

Expression of claudin-4 molecule in canine exocrine pancreatic acinar cell carcinomas

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Summary. *Aims:* Claudins, integral membrane proteins are components of the tight junction structures between epithelial and endothelial cells. These transmembrane proteins create a primary barrier to prevent paracellular transport of solutes, and also restrict the lateral diffusion of membrane lipids and proteins to maintain the cellular polarity. The aim of the present study was to characterise the expression pattern of claudin-4 tight junction molecule in canine normal pancreatic tissues and in the well-differentiated and poorly-differentiated pancreatic acinar cell carcinomas in canines.

Methods and results: The necropsy samples included canine intact pancreatic tissues, and canine well-differentiated and poorly-differentiated pancreatic acinar cell carcinomas samples. Claudin-4 was detected as an intense lateral membrane labelling of acinar cells in all intact pancreatic tissues. The intact epithelial cells of the different ducts were negative for the claudin-4 molecule. All primary and secondary canine well-differentiated exocrine pancreatic acinar cell carcinoma tissues showed intense apical lateral positivity for the claudin-4 molecule. All primary and secondary poorly-differentiated pancreatic acinar cell carcinoma tissues showed diffusely the loss of claudin-4 expression.

Conclusion: Consequently, we hypothesize that the loss of expression of claudin-4 plays a role in the progression of canine pancreatic acinar cell carcinoma and may lead to cellular detachment, disorientation and invasion of these pancreatic cancers. Furthermore, claudin-4 can be used as an immunohistochemical marker to distinguish canine well-differentiated and undifferentiated exocrine pancreatic acinar cell carcinomas.

Key words: Canine pancreas, Canine well-differentiated and poorly-differentiated pancreatic acinar cell carcinoma, Immunohistochemistry, Claudin-4

Introduction

Malignant tumours of the exocrine pancreas (pancreatic acinar cell carcinoma) have been reported in domestic animals such as *canine* (Anderson and Johnson, 1967; Rowlatt, 1967; Chang et al., 2007; Dennis et al., 2008), *feline* (Priester, 1974; Seaman, 2004), *horse* (Rowlatt, 1967; Priester, 1974), *cattle* (Rowlatt, 1967; Priester, 1974), *swine* (Anderson et al., 1956), *guinea fowl* (Toshkov et al., 1991), *guinea pig* (Reddy and Rao, 1975) and in experimental or exotic animals such as *rat* (Parsa et al., 1981), *mouse* (Plentz et al., 2009) and *ferret* (Whittington et al., 2006). The canine exocrine pancreatic acinar cell carcinoma can be divided into two main groups from a histological point of view: (1) well-differentiated and (2) poorly-differentiated (undifferentiated) carcinomas (Anderson and Johnson, 1967; Rowlatt, 1967; Head et al., 2002). Dennis et al. (2008) described a distinct variant of canine exocrine pancreatic carcinoma which was diagnosed in 6 dogs. These tumours were termed hyalinizing pancreatic adenocarcinoma (Dennis et al., 2008).

Claudins are a family of about 17–27 kDa integral membrane tight junction proteins which determine the size of molecules that can pass through the paracellular space in epithelial, mesothelial and endothelial tissues (Furuse et al., 1998). Claudin-4 tight junction integral protein consists of 209 amino acids and contains four putative transmembrane segments (Katahira et al., 1997). Claudin-3 and -4 are the receptors of *Clostridium perfringens* enterotoxin (Katahira et al., 1997; Morita et al., 1999), which is able to directly and rapidly lyse mammalian cells (McClane et al., 1988), suggesting that

these molecules can be evaluated as a possible therapeutic target to claudin-3 or/and -4 expressing epithelial tumours (Hough et al., 2000; Long et al., 2001; Michl et al., 2001). Claudin-4 has been utilized to differentially diagnose mesotheliomas from carcinomas and other tumour types such as serosal metastasis of the lung, breast, gastrointestinal tract, pancreas, ovary gland carcinomas and primary serous papillary carcinoma of peritoneum (Facchetti et al., 2007). There are several humanized immunohistochemical markers cross reactive with the same animal antigens (Frost et al., 2000; Rhind, 2002). The cross-reactive humanized anti-claudin antibodies can help the correct pathological differential diagnosis of different canine neoplastic lesions (Jakab et al., 2008b, 2009a,b, 2010b). Previous veterinary studies based on immunohistochemical analysis by humanized anti-claudin-4 antibody described that claudin-4 is expressed in different canine intact epithelial tissues, such as the luminal cells of the mammary gland lobules and ducts (Jakab et al., 2008a), the apocrine cells and duct epithelial cells in the sweat glands, the sebocytes and duct cells in the sebaceous glands (Jakab et al., 2008c), in the surface and crypt epithelial cells of the intact colorectal mucosa (Jakab et al., 2010a), and in canine normal hepatoid gland cells (Jakab et al., 2009b).

The aim of the present study was to evaluate the immunohistochemical detection of the claudin-4 molecule in formalin fixed paraffin-embedded canine intact pancreatic tissues, and well-differentiated, and poorly-differentiated exocrine pancreatic acinar cell carcinomas. To our knowledge, this is the first study that has examined claudin-4 molecule expression in canine intact pancreatic tissues and canine well-differentiated and poorly differentiated exocrine pancreatic acinar cell carcinomas by humanized anti-claudin-4 antibody.

Materials and methods

Histopathology

A total of 40 necropsy samples of canine pancreas were collected at Szent István University, Faculty of Veterinary Science, Department of Pathology and Forensic Veterinary Medicine between 2002 and 2010. We investigated 10 canine intact pancreatic samples collected during necropsy from 6 females and 4 males with an average age of 6.4 years (range: 4-10 years). The canine well-differentiated exocrine pancreatic acinar cell carcinoma samples (n=10) were collected from 2 females and 8 males with an average age of 11 years (range: 8-14 years). The canine poorly-differentiated exocrine pancreatic acinar cell carcinoma samples (n=10) were collected from 5 females and 5 males with an average age of 10.1 years (range: 8-16 years). Furthermore we investigated 5 intrahepatic metastases of well-differentiated and 5 intrahepatic metastases of poorly-differentiated acinar cell carcinomas.

Immunohistochemistry

All samples were fixed in 8% neutral buffered (in PBS, pH 7.0) formaldehyde solution for 24 hours at room temperature, dehydrated in a series of ethanol and xylene and embedded in paraffin. 3-4 μ m thick sections were cut, and routinely stained with haematoxylin and eosin. The slides for the humanized claudin-4 immunohistochemical reaction were deparaffinized in xylene and graded ethanol. The deparaffinized sections were treated with primary antibody humanized claudin-4 (diluted 1:100, mouse monoclonal, Zymed Inc., San Francisco, CA, USA) for 60 minutes at room temperature after treatment with appropriate antigen retrieval (Target Retrieval Solution, DAKO, Glostrup, Denmark, pH 6; microwave oven for 30 minutes). Immunohistochemical staining was performed using the streptavidin-peroxidase procedure. Antigen-bound primary antibody was detected using standard avidin-biotin immunoperoxidase complex (DAKO LSAB2 Kit). The chromogen substrate was aminoethylcarbazol (AEC). Mayer's haemalaun was used for counter-staining. Negative control was performed by the omission of the primary antibody. The external positive control was the low grade colorectal adenocarcinoma from a canine (Jakab et al., 2010a). The staining pattern was intense membranous.

Ten randomly selected areas of each slides were analysed by two independent pathologists (CSJ, JK) at high magnification (x400) with one hundred cells counted per field. Cross reactions were scored positive where linear membrane staining was seen.

Results

Claudin-4 was detected as an intense, lateral membrane labelling of acinar cells in all canine intact pancreas samples (Fig. 1A,B). In all canine normal pancreatic tissues the endocrine cells of the islet of Langerhans (Fig. 1C), the epithelial cells of the different ducts (Fig. 1D) and the stromal cells, the endothelial cells and lipocytes did not show positivity for the claudin-4 molecule (internal negative controls for the claudin-4).

All neoplastic acinar cells of canine well-differentiated pancreatic acinar cell carcinomas showed an intense, apical and lateral positivity for the claudin-4 molecule in the absence of intra-cytoplasmic positivity (Fig. 2A,B). All poorly-differentiated pancreatic acinar cell carcinoma samples showed diffusely the loss of claudin-4 expression. The pericarcinomatous normal exocrine pancreatic tissues were the internal positive controls in these samples (Fig. 2C,D). The hepatic metastases of well-differentiated acinar cell carcinomas showed an intense apical lateral positivity for the claudin-4, but the poorly-differentiated carcinoma cells were diffusely claudin-4 negative. The healthy liver cells, the biliary epithelial cells of the bile ducts, the

Claudin-4 expression in canine pancreatic cancer

Table 1. Summary of claudin-4 expression in canine and human hepatobiliary-pancreatic tissues and tumours.

Tissue/cell type/Tumour type	Canine expression	Human expression	Authors of human literature
Hepatocytes	-	-	Lódi et al., 2006.
Biliary ducts	-	+	Lódi et al., 2006.
Hepatocellular carcinoma	Not done	-	Lódi et al., 2006.
Biliary duct carcinoma	Not done	+	Lódi et al., 2006.
Pancreatic exocrine acini	+	+	Tsukahara et al., 2005.
Pancreatic ducts	-	+	Tsukahara et al., 2005.
Pancreatic Langerhans islets	-	-	Tsukahara et al., 2005.
Pancreatic carcinoma	+ (well-differentiated acinar carcinoma is positive, but the poorly-differentiated cancer is negative)	+/- (acinar carcinoma in some reports is negative while adenocarcinoma is positive but do not specify acinar or tubular)	Katalin B., 2009; Hewitt et al., 2006; Ouban and Ahmed, 2010.
Pancreatic ductal carcinoma	Not available*	+ (92%)	Tsukahara et al., 2005.

*Spontaneous pancreatic ductal adenocarcinoma (like human pancreatic ductal adenocarcinoma) and pancreatic intraepithelial neoplasias have not been reported in canine.

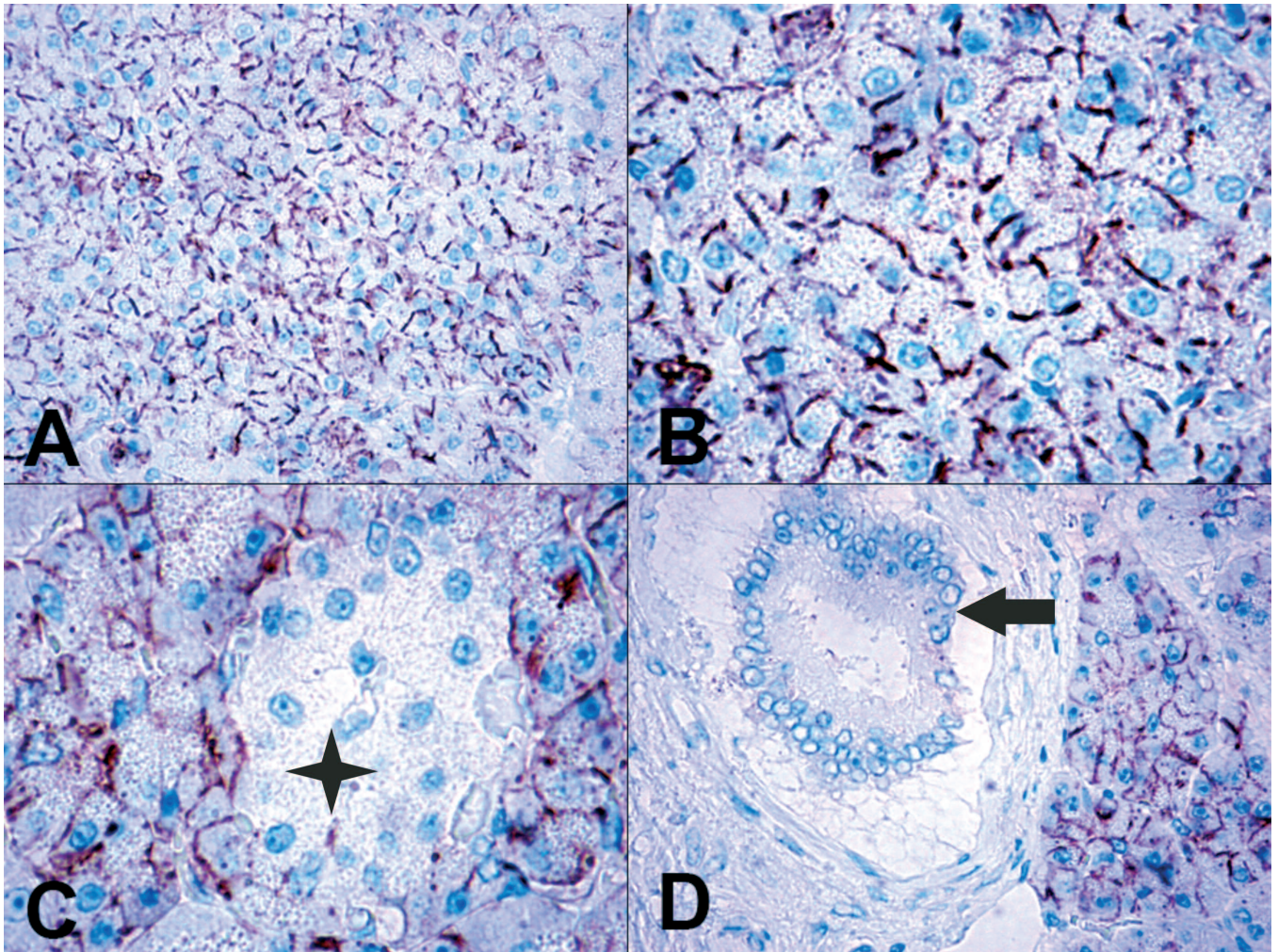


Fig. 1. A. Immunohistochemical labelling for claudin-4 demonstrated as an intense, membranous labelling of the canine intact acinar cells of the pancreas. B. Higher magnification. C. Absence of claudin-4 expression in the cells of the islet of Langerhans (asterisk) in the canine intact pancreatic tissue. D. The intact epithelial cells of the ducts (arrow) were negative for this claudin. IH. A, D, x 200; B, C, x 400

portal fibroblasts and the endothelial cells never expressed the claudin-4 molecule (internal negative controls for the claudin-4).

Discussion

Exocrine pancreatic carcinoma could derive from ductal epithelium or from acinar cells. Human pancreatic acinar cell carcinoma is a rare tumoural lesion, accounting for only about 1-2 % of all exocrine pancreatic neoplasms. This tumour most frequently occurs in middle-aged or elderly Caucasian men. Four histopathological groups of growth pattern have been described: acinar, solid, trabecular and glandular (Klimstra et al., 1992). The neoplastic cells resemble intact pancreatic acinar cells. Immunohistochemically, pancreatic acinar cell carcinoma recapitulates the secretory products of intact pancreatic acinar cells, including frequent production of digestive enzymes such as trypsin, lipase, chymotrypsin, and amylase (Caruso et

al., 1994). Banner et al. (1978) described that a canine exocrine pancreatic carcinoma is not a good model for a human exocrine pancreatic ductal carcinoma, because the most common canine exocrine pancreatic carcinomas are derived from acinar cells not from ductal epithelial cells. This means that in contrast to exocrine pancreatic ductal carcinomas the acinar variety of canine pancreatic carcinomas is more common in these species than previously thought (and than in human patients) and canine acinar cell carcinoma may serve as a model for human acinar cell carcinoma (Banner et al., 1978; Chang et al., 2007). However, based on the current results and on the human literature, claudin-4 expression shows differences among human and canine tissues (Table 1.). The pancreatic ductal carcinoma is the most important and constitutes the vast majority (more than 85 %) of human pancreatic tumours. Human oncopathology divides the ductal carcinoma of the exocrin pancreas into a well-differentiated and a poorly-differentiated type (Morohoshi et al., 1983). In the veterinary oncology,

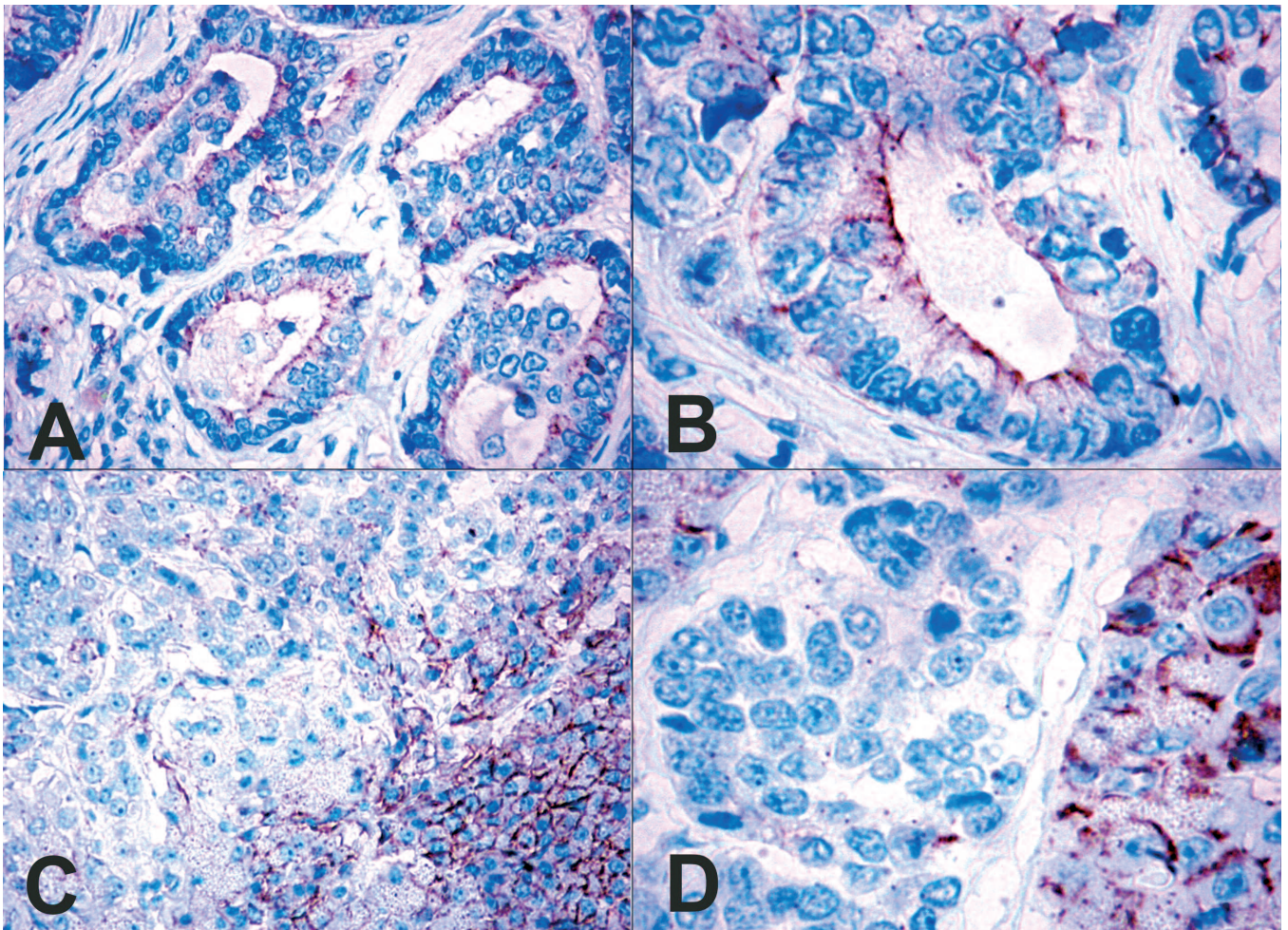


Fig. 2. A, B. The well-differentiated exocrine pancreatic carcinomas from canine showed intense apical lateral positivity for claudin-4. C, D. Solid island of the claudin-4 negative poorly-differentiated exocrine pancreatic carcinoma (left upper part of the picture) with pericarcinomatous claudin-4 positive exocrine pancreatic tissue (right lower part of the picture). IH. A, C, x 200; B, D, x 400

primary pancreatic ductal carcinomas have not been described in canines. This tumour was only experimentally induced by the administration of different carcinogenic material, such as N-ethyl-N'-nitro-N-nitrosoguanidine, through the drainage tube inserted into the pancreatic duct (Kamano et al., 1988).

In the present study we describe that in all canine intact pancreatic samples the pancreatic acinar cells (first type of the exocrine cells of the pancreas) showed diffuse high intense membranous claudin-4 positivity. Borka et al. (2007) described that claudin-4 protein was expressed in the acinar cells of the normal human pancreatic tissues (similar to our result) as well as in the epithelial cells of the healthy pancreatic ducts (second type of the exocrine cells of the pancreas). However, the endocrine cells of the islet of *Langerhans* were claudin-4 negative. They used a similar anti-claudin-4 antibody (mouse monoclonal, Zymed Inc., San Francisco, CA, USA) as we did and a similar dilution (1:100) to ours (Borka et al., 2007). Contrary to the result of the human study, in which the epithelial cells of the normal pancreatic ducts expressed the claudin-4 protein (Borka et al., 2007), the epithelial cells of canine normal pancreatic ducts were negative for claudin-4. This is the first difference in claudin-4 expression pattern between human and canine intact pancreas. In the present study 100 % of the intact duct epithelial cells showed diffuse claudin-4 negativity. It seems that the claudin-4 molecule is not a constitutive component of the tight junction structures in epithelium of the canine healthy pancreatic ducts. In human studies (Borka et al., 2007) and in the present veterinary studies the endocrine cells were negative for claudin-4 protein.

Banner et al. (1978) studied canine pancreatic acinar cell carcinomas by electron microscopy. They described that there was a decrease in the number and size of gap junctions; focal proliferation, fragmentation, and discontinuation of the tight junctions in these carcinomas compared with normal acinar cells (Banner et al., 1978). Alroy et al. (1978) described that the neoplastic cells of the canine exocrine pancreatic carcinomas displayed proliferation and fragmentation of tight junctions and reduction in size and number of gap junctions (Alroy et al., 1978). Claudin-4 tight junction molecule is expressed strongly in benign and malignant tumours of canine mammary gland, especially in the case of primary *in situ* and infiltrating simple carcinomas (Jakab et al., 2008a), in the secondary simple carcinomas of mammary gland (Jakab et al., 2009c), in benign and malignant hepatoid gland neoplastic lesions (Jakab et al., 2009b) and in low grade colorectal adenocarcinomas (Jakab et al., 2010a). Here we describe that all primary and secondary canine well-differentiated exocrine pancreatic acinar cell carcinomas showed intense claudin-4 positivity without cytoplasmic positivity and it localized specifically to the region of the tight junction, but all primary and secondary poorly-differentiated pancreatic acinar cell carcinoma samples showed diffusely the loss of claudin-4 expression. Human studies have described that pancreatic acinar cell

carcinomas show negativity for claudin-4 (Borka et al., 2007). In epithelial neoplastic cells, the formation of tight junctions and adherent junctions has been shown to reduce invasive potential (Rajasekaran et al., 1991). Our result suggests that the loss of expression of claudin-4 may lead to cellular detachment, disorientation and invasion of canine pancreatic acinar cell carcinomas. Furthermore claudin-4 can be used as an immunohistochemical differentiation marker between canine well-differentiated and undifferentiated pancreatic acinar cell carcinomas.

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