitium of the gastrointestinal tract, with additional deposition in other tissues, including the lung and urinary bladder. Notably, no amyloid deposition was observed in the liver, heart or kidneys, which is consistent with the distribution pattern reported in human EFEMP1-derived amyloidosis [3]. In contrast, a reported case of EFEMP1-derived amyloid deposits in a Tsushima leopard cat was associated with amyloid deposition in the arterial wall and interstitium of systemic organs, including the liver, kidney, spleen and heart, in addition to tissues affected in the present case [6]. This difference in the extent of amyloid deposition may reflect the severity of amyloidosis. Regardless of the severity, amyloid deposition in the stomach presents a risk of death-related haemorrhage. However, since only a very limited number of cases have been reported, further investigations involving a larger case series are warranted.

Based on the results of IHC and LC-MS/MS, it is suggested that the amino acid regions spanning residues 112–220 and 423–446 were included in the amyloid deposits. In a previous study, EFEMP1-derived amyloid deposits in a Tsushima leopard cat consisted of N-terminal peptides [6]. In contrast, both N- and C-terminal peptides were detected in the amyloid deposits of the current case. Also, N-terminal peptides were identified across a wider range of sequences than the C-terminal peptides. These findings may support the hypothesis proposed in a report of EFEMP1-derived amyloidosis in a rhesus macaque, which suggested that full-length EFEMP1 initially deposits in tissues, followed by self-association of the amyloidogenic region that leads to amyloid formation [7].

EFEMP1-derived amyloid deposition or amyloidosis has been described in aged humans [3,5,12] and animals, such as a 15-year-old Tsushima leopard cat [6] and a 37.5-year-old rhesus macaque [7]. The mean age at death of 58 captive Tsushima leopard cats was 12.1 years in

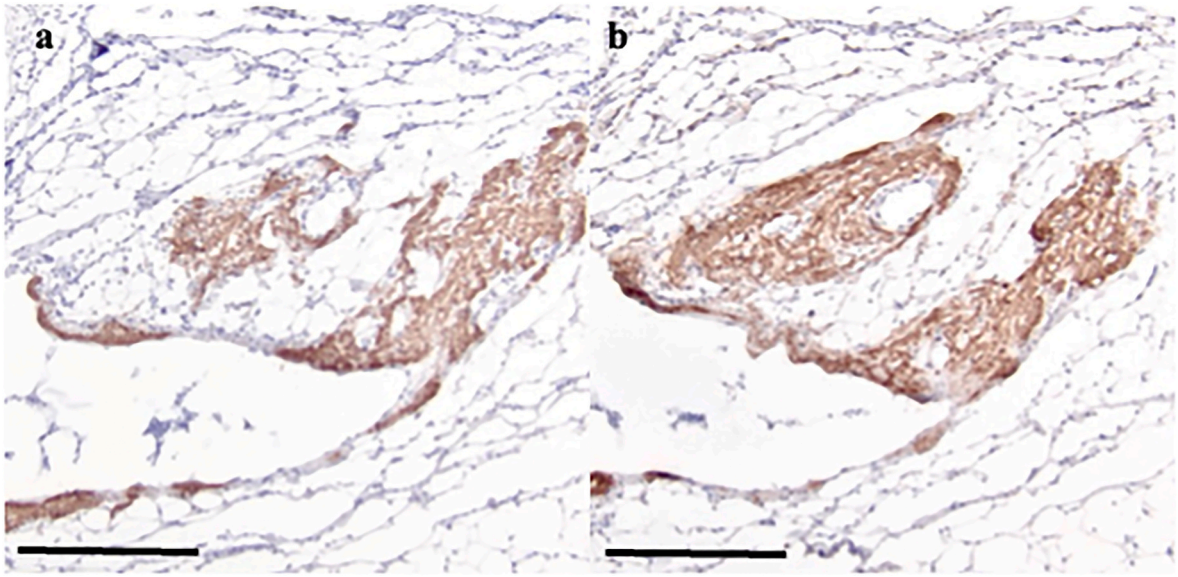

Fig. 3. EFEMP1-derived amyloidosis, Tsushima leopard cat. Amyloid immunopositive with both anti-N-terminal (**a**) and anti-C-terminal (**b**) EFEMP1 antibodies. IHC. Bar, 250 μm.

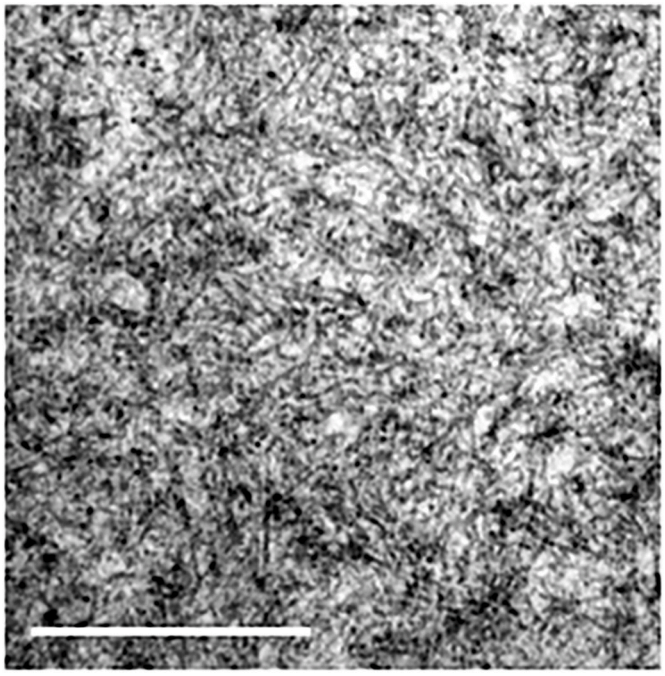

Fig. 4. EFEMP1-derived amyloidosis, stomach, Tsushima leopard cat. Randomly arranged amyloid fibrils. TEM. Bar, 500 nm.

a post-mortem analysis of this species [13]. Thus, the current patient was considered to be elderly and it seems that EFEMP1-derived amyloid may be deposited with age, as in humans. In addition, Ichimata *et al* [12] reported that enterocolic granulomatous phlebitis is associated with EFEMP1-derived amyloidosis in humans. In the current case, venous inflammation was not seen. Furthermore, although various lesions were observed in the systemic organs, EFEMP1-derived amyloid was not seen to co-localize with these lesions histopathologically. Thus, these lesions did not appear to involve EFEMP1-derived amyloidosis and were considered incidental changes.

We describe the first case of fatal gastric bleeding caused by EFEMP1-derived amyloidosis in animals. The Tsushima leopard cat is an endangered species in which there is a need for disease control. EFEMP1-derived amyloidosis should be suspected when gastrointestinal ulcers and bleeding are observed in this species. Further research on EFEMP1-derived amyloid may contribute not only to human medicine but also to the conservation of this species.

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Declaration of competing interest

The authors declared no conflicts of interest in relation to the research, authorship or publication of this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcpa.2025.07.009.

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