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EXPRESSION OF CLAUDINS IN THE NORMAL CANINE GASTRIC MUCOSA

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The aim of the present study was to investigate the expression pattern of claudin-1, -2, -3, -4, -5, -6, -7, -8, -10 and -18 in the intact fundic and pyloric gastric mucosa of dogs. Intense, linear, membranous claudin-18 positivity was detected in the surface gastric cells and in the epithelial cells of the gastric glands both in the fundic and pyloric stomach regions. The mucous neck cells in the apical part of the glands, furthermore the parietal cells and chief cells of the basal part of the gland were all positive for claudin-18, in the same way as the enteroendocrine cells. Cells of the basal part of the pyloric glands showed intense, linear, membranous claudin-2 positivity, but cells of the superficial portion of these glands and the surface gastric cells in this region were claudin-2 negative. Fibroblasts, endothelial cells, lymphocytes of the propria layer, smooth muscle cells and vegetative neurons were all negative for claudin-2. All gastric epithelial cells were negative for claudin-1, -3, -4, -5, -6, -7, -8 and -10. The endothelial cells of the propria layer had intense claudin-5 positivity. We assume that claudin-18 forms a paracellular barrier against gastric acid in the healthy canine stomach, in the same way as in mice.

Key words: Gastric mucosa, immunohistochemistry, claudins, dogs

Claudins are a family of functional tight junction (TJ) proteins expressed by epithelial and endothelial cells that have four transmembrane domains, with the N-terminus and the C-terminus in the cytoplasm, and with the hydrophobic loops in the extracellular space (Morita et al., 1999; Tsukita and Furuse, 1999). It has been established that TJs are the most apical intercellular junctions and have an impact on paracellular permeability by creating selective barriers to lipids and proteins between the apical and basolateral membrane domains (Furuse et al., 1998). Several human studies have revealed that claudins are involved in the

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epithelial-to-mesenchymal transition, and claudin proteins have a significant influence on tumour progression in several types of cancer (Sakamoto et al., 2010; Jung et al., 2011).

The expression pattern of claudin-1, -3, -4, -5 and -7 tight junction proteins were studied in dogs in the normal colorectum and in low-grade tubulopapillary colorectal carcinomas (CLGCCs) by Jakab et al. (2010). The normal colorectal mucosa was negative for claudin-1, -5, showed punctate positivity for claudin-3, -4, and diffuse lateral membranous pattern of staining for claudin-7. Claudin-1 was detected as a non-diffuse intense membrane labelling of neoplastic epithelial cells in canine low-grade colorectal cancer and 55% of the tumours showed a weak cytoplasmic pattern (of staining for claudin-1 protein). Claudin-3, -4 proteins were detected as a lateral labelling of neoplastic cells in CLGCCs. Claudin-7 protein was detected as an intense membrane labelling of neoplastic cells and showed weak cytoplasmic positivity in CLGCCs.

Clear expression of claudin-3, -5, and weak expression of claudin-7 protein were detected in healthy canine duodenal biopsy specimens obtained via endoscopy (Ohta et al., 2011).

The expression of claudin-1, -2, -3, -4, -5 and -7 was investigated in the normal canine mammary gland and in hyperplastic and neoplastic mammary lesions (Jakab et al., 2008). The results suggest that loss or reduction of expression of claudin-1, -2, -5 and -7 may lead to cellular disorientation, detachment and invasion in canine mammary neoplasia.

Positive claudin-18 expression is an independent risk factor for lymph node metastasis in human intrahepatic, intraductal papillary neoplasms (Shinozaki et al., 2011).

Although no data have yet been published in the veterinary literature on the occurrence of claudin proteins in the healthy canine gastric mucosa, the cross-reaction of humanised anti-claudin antibodies with the different canine tissues has been proved in several studies (Yu et al., 2003; Jakab et al., 2008, 2010; Sas et al., 2008).

The objective of the present study was to examine the expression and localisation of claudin-1, -2, -3, -4, -5, -6, -7, -8, -10 and -18 proteins in normal gastric mucosa of the fundic and pyloric regions in dogs. The results may provide valuable information for use in evaluating the importance of these TJ proteins in the pathogenesis of inflammatory bowel disease and gastric cancer in dogs.

Materials and methods

Histopathology

Twenty adult dogs were examined during the study (age: 3–14 years, mean: 8 years; 11 males, 9 females). Tissue samples 2 × 2 × 0.5 cm in size were

excised during necropsy from the fundus, body and antral region of the investigated canine stomachs.

The tissue samples were fixed in 8% neutral phosphate-buffered formalin solution at room temperature for 24 h, dehydrated in a series of ethanol and xylene, and embedded in paraffin. The 3–4 μm thick sections were routinely stained with haematoxylin and eosin (HE). The HE-stained slides were examined, and only the intact, non-neoplastic, non-inflammatory samples were chosen for further investigations.

Immunohistochemistry

Immunohistochemical detection of claudins was performed in formalin-fixed, paraffin-embedded sections. Three to four μm thick sections were cut from each paraffin block, deparaffinised, rehydrated and stained with antibodies to claudin-1, -2, -3, -4, -5, -6, -7, -8, -10 and -18. The slides were deparaffinised in xylene and graded ethanol. Prior to the application of the primary claudin antibodies (Table 1) at room temperature for 60 min, an appropriate antigen retrieval solution was used (Target Retrieval Solution, DAKO, Glostrup, Denmark, pH 6; microwave oven for 30 min). Immunohistochemical staining was performed with the streptavidin-peroxidase procedure (DAKO LSAB2 Kit). The chromogen substrate was 3, 3-diaminobenzidine (DAB). Mayer's haemalaun was used for counterstaining. In case of each claudin, appropriate tissue blocks were included for positive control (Table 1), and omission of the primary antibody was used as negative control.

The study protocol conformed to the relevant veterinary law and ethical regulations.

Results

The fundic surface gastric cells, cells of the apical portion of the fundic gland such as mucous neck cells, and cells of the basal part of the fundic gland including parietal cells, chief cells and enteroendocrine cells showed intense, linear, membranous claudin-18 positivity (Fig. 1). Fibroblasts, endothelial cells, lymphocytes of the propria layer, smooth muscle cells and vegetative neurons were all negative for claudin-18 (internal negative controls).

The pyloric surface gastric cells, cells of the apical and basal portions of the pyloric glands showed intense, linear, membranous claudin-18 positivity (Fig. 2).

Cells of the basal portion of the pyloric glands showed intense, linear, membranous claudin-2 positivity, but cells of the superficial portion of these glands and the surface gastric cells in this region were claudin-2 negative (Fig. 3). Fibroblasts, endothelial cells, lymphocytes of the propria layer, smooth muscle cells and vegetative neurons were all negative for claudin-2 (internal negative controls).

Table 1

Claudin-1, -2, -3, -4, -5, -6, -7, -8, -10 and -18 antibodies and positive control tissues used during immunohistochemistry

	Provider	Concentration	Positive control tissue	Staining pattern
Claudin-1	Zymed (polyclonal rabbit antibody)	1:100	Canine intact mammary gland	Membrane
Claudin-2	Zymed (monoclonal mouse antibody)	1:80	Canine intact mammary gland	Cytoplasm/ membrane
Claudin-3	Zymed (polyclonal rabbit antibody)	1:80	Canine intact mammary gland	Membrane
Claudin-4	Zymed (polyclonal rabbit antibody)	1:100	Canine intact mammary gland	Membrane
Claudin-5	Zymed (monoclonal mouse antibody)	1:100	Endothelial cell of the blood vessel in canine skin	Membrane
Claudin-6	Abcam (polyclonal rabbit antibody)	1:50	Human germ cell tumour	Membrane
Claudin-7	Zymed (polyclonal rabbit antibody)	1:80	Canine intact mammary gland	Membrane
Claudin-8	Zymed (polyclonal rabbit antibody)	1:100	Human kidney	Membrane
Claudin-10	Zymed (polyclonal rabbit antibody)	1:50	Canine intact mammary gland	Membrane
Claudin-18	Zymed (polyclonal rabbit antibody)	1:100	Human signet-ring cell carcinoma	Membrane

Surface gastric cells, cells of the fundic gland such as mucous neck cells, parietal cells, chief cells, enteroendocrine cells, and cells of the apical and basal parts of the pyloric glands were all negative for claudin-1, -3, -4, -5, -6, -7, -8 and -10.

Endothelial cells of the propria layer in both the fundic and pyloric regions showed intense claudin-5 positivity (Fig. 4).

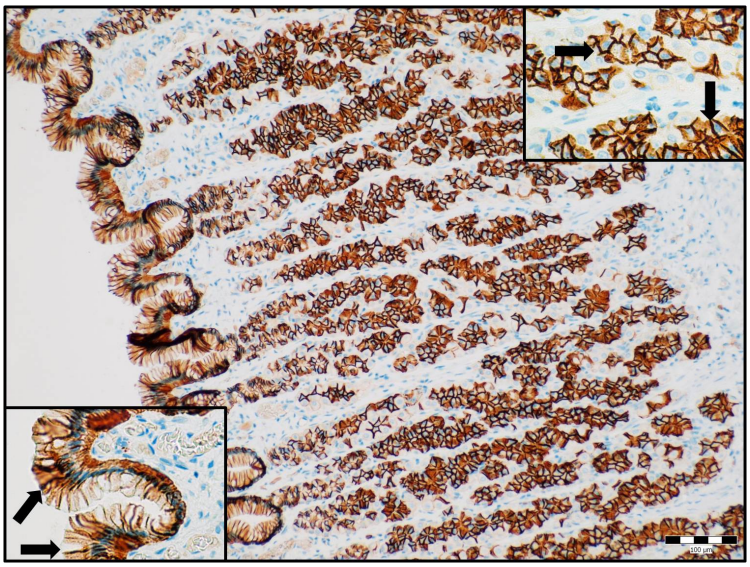

Fig. 1. Canine intact fundic surface gastric cells and intact cells of the fundic glands showed intense, linear, lateral, membrane claudin-18 positivity. Immunohistochemical staining (IHC), × 100, Bar = 100 µm. Inserts: fundic surface gastric cells (below) and cells of the fundic glands (above) with intense, linear, lateral, membrane claudin-18 positivity (arrows). IHC, × 400

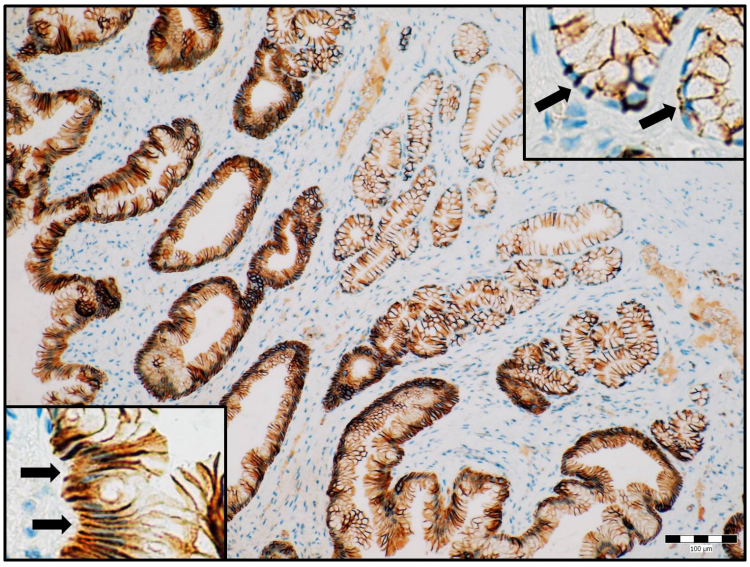

Fig. 2. Canine intact pyloric surface gastric cells and intact cells of the pyloric glands showed intense, linear, lateral, membrane claudin-18 positivity. IHC, × 100, Bar = 100 µm. Inserts: intact pyloric surface gastric cells (below) and intact cells of the basal part of the pyloric glands (above) with intense, linear, lateral, membrane claudin-18 positivity (arrows). IHC, × 400

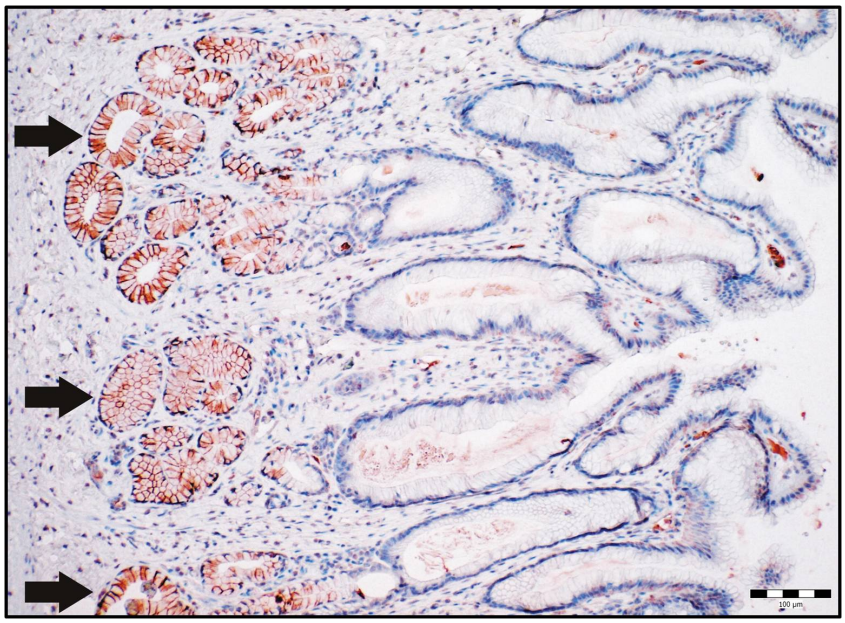

Fig. 3. Cells of the basal part of the pyloric glands (arrows) were positive for claudin-2. IHC, × 100, Bar = 100 µm

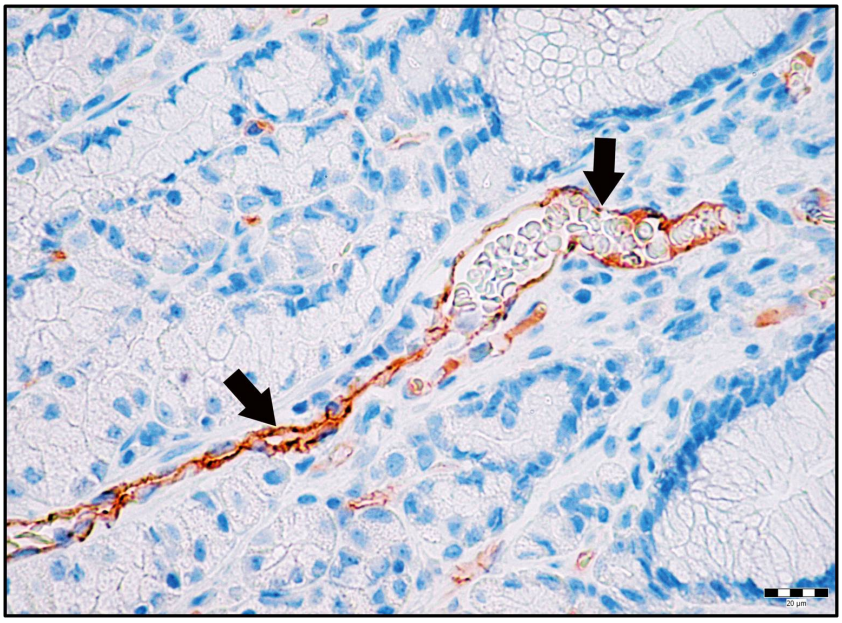

Fig. 4. Intense membrane claudin-5 positivity in the endothelial cells (arrows) of the gastric mucosa. IHC, × 400, Bar = 20 µm

Discussion

Dogs are simple-stomached animals. Their gastric mucosa is histologically and immunohistochemically diverse with regard to the types of gastric glands. These glands are divided into three groups according to the anatomical regions, such as cardiac, fundic and pyloric. Epithelial cell types of the fundic-pyloric gastric mucosa in dogs include bicarbonate-secreting surface epithelial (gastric) cells, mucous neck cells of the fundic gland (cells of the apical portion), acid-secreting parietal cells of the fundic gland (cells of the basal portion), pepsinogen-secreting chief cells (cells of the basal portion), neuroendocrine (enteroendocrine) cells, and cells of the apical and basal portions of the pyloric glands (Samuelson, 2007).

Claudins are key components of epithelial, mesothelial, perineural and endothelial tight junctions which act as a barrier to paracellular flow of water and solutes, and to the transmigration of other cells (Tsukita et al., 2001).

Only one study has investigated claudin expression in the gastric mucosa in dogs. This study was based on RT-PCR and immunohistochemistry (Türeci et al., 2011). The researchers demonstrated that the claudin-18 amino acid sequence is highly conserved in dog, mouse, rat, rabbit, monkeys, lizards and humans. Gene structure, promoter elements and RNA expression pattern of the lung-tissue-specific claudin-18 are all homologous across species.

In our present immunohistochemical study we investigated the claudin-1, -2, -3, -4, -5, -6, -7, -8, -10 and -18 expression pattern in the intact fundic and pyloric gastric regions of dogs.

The role and importance of these claudin proteins in tumour progression have been investigated in several studies in different human and animal tissues. Increased expression of claudin-6, -7 and -9 is sufficient to enhance tumorigenic properties of a gastric adenocarcinoma cell line (Zavala-Zendejas et al., 2011). Claudin-1 is not expressed in the epithelium of normal canine colorectal mucosa, but increased expression of this protein was observed in canine low-grade colorectal adenocarcinoma (Jakab et al., 2010). The reduced expression of claudin-1, -3, -4 and -5 plays a role in the carcinogenesis of canine pancreatic exocrine acinar cells. Claudin-7 is an immunohistochemical marker of benign and proliferative lesions of canine biliary epithelial cells, and has diagnostic value in the differentiation of cholangiocarcinomas from hepatocarcinomas in dogs (Jakab, 2012).

To the best of our knowledge, this is the first immunohistochemical study which analysed the expression of claudins in the canine stomach. According to our results, the surface gastric cells, the epithelial cells of the fundic and pyloric glands, and the enteroendocrine cells showed claudin-18 positivity.

In another immunohistochemical study gastritis was described in a stomach-specific claudin-18-knockout mouse line. The investigators demonstrated that the stomach-type claudin-18 is responsible for proton barrier function in the

stomach (Tamura et al., 2012). Its deficiency causes paracellular hydrogen ion leak, a persistent up-regulation of proinflammatory cytokines, chronic recruitment of neutrophils and atrophic gastritis in mice (Hayashi et al., 2012).

In this study we demonstrated claudin-18 positive immunoreaction in the normal canine gastric mucosa both in the fundic and pyloric regions. We suppose that claudin-18 normally forms a paracellular barrier against gastric acid in the canine stomach, in the same way as in mice and possibly in other species.

Lymphocytic-plasmacytic gastritis is the most common type of chronic gastritis in the dog (Day et al., 2008; Shabestari et al., 2008). Infection with gastric *Helicobacter*-like organisms (GHLO) can be responsible for some cases of gastritis and hyperplasia of parietal cells in dogs (Sapierzyński et al., 2006). The presence of GHLO colonisation, as well as the inflammatory state of the mucosa can alter the rate of gastric epithelial cell proliferation in dogs (Sapierzyński and Malicka, 2004). *Helicobacter pylori* activated myosin light-chain kinase in epithelial cells and increased the permeability of the gastric mucosa by disrupting claudin-4 and claudin-5 in C57BL/6 mice (Fedwick et al., 2005).

In our next immunohistochemical study we wish to investigate whether GHLO are able to disrupt the tight-junctional proteins, such as claudin-2 and -18 in the stomach of dogs with chronic gastritis.

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