Expression of claudin-1, -3, -4, -5 and -7 proteins in low grade colorectal carcinoma of canines

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Summary. The aim of the present study was to characterise the expression pattern of claudin-1, -3, -4, -5 and -7 tight junction proteins in canine normal colorectum and in the low-grade, tubulopapillary colorectal carcinoma in canines. *Methods and results*: The biopsy samples included 10 canine normal colorectal tissues and 20 canine low grade colorectal carcinomas (CLGCCs). The canine normal colorectal mucosa was negative for claudin-1. Claudin-1 was detected as a non-diffuse intense membrane labelling of neoplastic epithelial cells in low grade colorectal cancer in canines. Fifty five per cent of all tumours showed a weak cytoplasmic pattern of staining for claudin-1 protein. The normal colorectal mucosa showed diffuse punctate positivity for claudin-3. Claudin-3 was detected as an intense lateral membrane labelling of tumour cells in CLGCCs. Claudin-4 expression in surface and crypt epithelial cells of the intact colorectal mucosa in canines was punctate. Claudin-4 molecule was detected as a lateral membrane labelling of neoplastic cells in CLGCCs. The epithelium of the CLGCCs and the low grade colorectal carcinoma were negative for claudin-5. The surface and crypt epithlial cells of the canine normal colorectal mucosa showed a diffuse lateral membranous pattern of staining for claudin-7. Claudin-7 molecule was detected as an intense membrane labelling of neoplastic cells in CLGCCs. Seventy per cent of all tumours showed weak cytoplasmic positivity for claudin-7. *Conclusion*: Consequently, we hypothesize that claudin-1 plays a role in the progression of CLGCCs. Further functional studies are needed to clarify the biological role of the mislocalization of the claudin-1 molecule from cell membrane to the cytoplasm in CLGCCs. Lower claudin-4 expression suggests that reduced expression of claudin-4 molecule may lead to cellular disorientation, detachment and invasion of CLGCCs. Further functional studies are needed to clarify the biological role of overexpression and mislocalisation of claudin-7 in CLGCCs.

Key words: Canine low grade colorectal carcinoma, Immunohistochemistry, Claudin-1, -3, -4, -5, -7

Introduction

Human colorectal carcinoma (HCRC) is an important cause of death by cancer worldwide and has variable geographical distribution. Virtually 98% of all cancers in the large intestine are adenocarcinomas (Jemal et al., 2002; Kinzler and Vogelstein, 2002). They usually arise in polyps and produce symptoms relatively early and at a stage generally curable by surgical resection (Burgart, 2002). Major predisposing factors in the etiopathogenesis of HCRC include the presence of pre-malignant conditions, such as familial polyposis syndrome, inflammatory bowel disease and dietary factors. A diet rich in refined carbohydrate and low in unabsorbable vegetable fibre content and fresh vegetables, the intake of red meat, and a decreased intake of protective micronutrients, all increase fecal transit time (Eaden et al., 2001; Jass, 2001; Wei et al., 2004; Kumar et al., 2005). The peak incidence for HCRC is between ages 60 and 79 years (Kumar et al., 2005). The histological grade of HCRCs is an important prognostic variable and depends on the degree of glandular differentiation and cellular polarity. High-grade or poorly differentiated HCRC's are usually more aggressive than their low-grade or well differentiated counterparts (Hermanek and Sobin, 1995).

In canines up to 60 percent of all intestinal tumours

are located in the colon and rectum (Holt and Lucke, 1985). Colorectal carcinomas develop in older canine, with an avarage of 8-9 years, and male canines have a higher incidence rate than females (Schaffer and Schieffer, 1968; Patnaik et al., 1977). Malignant transformation of adenoma to carcinoma, observed in humans, may be encountered in canines (Silverberg, 1971). Canine colorectal carcinoma (CCRC) can spread in the lymphatics to lymph nodes and produces peritoneal seeding (Brunnert et al., 1993) and distant metastases (Stampley et al., 1987; Hampson et al., 1990). In the veterinary oncopathology the four categories of CCRC, namely colorectal adenocarcinoma, mucinous adenocarcinoma, signet ring cell carcinoma, and undifferentiated or solid carcinoma, can be divided into two mainly histological groups: (1) low grade CCRC, well or moderately differentiated and (2) high grade CCRC, undifferentiated or poorly differentiated (Head et al., 2002).

Adhesion between neighboring epithelial cells is a crucial and tightly controlled process. In epithelial cells, specialized structures, such as tight junctions, adherent junctions and desmosomes are responsible for the establishment of contacts between neighboring cells. Claudins are key components of epithelial and endothelial tight junctions, which act as a barrier to paracellular flux of water, solutes, and transmigration of other cells. There are currently at least 24 known members of the claudin family, which are expressed in a tissue specific pattern. These transmembrane proteins are connected to the actin cytoskeleton via a network of proteins, such as zonula occludens-1 (ZO-1) (Furuse et al., 1999; Tsukita et al., 2001). In carcinogenesis, it has been proposed that the loss of cell polarity is followed by an abnormal influx of different growth factors, which gives rise to auto- and paracrine stimulation of neoplastic epithelia (Mullin, 1997). The loss of claudins and other tight junction proteins has been suggested to account for the loss of cell adhesion and to be an important step in the development of tumour invasion and metastasis (Karen et al., 2005). Several previous studies have examined the role of claudins in human colorectal carcinogenesis (Miwa et al., 2001; De Oliveira et al., 2005; Dhawan et al., 2005; Resnick et al., 2005; Grone et al., 2007; Kinguasa et al., 2007; Nakayama et al., 2008; Oshima et al., 2008; Weber et al., 2008).

In our previous study we described 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, and we deteceted a strong expression of claudin-3 and -4 in carcinoma in situ and grade I, II and III simple infiltrating carcinomas (Jakab et al., 2008d). In addition to our previous studies we investigated the microvessel density in canine mammary gland tumours by quantitative claudin-5 molecule immunohistochemistry (Jakab et al., 2008c), claudin-5 protein expression and localization in the normal canine mammary gland (Jakab et al., 2008a) and neoplastic lesions of mammary gland (Jakab et al., 2008d).

The aim of the present study was to characterise the expression pattern of claudin-1, -3, -4, -5 and -7 tight junction proteins in canine normal colorectum and in the low-grade, tubulopapillary colorectal carcinoma in canines. The hypothesis of the present study was that similarly to mammary gland carcinomas (Jakab et al., 2008d), in the case of CLGCCs there is a change in the expression pattern of the claudin proteins, which could facilitate the detachment and cellular disorientation and invasiveness of these tumours. We also wanted to investigate whether colorectal epithelial cells and CLGCC cells express claudin-5 endothel-specific protein, and whether it could be used for microvessel density assessment. To our knowledge, this is the first study that has examined claudin-1, -3, -4, -5 and -7 molecule expression in canine normal colorectum and low-grade colorectal cancer.

Materials and methods

Samples of canine tumours

Tissue samples were collected between 2005 and 2008 at Szent István University, Faculty of Veterinary Science, Department of Pathology and Forensic Veterinary Medicine (Budapest, HU). The samples included 10 canine normal colorectal tissue samples and 20 canine low grade colorectal carcinoma (CLGCC). Samples were fixed in 8% neutral buffered formalin for 24 hours at room temperature, dehydrated in a series of ethanol and xylene, and embedded in paraffin. The 3-4 µm thick sections were routinely stained with hematoxylin and eosin (HE). Each neoplastic case was classified by the pathologists (CSJ and JK) according to grade (Greene et al., 2002) (Fig. 1A-D).

Immunohistochemistry

Serial sections (3-4 µm) were initially dewaxed in xylene and graded ethanol. After treatment with appropriate antigen retrieval (Target Retrieval Soluton, DAKO, Glostrup, Denmark, pH 6; microwave - 800W - oven for 30 min), the sections were incubated with primary antibodies against claudin-1 (rabbit polyclonal, diluted 1 in 100), claudin-3 and claudin -7 (both rabbit polyclonal and diluted 1 in 80), claudin-4 and claudin-5 (both mouse monoclonal and diluted 1 in 100) for 60 min at room temperature. All primary antibodies were from Zymed Inc., San Francisco, CA, USA. Immunohistochemical labelling 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 3, 3'-diaminobenzidine tetrahydrochloride (DAB substrate-chromogen, DAKO). Sections were counterstained with Mayer's hematoxylin.

Negative controls were performed by omission of the primary antibody and positive controls was canine normal mammary gland for claudin-1, -3, -4 and -7

(Jakab et al., 2008a,b), endothelial cells of microvessels in canine mammary gland cancer for claudin-5 (Jakab et al., 2008c), and canine normal mammary gland for claudin-7 (Jakab et al., 2008a). Claudin labelling patterns were compared with adjacent normal colonic epithelium. The internal positive controls were the peritumoural normal colonic epithelial tissues for claudin-3, -4, and -7, and the endothelial cells of the peritumoural lymphatic vessels, small arteries and veins for claudin-5. Peritumoural fibroblasts and smooth muscle cells served as internal negative controls since these cells do not express claudin proteins.

Immunohistochemistry assessment

In each case, two independent observers (CSJ and JK) recorded the distribution and intensity of labelling. The immunoreactivity was assesed as follows: negative (-), no immunostaining present; (+), <25% of cells positive; (++), 25-50% of cells positive; (+++), 50-75% of cells positive; (++++), 75-100% of cells positive.

For scoring purposes, the intensity of staining of claudin molecules expressing neoplastic cells was compared to that of normal colonic mucosa. Low grade adenocarcinomas that displayed a well-localized linear membranous staining pattern equal to that of normal colonic mucosa were scored as 2. CCRCs showing a more intense staining pattern than that of normal colonic mucosa were scored as 3; and CCRCs showing a considerably lower intensity than normal colonic mucosa were scored as 1.

Results

The canine normal colorectal mucosa was negative for claudin-1 (Fig. 2A). Claudin-1 was detected as a non-diffuse intense membrane labelling of neoplastic epithelial cells in low grade colorectal cancer in canines. The CLGCC showed ++ claudin-1 positivity and the tumours showed a more intense staining pattern than that of normal colorectal mucosa (3 immunopositivity) (Fig. 2B-D). A few tumours showed (11:20; 55%) a weak cytoplasmic pattern of staining for claudin-1 protein.

The normal colorectal mucosa showed a diffuse punctate positivity for claudin-3 molecule. This claudin protein localized specifically to the region of the tight junction in surface and crypt epithelium (Fig. 3A,B). The intact surface and crypt colonocytes did not show a cytoplasmic positivity for claudin-3 molecule. The claudin-3 protein was detected as an intense lateral membrane labelling of tumour cells in low grade colorectal cancer in canines. The CLGCC showed a ++++ positivity for claudin-3 and it showed a more intense staining pattern than that of normal colorectal mucosa (3 immunopositivity) (Fig. 3C-D). We did not detect claudin-3 cytolplasmic positivity in the neoplastic cells.

Claudin-4 expression in surface and crypt epithelial cells of the intact colorectal mucosa from canine was punctate, without cytoplasmic positivity and it localized specifically to the region of the tight junction, like claudin-3 protein. Claudin-4 molecule was detected as a lateral membrane labelling of neoplastic cells in low
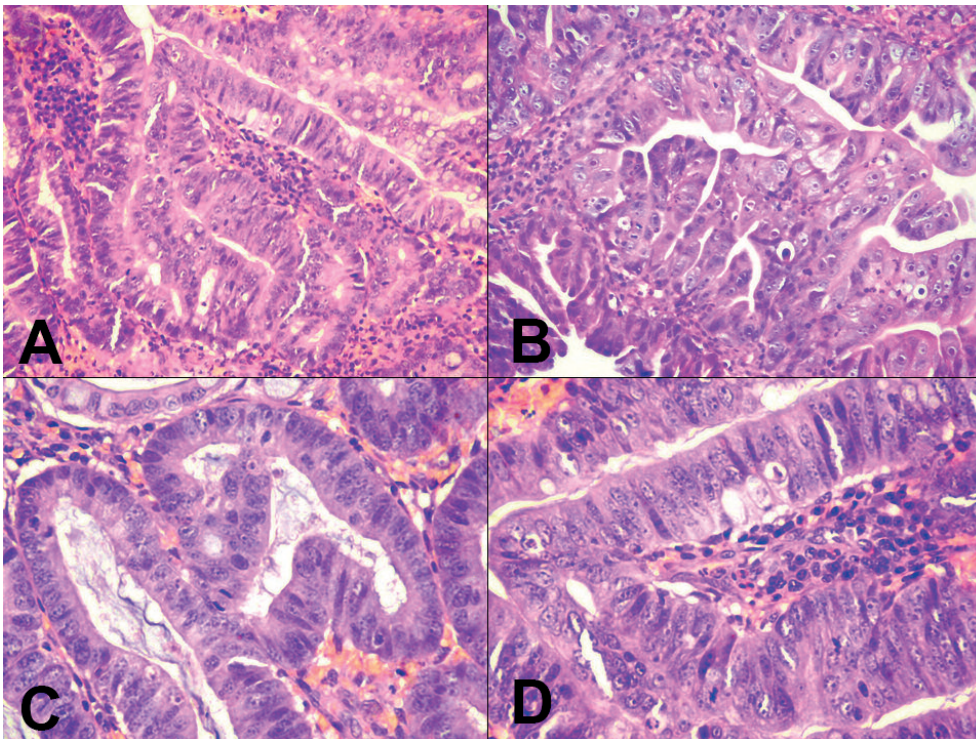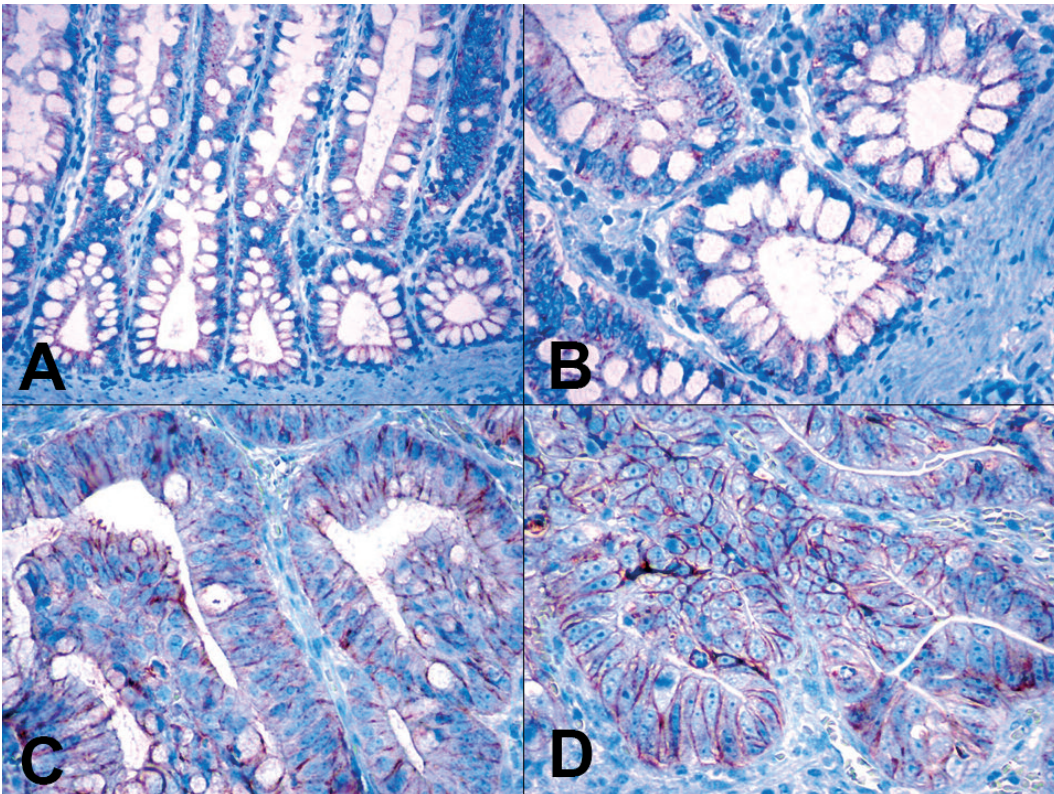

Fig. 1. Canine low grade colorectal carcinoma. Tubular and tubulopapillary adeno-carcinoma in the colorectum of the canine show irregular branching tubules and papillary projections embedded in a moderate amount of fibrous stroma. In the pictures some mitotic neoplastic cells can be seen. A-C, x 200; D, x 400
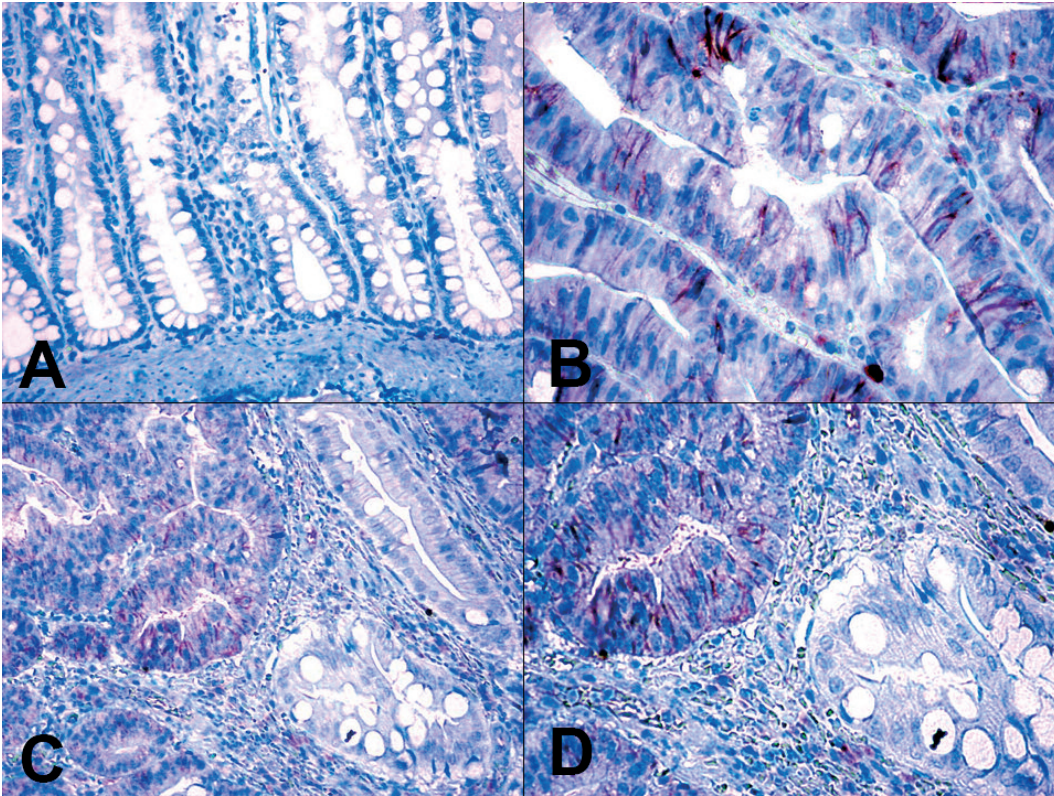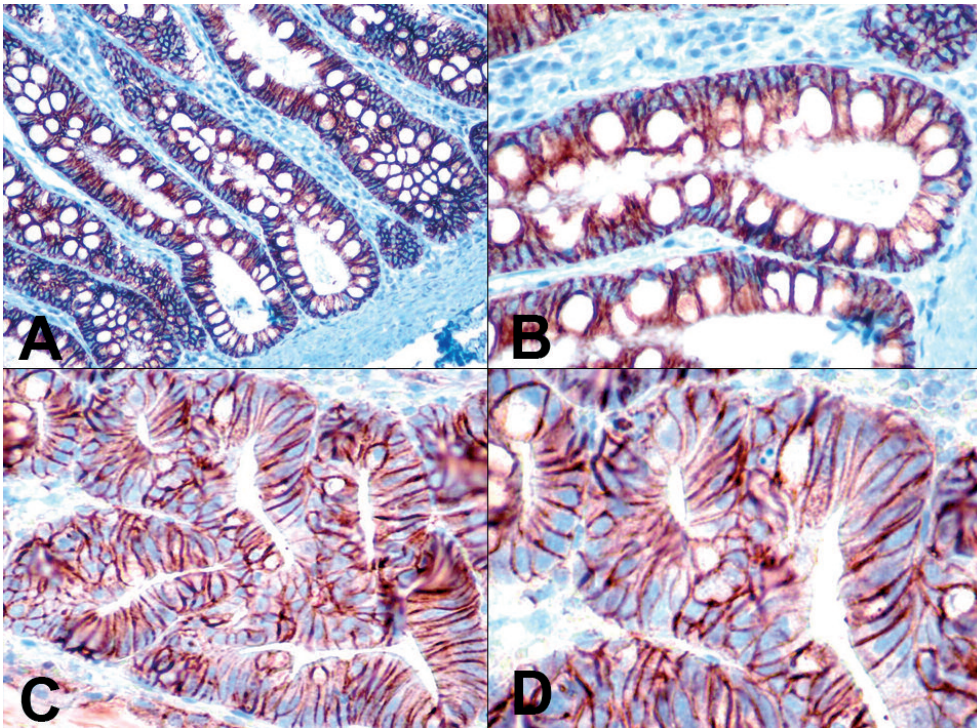
Fig. 2. A. The canine normal colorectal mucosa was negative for claudin-1 protein. **B.** Increased claudin-1 expression in the canine low grade colorectal carcinoma. **C.** On the right hand side of the picture normal, claudin-1 negative glands of canine colorectum and on the left hand side claudin-1 positive canine low grade colorectal cancer can be seen. **D.** Similar picture at higher magnification A, C, x 200; B, D, x 400

Fig. 3. A-B. The canine normal colorectal mucosa showed a diffuse punctate positivity for claudin-3 molecule. **C-D.** A strong diffuse membranous claudin-3 expression in canine low grade colorectal cancer A, x 200; B-D, x 400

Fig. 4. A-B. Canine normal colorectal mucosa showed diffuse linear membrane positivity for claudin-7 molecule. **C-D.** Strong diffuse claudin-7 expression in canine low grade colorectal cancer A, C, x 200; B, D, x 400

grade colorectal cancer in canines. The CLGCC showed +++ positivity for claudin-4 and the low grade carcinomas showed an equally intense staining pattern when compared with normal colorectal mucosa (2 immunopositivity). We did not detect claudin-4 cytolplasmic positivity in the tumour cells.

The epithelium of the canine normal colorectal mucosa and the CLGCC were negative for claudin-5, but the endothelial cells of the normal vessels, lymphatics and the intra- and peritumoural tumoural microvessels showed intense claudin-5 positivity.

The surface and crypt epithlial cells of the canine normal colorectal mucosa showed a diffuse lateral membranous pattern of staining for claudin-7, without cytoplasmic positivity (Fig. 4A-B). Claudin-7 molecule was detected as an intense membrane labelling of neoplastic cells in low grade colorectal cancer in canines.

The CLGCC showed ++++ positivity for claudin-7 and the low grade carcinomas showed an equally intense staining pattern when compared with normal colorectal mucosa (2 immunopositivity) (Fig. 4C-D). Seventy percent of all tumour samples (14:20) showed weak cytoplasmic positivity for the claudin-7 protein.

Discussion

In epithelial cells, specialized structures, such as tight junctions and adherent junctions are responsible for the establishment of contacts between neighboring cells. Claudins are key components of epithelial and endothelial tight junctions, which act as a barrier to paracellular flux of water, solutes, and transmigration of other cells. Claudins are responsible for the formation of tight junction strands, and the second function of these proteins is the preservation of cell polarity in the epithelial and endothelial barriers (Furuse et al., 1999; Tsukita et al., 2001). Within tight junctions, claudins form homophilic and heterophilic complexes on apposing cells (Furuse et al., 1999). These transmembrane proteins are connected to the actin cytosceleton via a network of proteins, such as zonula occludens-1 (ZO-1) (Furuse et al., 1999; Tsukita et al., 2001). Claudin proteins have been proposed to have important roles in different biological processes, including embryogenesis, development, tissue remodelling, and oncogenesis. The preservation of cell polarity and paracellular flux by many claudin molecules suggests that these proteins contribute to tumour suppressive functions in epithelial neoplasia. The loss of claudins and other tight junction proteins has been suggested to underlie the loss of cell adhesion and be an important step in the development of tumour invasion and metastasis (Karen et al., 2005). Claudins have been shown to modify tumour invasion by the regulation of matrix metalloproteinases (Miyamori et al., 2001).

In our present study we investigated the expression of claudin-1, -3, -4, -5 and -7 tight junction proteins in the surface and crypt epithelial cells of the canine normal colorectal mucosa and in the neoplastic epithelial cells of CLGCC. Ridyard et al. (2007) described increased expression of claudin-2 by the colonic epithelium of canines with idiopathic lymphocytic-plasmacytic colitis.

HCRC is the second most common cause of death by cancer (Kumar et al., 2005). Several studies have described that claudin-1 expression is progressively increased in human colon carcinogenesis, with the highest expression in metastatic samples and cell lines derived from metastatic sources (Resnick et al., 2005). Manipulation of claudin-1 levels in colon cancer cells showed a positive correlation between claudin-1 expression and tumour growth and metastasis (Dhawan et al., 2005). Claudin-1 was identified as a probable target of ß-catenin/Tcf signaling, which supports a potential role for claudin-1 dysregulation in human colorectal carcinogenesis (Miwa et al., 2001). Claudin-1 expression is a positive prognostic indicator and correlates with lower tumour grade, absence of lymphovascular invasion, and increased patient survival (Resnick et al., 2005). Human preliminary studies, using non-transformed RIE cells and normal colon tissue samples observed that claudin-1 protein is not expressed. However, it is highly expressed in colon tumour tissues and the expression increases further in the metastatic colon cell lines and tissue samples (Dhawan et al., 2005). In our present study we have detected that the epithelium of the canine normal colorectal mucosa was negative for claudin-1 molecule, but claudin-1 was detected as a non-diffuse intense membrane labelling of neoplastic epithelial cells in CLGCC. We detected that claudin-1 expression increased in the CLGCC compared to normal mucosa. Miwa et al. (2001) reported that claudin-1 was frequently upregulated in neoplastic tissue when compared to normal mucosa. Our result suggests that increased expression of claudin-1 may play an important role in the colorectal carcinogenesis in canines. However, in our previous study we described that the reduction of expression of claudin-1 protein may lead to cellular disorientation, detachment and invasion in canine mammary neoplasia (Jakab et al., 2008d). Further functional studies are needed to clarify the biological role of claudin-1 expression in these histologic subtypes of canine colorectal tumour. In the present study a few tumours showed a weak non-junctional, cytoplasmic pattern of staining for claudin-1 protein. A previous human study described increased expression of claudin-1 in human colon cancers, with mislocalization from cell membrane to the nucleus and cytoplasm. Metastatic colon cancer cells expressed the highest levels of claudin-1 and the highest rate of mislocalization (Dhawan et al., 2005).

In the present study we detected a diffuse punctate positivity for claudin-3 and claudin-4 molecules in the epithelium of the canine normal colorectal mucosa and overexpression of claudin-3 protein in the CLGCC. According to a human study, normal colonic epithelium exhibited a membranous pattern of staining for claudin-4 molecule (Resnick et al., 2005). Of note is the fact that claudin-3 and -4 act as receptors for *Clostridium perfringens* enterotoxin (CPE) (Katahira et al., 1997; Morita et al., 1999) which is able to directly and rapidly lyse mammalian cells (McClane et al., 1988; Kominsky et al., 2004).

The first endothel-specific claudin was claudin-5, also known as transmembrane protein deleted in velo-cardio-facial syndrome (TMVCF) (Morita et al., 1999; Sirotkin et al., 1997). Several members of the claudin-family, such as claudin-2 and claudin-5 are able to activate membrane-type-1-MMP-mediated pro-MMP-2 processing (Miyamori et al., 2001). In our previous studies we investigated the microvessel density in canine mammary gland tumours by quantitative claudin-5 molecule immunohistochemistry (Jakab et al., 2008c), claudin-5 protein expression and localization in the normal canine mammary gland (Jakab et al., 2008a), and neoplastic lesions of mammary gland (Jakab et al., 2008d). The immunohistochemical detection of claudin-5 protein had a higher sensitivity than CD31, and vWf antigen in case of canine hemangiosarcomas (Jakab et al., 2009a). In the present study we detected that the surface and crypt epithelial cells of the canine normal colorectal mucosa and the neoplastic cells of the CLGCC were negative for claudin-5 molecule, but the endothelial cells of the normal vessels of the intact colorectum and the intra- and peritumoural microvessels showed intense claudin-5 positivity.

Claudin-7 is expressed normally in the distal nephron epithelium of the kidney (Li et al., 2004). This protein is a novel immunohistochemical marker for renal tumour classification. The distal nephron marker, claudin-7, is overexpressed in human chromophobe renal cell carcinoma versus oncocytoma and other tumour subtypes (Hornsby et al., 2007). Claudin-7 may play an important role in human immunodeficiency virus (HIV) infection of CD4(-) cells. This protein can serve as a receptor for HIV-1 infection of CD4(-) cells or as a ligand on the viral envelope (Zheng et al., 2005). Human studies have desrcibed that claudin-7 is overexpressed in gastric dysplasia and adenocarcinoma but it is not in the surrounding nonneoplastic epithelial cells. These results suggest that claudin-7 expression is an early event in gastric tumourigenesis and is maintained throughout tumour progression (Johnson et al., 2005). The increased expression of claudin-7 in HCRC cells leads to a loss of polarization and to an increase of h-catenin/Tcf-4 activity and proliferation in colorectal cancer cells, resulting in an enhancement of their tumorforming ability in vivo (Darido et al., 2008). We reported previously that claudin-7 is one of the integral constituents of tight junction structures of canine normal mammary gland (Jakab et al., 2008a) and canine primary (Jakab et al., 2008d), secondary epithelial mammary gland tumours (Jakab et al., 2009b). In our present study we detected that the surface and crypt epithlial cells of the canine normal colorectal mucosa showed a diffuse lateral membranous pattern of staining for claudin-7 and the CLGCC overexpressed this claudin protein. This tight junction protein overexpressed in human squamous cell carcinomas of the uterine cervix (Sobel et al., 2005), as well as in the adenocarcinoma of the esophagus (Montgomery et al., 2006). In contrast, it is underexpressed in ductal carcinoma of the breast (Kominsky et al., 2003). In this present study more than

50 % of the CLGCCs showed a lateral membrane and cytoplasmic positivity for claudin-7 molecule. Further functional studies are needed to clarify the biological role of mislocalisation of claudin-7 from the cell membrane to the cytoplasm in canine low grade carcinoma.

In conclusion, the results of the present study have shown that claudin-1 is not expressed in the epithelium of canine normal colorectal mucosa, but increased expression of this protein is observed in CLGCCs. Consequently, we hypothesize that claudin-1 plays a role in the progression of canine colorectal carcinoma. Further functional studies are needed to clarify the biological role of the mislocalization of claudin-1 molecule from the cell membrane to the cytoplasm in these histologic subtypes of canine colorectal tumour. The canine intact colorectal epithelium showed diffuse punctate positivity for claudin-3 and claudin-4 molecules, and the low grade colorectal cancer overexpressed the claudin-3 protein. The CLGCCs showed a lower claudin-4 expression and this result suggests that reduced expression of claudin-4 molecule may lead to cellular disorientation, detachment and invasion of canine colorectal cancers. In the present study we detected that the epithelial cells of the canine normal colorectal mucosa and the neoplastic cells of the CLGCCs were negative for claudin-5 molecule, but the endothelial cells of the intact vessels, lymphatics and intratumoural, peritumoural microvessels showed strong claudin-5 positivity. This can help during light microscopic analysis in the detection of lymphovascular invasion of the colorectal cancer cells (claudin-5 positive vessels, lymphpatics with claudin-5 negative tumour emboli), as well as in the case of canine mammary gland cancer intravasation (Jakab et al., 2008e). In addition, claudin-5 can be used for the intra-, and peritumoural microvessel density assessment of canine colorectal cancers. The epithelial cells of the canine normal colorectal mucosa showed a diffuse lateral membranous positivity for claudin-7, and the CLGCCs overexpressed this claudin. More than 50% of canine low grade cancer showed a lateral membrane and cytoplasmic positivity for claudin-7 molecule. Further functional studies are needed to clarify the biological role of overexpression and mislocalisation of claudin-7 in CLGCCs.

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