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#### E-cadherin and b-catenin expression in canine colorectal adenocarcinoma

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## Abstract

E-cadherin and its associated cytoplasmic proteins, including b-catenin, have been examined as poten-tial oncogenic markers due to the significant correlation between tumour dedifferentiation and the invasive capacity of epithelial tumours. The purpose of this study was to evaluate the expression of E-cadherin and b-catenin in canine colorectal cancer using immunohistochemistry and to examine the relationship between this expression and various clinicopathological variables. The expression pat-tern of E-cadherin and b-catenin was investigated in 44 colorectal canine carcinomas. In the intestinal mucosa of noncancerous areas, epithelial cells demonstrated equally strong membranous expression of E-cadherin and b-catenin localised to the cell-cell junctions. Reduced expression of E-cadherin and b-catenin was demonstrated in 75% and 81.8% of the colorectal carcinoma cases, respectively. The down-regulation of both E-cadherin and b-catenin was correlated with decreased differentiation and increased tumour grade. In addition, the expression of b-catenin was correlated with tumour size. These results suggest that dysfunction of the E-cadherin-catenin complex starts in the early stages of carcinogenesis and that the disruption of the tissue architecture is progressively associated with the invasion of the tumour.

## **1. Introduction**

Worldwide, intestinal adenocarcinoma is a common solid tumour in humans, and it is characterised by a poor prognosis and reduced survival (McEntee and Brenneman, 1999; El-Bahrawy et al., 2001). The prognosis depends upon the potential invasive-ness of the tumour, which is measured by its ability to invade the basement membrane. The molecular mechanisms through which the tumour acquires this invasive potential remain poorly understood (Bondi et al., 2006). In domestic animals, including the dog and cat, pathogenesis seems to be a multistage process, beginning as a benign lesion and progressing to a carcinoma through invasion of the basement membrane (Aust et al., 2001). Furthermore, the loss of intercellular adhesions and increased cell motility seem to promote tumour cell invasion (Oyama et al., 1994). Importantly the epithelial cell tight junctions and adherens junction complexes serve as a paracellular barrier in polarised epithelial cells. Abnormalities in the adhesion molecules that make up these junctions have been associated with aggressive histopathol-ogical characteristics of malignant tumours and have been extensively studied as major factors in cancer progression and metastasis (Joo et al., 2002; Salon et al., 2004). A variety of human cancers exhibit dysregulation of E-cadherin and b-catenin expression that can be correlated with a high grade and advanced tumour stage. The cadherins are transmembrane glycoproteins that mediate calcium-dependent adhesion between cells (Elangbam et al., 1997; Alattia et al., 1999). They play an important role in morpho-genesis during embryonic development and in maintaining integ-rity in developed tissues. Cadherins are cell surface glycoproteins involved in homotypic Ca2+-dependent cell-cell adhesion. Three cadherins, E-, N-, and P-cadherin, share a common basic structure and mediate cell-cell binding in a homophilic and subclass-specific manner. E-cadherin is expressed in all epithelial cells (Elangbam et al., 1997; Alattia et al., 1999; Jungck et al., 2004). The cytoplasmic domains of the cadherins are linked to b-catenin, which con-nects them to the actin cytoskeleton. Binding to catenins is crucial for E-cadherin function, and loss of this interaction reduces cell-cell adhesion even when the binding activity of the extracellu-lar domains of the cadherins remains intact (Harrington and Syrigos, 2000; Masszi et al., 2004).

Intestinal carcinoma in dogs has a very poor prognosis due to the high incidence of local recurrence, lymph node metastasis, and peritoneal dissemination. The present study was designed to assess the relationship between E-cadherin or b-catenin expression and the staging of intestinal adenocarcinomas in dogs.

#### 2. Materials and methods

### 2.1. Tissue selection and pathological examination

Paraffin-embedded canine intestinal adenocarcinomas (n = 44) from the Veterinary Pathology Diagnostic Service of Padua and Turin University were included in this study. These specimens were obtained from surgical biopsies. In addition, as positive con-trols, normal intestinal samples were obtained from five dogs, aged 10-48 months, which had been euthanised due to neurological problems. The tumours were classified based on haematoxylin and eosin-stained sections following the diagnostic criteria of the World Health Organization classification of tumours in domestic animals. The human grading system was used to classify the inva-siveness of the tumour: T1 (invading the submucosa), T2 (invading the muscularis propria), T3 (extending beyond the muscularis propria), or T4 (invading the free surface of adjacent organs).

## 2.2. Immunohistochemistry

An immunohistochemical panel was performed to assess changes in the intestinal epithelial cells. The following canine anti-gen-specific antibodies were used: mouse anti-E-cadherin (clone 36, Transduction Laboratories, Lexington, USA) and mouse anti-b-catenin (clone 14, Transduction Laboratories). For the immuno-staining, paraffin-embedded intestinal sections (4 lm) were de-waxed and rehydrated, and antigen retrieval was performed using microwave exposure for 3 min at 600 W (three times) in a citrate buffer (pH 6.0). When the temperature of the buffer reached room temperature, the slides were rinsed in phosphate-buffered saline (PBS) and incubated with 3% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in methanol for 15 min to block endogenous peroxidase activity. The slides were rinsed in PBS and blocked in PBS supplemented with 10% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA) for 20 min. The sections were then incubated with the primary antibody diluted in 10% BSA in PBS for 60 min. The anti-E-cadherin and anti-b-catenin antibodies were used at a concen-tration of 3.6 lg/ml. After two washings with PBS, the sections were incubated with the secondary antibody provided in a commercial kit (Real EnVision Peroxidase Detection System, Dako) for 30 min and then washed twice with PBS. The reaction was then developed using the DAB solution provided in the kit. The slides were washed in tap water and counterstained with haematoxylin and PAS.

#### 2.3. Evaluation of immunohistochemistry

To assess E-cadherin and b-catenin expression, a semi-quantitative evaluation of the immunostained sections was performed according to previously published methods (Kwak et al., 2007). Normal intestinal epithelial cells present in normal tissues or adjacent to the lesions were used as internal staining controls. The normal intestinal epithelial expression of E-cadherin and b-catenin seen in the control tissue was defined as exclusively membranous. The percentage of tumour cells with normal membranous expression of E-cadherin and b-catenin in each section was assessed, and the specimens were scored using a five-tiered grading system: (0) no staining, (1) 1-20%, (2) 20-50%, (3) 50-80% and (4) >80%. For the scoring of the b-catenin expression, the expression was further subdivided into either cytoplasmic or nuclear localisation. b-Cate-nin expression in tumour cells was considered nuclear positive (+) when more than 10% of the cells demonstrated nuclear staining. b-Catenin expression was considered cytoplasmic positive (+) when more than 10% of the tumour cells demonstrated cytoplasmic staining. For each case examined, the analysis

was performed by counting ten fields (200 x). The semi-quantitative evaluation was carried out using an electronic image analysis system (Adobe Photoshop CS3). The images were digitalised using a video camera connected to a single microscope and a computer equipped with a frame grabber (Neotech Ltd., Eastleigh Hampshire, UK).

#### 2.4. Statistical analysis

Statistical analyses were performed using GraphPad-InStat software (GraphPad Software, San Diego, CA, USA). The correlation be-tween the expression of E-cadherin or b-catenin and the clinicopathological parameters was evaluated using the chi-squared test or Fisher's exact test. To analyse the age distribution, an unpaired t-test was used. A p value less than 0.05 was considered statistically significant.

## 3. Results

## 3.1. Clinicopathological characteristics

Based on the histological subtype, the adenocarcinomas were divided as follows: 27 acinar adenocarcinomas, characterised by variable sized acinar structures arising from crypts that replaced the intestinal mucosa; 13 papillary adenocarcinomas, characterised by papillary projections lined by multiple layers of anaplastic columnar cells with little stroma; four mucinous adenocarcinomas, mucin-producing tumours with an acinopapillary pattern; and one adenosquamous carcinoma, characterised by squamous differenti-ation. Of the 44 cases, eight were graded as T2, 18 were graded as T3, and 18 were graded as T4. The mean age of the animals was  $8.4 \pm 2.6$  years. No statistically significant correlations between the histological type of tumours, the grade and the size of the tumours, and the gender of the animals were observed.

## 3.2. Immunohistochemical results

In normal intestinal mucosa, the expression of E-cadherin and b-catenin was located uniformly at intercellular borders, and no cytoplasmic or nuclear immunoreactivity was observed in normal epithelia (Figs. 1a and 2a).

The expression of E-cadherin in the membrane was preserved in 11 tumour lesions. Four of these (36.4%) were from male dogs, and seven (63.3%) were from female dogs (Table 1). E-cadherin expression in the membrane was reduced in the remaining tumours (75.0%): 18 (54.5%) of these were from male dogs, and 15 (45.4%) were from female dogs (Fig. 1b-d) (Table 1). No statistically significant correlations were evident either between the E-cadherin expression level and the gender of the animals or between the histological types of tumours and the expression of E-cadherin. However, the tumour grade could be correlated with the expression of E-cadherin (p < 0.01), with a greater loss of E-cadherin expression in

grade 4 tumours (Table 1). E-cadherin immunoreactivity could also be correlated with age (p < 0.01), with a higher mean age associated with reduced expression (Table 1). The relationship between the number of cases and the E-cadherin expression scores is shown in Table 4.

b-Catenin expression in the tumour lesions was detected on the cellular membrane, in the cytoplasm, and in the nucleus. The expression of b-catenin in the membrane was preserved in eight tumours (18.2%): five of these (62.5%) were from male dogs, and three (37.5%) were from female dogs (Table 2). In 81.8% of the tumours (36 cases), b-catenin expression in the membrane was reduced: 17 cases (47.2%) were from male dogs, and 19 (52.8%) were from female dogs (Tables 2 and 3). No statistically significant correlation between b-catenin expression and the gender of the animals or the histological types of tumours was observed, but a correlation between the membrane expression of b-catenin and the tumour grade was evident (p<0.05). As seen in Table 2, a greater reduction in the membrane expression of b-catenin is evident in the tumours with a higher grade. In contrast to the expression of E-cadherin, membrane b-catenin immunoreactivity did not correlate with age. The number of cases associated with the differ-ent membrane expression scores is reported in Table 4.

In 6.8% of tumours, cytoplasmic expression of b-catenin was observed, and in 20.5% of the colorectal carcinomas, nuclear staining was apparent (Fig. 2b-d). No statistically significant correlation between the nuclear and cytoplasmic expression of b-catenin and the tumour grade or the histological type was evident.

The expression of E-cadherin was not significantly correlated with tumour size; however, the reduced expression of b-catenin was statistically associated (p < 0.01) with increased tumour size (>2 cm) (see Fig. 3a,b).



Fig. 1. Expression of E-cadherin. (a) A photomicrograph taken under low-power magnification demonstrates membranous localisation in normal epithelia. The loss of E-cadherin expression in epithelial tumour cells in T2 (b), T3 (c), and T4 (d) tumours.



Fig. 2. Expression of b-catenin. (a) A photomicrograph taken under low-power magnification demonstrates membranous localisation in non-neoplastic epithelia. (b) The loss of b-catenin expression in a T3 colon carcinoma. (c) Cytoplasmic and nuclear localisation in scattered neoplastic cells. (d) The complete loss of expression in a T4 carcinoma.

#### 4. Discussion

Cadherins are transmembrane proteins with an extracellular domain located in the N-terminal part of the molecule and an intracellular domain that is linked to actin filaments via a- and b-catenin (Alattia et al., 1999; Conacci-Sorrell et al., 2003). The interactions between cadherins and cytoskeletal proteins that are mediated through catenins confer stability on the cell-cell adher-ens junction. During development, E-cadherin is involved in the control of the epithelial-mesenchymal conversion in embryogene-sis; however, during life, it has been implicated in pathological processes including organ fibrosis and neoplasia (Masszi et al., 2004). Different studies in vitro and in vivo have shown that alterations in E-cadherin and b-catenin expression are associated with an invasive tumour phenotype (Morton et al., 1993; Richmond et al., 1997; Schandl et al., 2000). Decreased cell adhesion, in-creased cell motility, and the secretion of proteolytic enzymes by tumour cells are all factors that are considered to be highly indicative of tumour invasion. In particular, the low expression of catenins has been associated with increased dedifferentiation, invasion, and metastasis in carcinomas (Takayama et al., 1996; Elangbam et al., 1997; Harrington and Syrigos, 2000; Masszi et al., 2004), and various studies have demonstrated that the down-regulation of b-catenin is significantly correlated with malignant transformation (Morin, 1999; Aust et al., 2001). Canine intestinal carcinomas have a very poor prognosis. Adenomas are infrequently diagnosed as they result in few clinical signs. Surgical resection of adenocarcinomas of the small and large intestine is common. The reported median survival time is 10 months, with 1- and 2-year survival rates of 40.5% and 33.1%, demonstrating the aggressive behaviour of this neoplasia.

In this study, 75.0% of colorectal adenocarcinomas demonstrated reduced expression of E-cadherin, and 81.8% exhibited reduced expression of b-catenin. The reduction in the membrane expression of E-cadherin and b-catenin was greatest in tumours with transmural invasion (p < 0.005) compared to tumours with invasion that was limited to the submucosa and muscular layer. In a previous study in the dog, the expression of cadherins and catenins was only evaluated based on staining intensity, and no data correlating modification of the staining sites with altered tumour invasiveness were presented (McEntee and Brenneman, 1999). In the current study, both cytoplasmic staining (6.8%) and nuclear staining (20.5%) for b-catenin were observed, although no statistically significant associations with the clinical data were found. Recent studies have reported that b-catenin interacts directly with the cytoplasmic domain of E-cadherin, thereby medi-ating the interaction with the actin filament network, and that the nuclear translocation of b-catenin disrupts the interaction between E-cadherin and a-catenin (Oyama et al., 1994; Hao et al., 1997; Wong et al., 2004). In normal epithelial cells, b-catenin is regulated by the upstream regulators in the Wnt signalling cascade (Tetsu and McCormick, 1999). Mutations in the b-catenin into the nucleus. Although b-catenin signalling appears to play an important role in colorectal carcinogenesis, the

contribution of the nuclear translocation of b-catenin to tumour progression is not known. A recent study has suggested that the nuclear translocation of b-catenin might be involved in the devel-opment of intramucosal and invasive colon cancer, but not adeno-mas (Wong et al., 2003). However, in dogs, this mechanism is still unclear. In our work, tumours from nine dogs demonstrated nuclear b-catenin localisation. Even though these data are not statisti-cally correlated with the clinical data, they should be taken into consideration for prognostic studies with follow-up and future studies examining benign neoplastic lesions. The decreased expression of the cadherin-catenin complex in this experiment is concordant with results obtained in previous studies examining human cancers, suggesting that the dysfunction of the E-cadherin-catenin complex in dogs occurs at early stage of carcinogenesis and that it is correlated with the progression of the tumour (Park et al., 2007). It has been shown that the variations in the behaviour of malignant tumours are related to their degree of dif-ferentiation, as defined using morphologic criteria and the size of the tumours. In dogs, poorly differentiated intestinal carcinomas, which invade and metastasize most rapidly, are associated with a poor prognosis (Kitagawa et al., 1999).

In summary, the present study shows that concomitant down-regulation of E-cadherin and b-catenin expression occurs in malignant neoplastic lesions of the colorectal tract in dogs and that this expression is significantly associated with the tumour grade and tumour invasiveness. These findings suggest that dysfunction of the E-cadherin-catenin complex starts at an early stage of carcino-genesis and that the disruption of the tissue architecture is pro-gressively associated with the invasion of the tumour. Blocking E-cadherin down-regulation in tumours is a goal of gene therapy, and dogs may represent a potentially important animal model in which to investigate new therapeutic approaches. Dogs might also represent an interesting model to study colorectal carcinoma due the similar clinical behaviour of the tumour compared to humans.

# Table 1

Relationship between clinicopathological parameters and expression of E-cadherin.

		Number of cases with preserved E-cadherin expression*	Number of cases with reduced E- cadherin expression <sup>**</sup>
Gender			
	Male	4 (36.4%)	18 (54.6%)
	Female	7 (63.6%)	15 (45.4%)
Histological type of tumour			
	Acinar	7 (63.6%)	20 (60.6%)
	Papillary	3 (27.3%)	9 (27.3%)
	Mucinous	1 (9.1%)	3 (9.1%)
	Adeonosquamous	0	1 (3.0%)
Tumour size			
	<2 cm	8 (72.7%)	16 (48.5%)
	>2 cm	3 (27.3%)	17 (51.5%)
Tumour grade***			
	T2	8 (72.7%)	0
	T3	3 (27.3%)	15 (45.5%)
	T4	0	18 (54.5%)

"Preserved" indicates more than 50% staining, while "reduced" indicates from 0% to 50% staining.

\* Percentage of cases with preserved E-cadherin expression. \*\* Percentage of cases with reduced E-cadherin expression. \*\*\* p < 0.01.

## Table 2

Relationship between clinicopathological parameters and expression of b-catenin.

		Number of cases with preserved β-catenin expression*	Number of cases with reduced β- catenin expression**
Gender			
	Male	5 (62.5%)	17 (47.2%)
	Female	3 (37.5%)	19 (52.8%)
Histological type of tumour			
	Acinar	4 (50.0%)	23 (63.9%)
	Papillary	4 (50.0%)	8 (22.2%)
	Mucinous	0	4 (11.1%)
	Adeonosquamous	0	1 (2.8%)
Tumour size			
	<2 cm	7 (87.5%)	17 (47.2%)
	>2 cm	1 (12.5%)	19 (52.8%)
Tumour grade"**			
	T2	4 (50.0%)	4 (11.1%)
	T3	1 (12.5%)	17 (47.2%)
	T4	3 (37.5%)	15 (41.7%)

"Preserved" indicates more than 50% staining, while "reduced" indicates from 0% to <sup>11</sup> Percentage of cases with preserved  $\beta$ -catenin expression. <sup>12</sup> Percentage of cases with reduced  $\beta$ -catenin expression. <sup>13</sup> p < 0.05.

# Table 3

The expression and localisation of E-cadherin and b-catenin.

	Localisation	Expression	Number of cases
E-Cadherin			
	Membrane	Preserved	11 (25.0%)
	Membrane	Reduced	33 (75.5%)
β-Catenin			
	Membrane	Preserved	8 (18,2%)
	Membrane	Reduced	36 (81.8)
	Cytoplasmic	(1)	3 (6.8%)
	Cytoplasmic	(0)	41 (93.2%)
	Nuclear	(1)	9 (20.5%)
	Nuclear	(0)	35 (79.5%)

(0), no staining; (1), 1–20% staining; (2), 20–50% staining; (3), 50–80% staining and (4), >80% staining.

"Preserved" indicates more than 50% staining, while "reduced" indicates from 0% to 50% staining.

# Table 4

The membrane expression of E-cadherin and b-catenin scores.

Scores of	Number of cases with	Number of cases with
membrane	membrane E-cadherin	membrane β-catenin
expression	expression	expression
(0)	5 (11.4%)	10 (22.7%)
(1)	16 (36.4%)	18 (40.9%)
(2)	12 (27.3%)	8 (18.2%)
(3)	9 (20.4%)	8 (18.2%)
(4)	2 (4.5%)	0 (0.0%)

(0), no staining; (1), 1–20% staining; (2), 20–50% staining; (3), 50–80% staining and (4), >80% staining.



Fig. 3. (a) Retained membrane expression of E-cadherin in epithelial tumour cells in a T3 tumour. (b) Nuclear and cytoplasmic expression of b-catenin in a T3carcinoma T3.

## 5. Confl ict of interest statement

All authors have disclosed any financial and personal relation-ships with other people or organisations that could have inappro-priately influenced their work.

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