Expression of E-Cadherin, β-Catenin and APC protein in Canine Colorectal Tumours

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Abstract. *The present study aimed at evaluating, through immunohistochemical staining, E-cadherin, β-catenin and adenomatous polyposis coli (APC) expression and distribution in normal, dysplastic and neoplastic canine colon and rectum, and at correlating the protein expression with the histological grade of malignancy. In order to obtain a more thorough evaluation of the APC/β catenin complex, both proteins were also assessed for colocalization in neoplastic cells through double immunofluorescence staining. Immunohistochemical investigation showed a marked decrease of E-cadherin and APC expression in malignant tumours, while a lack of membranous β-catenin distribution and a cytoplasmic positivity, rather than a decrease of expression, was observed in less differentiated carcinomas. Confocal laser microscopical observation showed cytoplasmic β-catenin distribution solely in APC-negative cells, demonstrating that the correct β-catenin distribution on the cell membrane can be APC dependent, as in human colorectal carcinomas. Therefore, the changes in adhesion molecules can play a very important role both in colorectal carcinogenesis and in malignant progression; moreover, these proteins can also be considered powerful prognostic tools in veterinary oncology.*

The adenomatous poliposis coli (*APC*) gene is an oncosuppressor gene involved in intestinal carcinogenesis (1). It codifies for a large protein which plays a role in the intercellular adhesion mediated by the E-cadherin/β-catenin complex. More precisely, APC protein competes with E-cadherin for binding with β-catenin, freeing it from the adhesion complex. Free β-catenin interacts with glycogen synthase kinase 3β (GSK3 β) and axin protein, which phosphorylate serine and threonine residues present on the *N*-terminal region of β-catenin (2), thereby allowing its degradation in opposition to *Wnt-1* gene action (3). In colorectal tumours, the Wnt signaling pathway is activated; some mutations accumulate in the *APC* gene, and mutated APC protein is not able to link β-catenin which thus cannot be degraded and therefore accumulates in the cytoplasm and can translocate into the nucleus (4, 5). The consequences could be: i) the impossibility of linking β-catenin to E-cadherin and the subsequent loss of intercellular adhesion; ii) the activation, by β-catenin, of the nuclear transcription factor transcriptional cell factor 4 (Tcf-4) (6). It is believed that the activation of target genes, secondary to a mutated APC/β-catenin/Tcf-4 pathway, may serve critical roles in mediating the transformation process of normal human colonic epithelium (7). This oncogenic mechanism, which is activated by nuclear β-catenin, has also been found to be triggered in other human tumour types such as breast cancer (8), pleural malignant mesothelioma (9) and pulmonary blastoma (10).

These possible mechanisms have received little investigation in veterinary medicine so far (11). The aim of the present study was to investigate the role played by APC protein and β-catenin in canine intestinal carcinogenesis and malignant progression. In this regard, an immunohistochemical evaluation of β-catenin, E-cadherin and APC protein was performed on a series of normal, dysplastic and neoplastic canine colon, and the protein expressions were assessed for their correlation with the histological grade of malignancy. In addition, to obtain a

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better evaluation of the APC/β catenin complex, these proteins were assessed for colocalization in colon cells through double immunofluorescence staining and confocal laser microscopical observation.

Materials and Methods

Samples. Thirty formalin-fixed and paraffin wax-embedded samples, consisting of five normal canine colon epithelia, five dysplasias and 20 colorectal neoplasms (six benign and 14 malignant) were examined.

Normal colon tissues came from necroscopies (three) and were collected soon after death in order to avoid autolysis, while the other two normal samples came from the same dogs suffering from neoplasias, as well all five dysplastic samples. All neoplastic samples were acquired through biopsies and surgical resections.

The colorectal neoplasms were classified according to WHO criteria (12) (Table I) and were considered as benign (adenomas and polyps) when the cell proliferation did not go beyond the muscolaris mucosae. The malignant neoplasms were graded by two observers as well-differentiated (W) when the infiltration involved the submucosa and poorly differentiated (P) when the muscular layer was also involved. The infiltration of intestinal wall by individual neoplastic cells was also considered as an expression of malignancy. Cell shape and size, nuclear characteristics and number of mitoses were also considered as parameters of differentiation. More detailed information is given in Table I.

Table I. *Histological type, localisation site and differentiation grade of canine colorectal tumours*.

Intestinal tumours	Localisation site	Differentiation grade
Benign tumours		
Adenomas	Colon	
	Colon	
	Rectum	
Polyps	Rectum	
	Rectum	
	Rectum	
Malignant tumours		
Papillary adenocarcinomas	Colon	W
	Colon	W
	Colon	W
	Colon	W
	Colon	P
Tubular adenocarcinomas	Colon	P
	Rectum	P
	Colon	P
	Colon	P
	Rectum	W
	Rectum	P
Solid carcinomas	Colon	P
	Colon	P
	Colon	P

W, Well-differentiated; P, poorly differentiated.

Immunoperoxidase labelling. Sections were dewaxed in xylene, dehydrated in graded alcohols and washed in 0.01 M phosphate-buffered saline (PBS), pH 7.2-7.4. Endogenous peroxidase was blocked with hydrogen peroxide 0.3% in absolute methanol for 30 min. The streptavidin-biotin-peroxidase method (LSAB Kit; Dako, Glostrup, Denmark) was used. Antigen enhancement was performed by pretreating with microwave heating in citrate buffer, pH 6.00, twice for 5 minutes at 750 W. The primary antibodies, a monoclonal mouse anti-human β-catenin and anti-E-cadherin (Transduction Laboratories, Lexington, USA) diluted 1 in 100 in an antibody diluent (Dako), and a polyclonal rabbit anti-APC (C-20; Santa Cruz Biotechnology, Santa Cruz, Ca, USA) diluted 1 in 200, were applied overnight at 4˚C. The immunolabelling procedure included negative control sections incubated with mouse and rabbit IgG, instead of primary antibody. A mixture of biotinylated anti-mouse, anti-rabbit and anti-goat immunoglobulins (LSAB Kit; Dako), diluted in PBS, was used as secondary antibody and was applied for 30 min. After being washed in PBS, the sections were incubated in streptavidin conjugated to horseradish peroxidase in Tris-HCl buffer containing 0.015% sodium azide (LSAB Kit; Dako) for 30 min. In order to reveal the immunolabelling, 3'3'-diaminobenzidine tetrahydrochloride was used as a chromogen, with haematoxylin as a counterstain.

Two-colour immunofluorescence labelling. The pre-treatment steps and primary antibodies were the same as those used for immunoperoxidase labelling and the procedure was the same as that used by Borzacchiello *et al*. (13). The primary mouse monoclonal anti-β-catenin antibody was diluted 1 in 10 in the same antibody diluent used for immunoperoxidase labelling and applied overnight at 4˚C. Slides were washed three times in PBS and incubated with a tetramethylrhodamineisothiocyanate (TRITC)-conjugated goat anti-mouse secondary antibody, diluted 1 in 100 in PBS, for two hours at room temperature. After three washes in PBS, anti-APC rabbit policlonal antibody, diluted 1 in 20, was applied and the sections were again incubated overnight at 4˚C. Slides were washed three times more in PBS and then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody (Chemicon, Germany) diluted 1 in 50 in PBS, for two hours at room temperature. Slides were rinsed with PBS and mounted in Fluorescent Mounting Medium (Dako). For scanning and photography, a laser scanning microscope (LSM 510; Zeiss, Göttingen, Germany) was used. Mouse monoclonal anti-β-catenin antibody bound to TRITC was irradiated at 543 nm and then detected with a 560 nm long pass filter. Rabbit polyclonal anti-APC bound to FITC was irradiated at 488 nm and detected with a 505-560 nm band pass filter.

Two-channel frame-by-frame multitracking was used for detection to avoid cross-talk signals. The different frames were scanned separately, with appropriate installation of the optical path for excitation and emission of each scan according to the manufacturer's instructions.

Scoring of immunoreactivity. The distribution of E-cadherin and β-catenin was scored as follows: M (membranous) when the positivity was localized on the intercellular borders of neoplastic cells; H (heterogeneous) when the immunolabelling was localized on the intercellular borders of some neoplastic cells mixed with negative cells; C (cytoplasmic) when the immunolabelling was evenly distributed throughout the cytoplasm.

Table II. *Correlation of E-cadherin, β-catenin and APC expression and distribution with histological features.*

	Distribution of immunolabelling and percentage (mean±SD) of immunolabelled cells					
	Normal colon (5)	Dysplasia (5)	Benign tumour (6)	Malignant tumour (14)		*P*-Value
				Well-differentiated (5)	Poorly differentiated (9)	
E-cadherin	M 96.2±2.5	M 94±5.7	H (3); M (3) 70±19.4	H (5) 35±15.7	H (5) 15±10.4 – (4)	<0.0001
β-catenin	M 98±2.12	M 96.4±2.5	H (3); M (3) 71±12.7	M (3); H (2) 42±16	H (3); C (6) 40±13.4	<0.0001
APC	96±2.4	92.2±5.2	43.2±16.4	(3) 25±8.9 –(2)	(5) 15±5.2 –(4)	<0.0001

M: membranous; H: heterogeneous; C: cytoplasmic; –, absence of immunolabelling.

The intensity of immunolabelling was scored (+) when the positivity was strong, (±) when the positivity was weak, and (–) when it was absent.

In order to quantify the E-cadherin, β-catenin and APC immunohistochemical labelling, twenty fields in each section were examined at x400 (x40 objective and x10 ocular) magnification. At least 1,000 cells were counted to obtain the percentage of positive cells. The mean positive cell percentage was related to histological features and grades (for malignant tumours) by analysis of variance (ANOVA).

Results

Immunohistochemistry

Normal samples. APC was strongly expressed by epithelial cells of the intestinal surface and colonic glands which showed a diffuse cytoplasm positivity; the percentage of positive cells ranged from 94 to 100% (96±2.4). β-Catenin and E-cadherin were evident with strong positivity localized on the intercellular borders. The proportion of positive cells ranged from 95 to 100% (98±2.1) for β-catenin and from 85 to 100% (96.2±2.5) for E-cadherin (Table II).

Dysplastic samples. APC was strongly expressed in the cell cytoplasm and the percentage of positive cells ranged from 87 to 100% (92.2±5.2). β-Catenin and E-cadherin exhibited strong intensity and a membranous distribution; the percentage of positive cells ranged from 94 to 100% (96.4±2.5) for β-catenin and from 96 to 100% (94±5.7) for E-cadherin (Table II).

Benign tumours. The proportion of APC-positive cells ranged from 24 to 67% (43.2±16.4) and the intensity of expression was strong and diffused in the cell cytoplasm. β-Catenin intensity was strong and the distribution was membranous in three samples and heterogeneous in the other three; the percentage of positive cells ranged from 46 to 80% (71±12.7). Three samples showed heterogeneous immunolabelling for E-cadherin while the other three were considered membranous. These features were superimposable on those immunolabelled with β-catenin. The proportion of positive cells ranged from 42 to 80% (70±19.4) (Table II).

Malignant tumours.

Well-differentiated carcinomas. In three out of five well-differentiated carcinomas, APC was expressed by many cells with weak cytoplasmic positivity (Figure 1A), whereas it was absent from the other two. The percentage of positive cells ranged from 25 to 62% (30±21.4). β-Catenin showed weak and membranous expression in three samples, while in the other two it was heterogeneous. A few cells with cytoplasmic distribution and nuclear positivity were also observed (Figure 1B). The percentage of positive cells ranged from 20 to 64% (42±16). E-Cadherin immunolabelling was weak and heterogeneous and the percentage of positive cells ranged from 22 to 60% (35±15.7).

Poorly differentiated carcinomas. In five out of nine poorly-differentiated carcinomas, APC expression was weak and cytoplasmatic. The percentage of positive cells ranged from 19 to 45% (25±13.2). The remaining four carcinomas were APC negative (Figure 1C). In six out of nine poorly differentiated carcinomas, β-catenin showed strong immunolabelling, which was diffuse in the cell cytoplasm and often also in the nuclei of many neoplastic cells (Figure 1D). These features were evident in less differentiated areas of neoplasms, whereas in their normal counterpart or in better differentiated areas, β-catenin retained its membranous localization. Four of these samples were the same four carcinomas which were shown to be APC negative. In the remaining three samples, β-catenin showed weak and heterogeneous expression. The percentage of positive cells

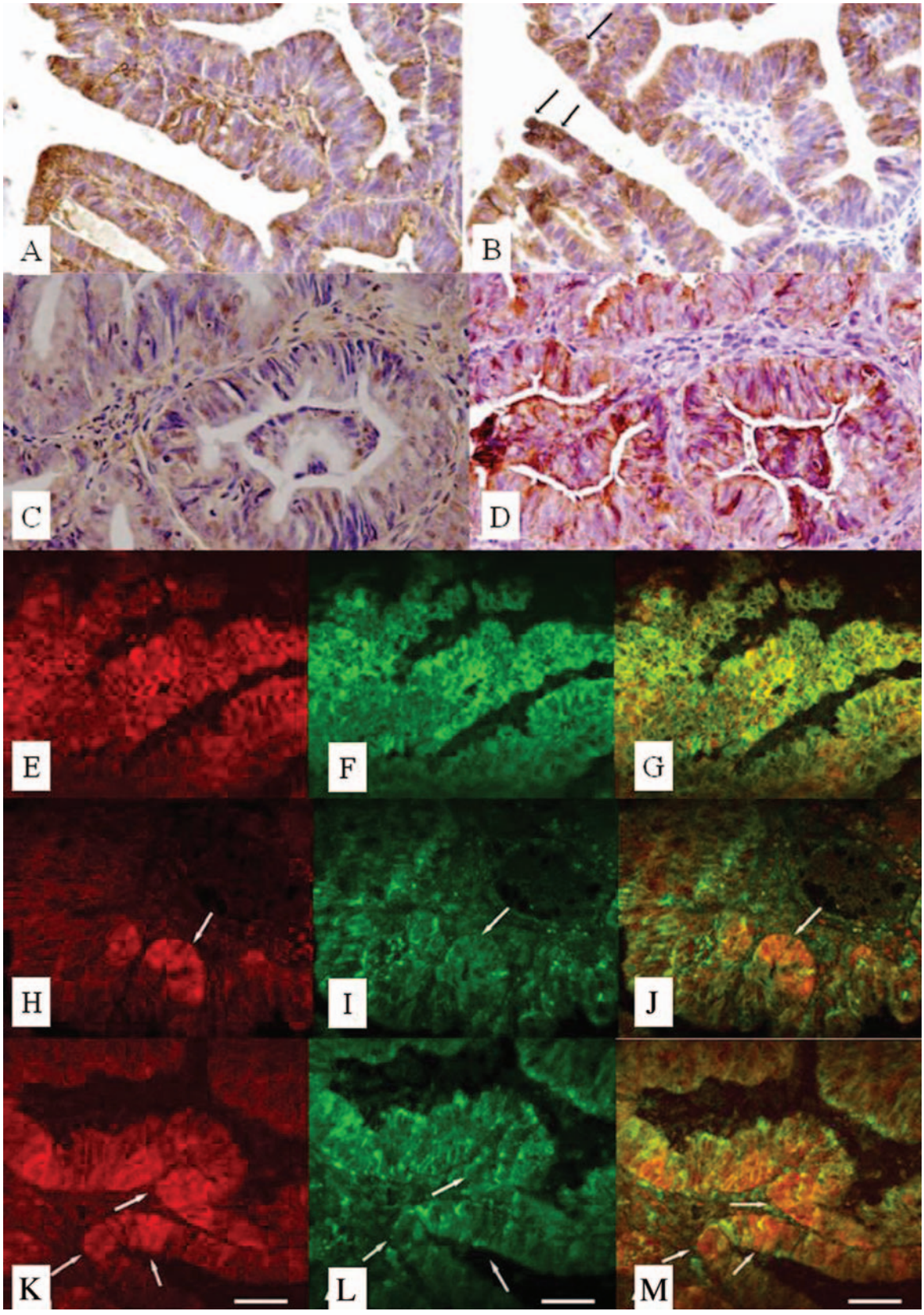

Figure 1.

ranged from 22 to 62% (42±13.4). E-Cadherin was weak and heterogeneous in five samples, with the percentage of positive cells ranging from 10 to 45% (25±10.4), and immunolabelling was absent from the remaining four. Results are summarized in Table II.

Two-colour immunofluorescence labelling

Normal and dysplastic samples. β-Catenin conjugated with TRITC was evident as red fluorescence localized on the intercellular borders of epithelial cells (Figure 1E), while FITC-conjugated APC expressed green fluorescence was distributed in the cytoplasm of many cells (Figure 1F). A colocalization of APC and β-catenin, evident as yellow fluorescence, was observed in many epithelial cells (Figure 1G).

Neoplastic samples. Immunofluorescence for β-catenin showed a weak intensity in many cells in which the membranous distribution was retained. Some groups of neoplastic cells expressed a cytoplasmic β-catenin distribution (Figure 1H and 1I). In these cells, APC was weakly expressed or absent (Figure 1K and 1L). A lack of colocalization of APC/ β catenin was also observed (Figure 1J and 1M).

Correlation of β-catenin, E-cadherin and APC expression in normal colon, dysplasia, benign and malignant, well and poorly differentiated tumour. A progressive decrease in the expression of β-catenin, E-cadherin and APC from non-neoplastic samples up to poorly differentiated malignant tumours, as determined by the percentage of immunolabelled cells, was observed (Figure 2). In particular, a decrease of E-cadherin and APC expression was evident in poorly differentiated malignant carcinomas. The differences between non-neoplastic samples and benign, well and poorly differentiated tumors were statistically significant ($p<0.0001$). β-Catenin expression decreased from normal, dysplastic samples to benign and malignant tumours ($p<0.0001$), while no statistical difference resulted from comparison between well- and poorly differentiated carcinomas ($p=0.96$) (Figure 2).

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Figure 1. *A, Well-differentiated papillary adenocarcinoma showing APC protein to be strongly expressed by many cells. The immunolabelling is evident at the luminal pole of neoplastic cells. (Streptavidin-biotin peroxidase; Bar=20 μm); B, Well-differentiated papillary adenocarcinoma. Cytoplasmic β-catenin distribution with nuclear positivity is evident in some cells (arrows). Membranous distribution is also shown. (Streptavidin-biotin peroxidase; Bar=20 μm); C, Poorly differentiated tubular adenocarcinoma. Immunostaining for APC protein showing complete negativity (Streptavidin-biotin peroxidase; Bar=20 μm); D, Poorly differentiated tubular adenocarcinoma showing β-catenin distributed in the cytoplasm of many neoplastic cells. (Streptavidin-biotin peroxidase; Bar=20 μm); E, Dysplasia. β-Catenin immunostaining, corresponding to red TRITC immunofluorescence, with a membranous distribution in many cells. Cytoplasmic distribution is evident in few cells. (Two-colour immunofluorescence. Bar=40 μm); F, Dysplasia. APC immunostaining, corresponding to green FITC imunofluorescence, with strong expression in many cells. (Two-colour immunofluorescence. Bar=40 μm); G, Dysplasia. Coexpression of APC and β-catenin, evident in yellow, shows the colocalization of both protein in many cells. (Two-colour immunofluorescence. Bar=40 μm); H, Well-differentiated tubular adenocarcinoma. β-Catenin immunostaining, corresponding to red TRITC immunofluorescence, is membranous in many cells. Cytoplasmic distribution is evident in some neoplastic cells (arrows). (Two-colour immunofluorescence. Bar=40 μm); I, Well-differentiated tubular adenocarcinoma. APC protein immunostaining, corresponding to green FITC imunofluorescence, with weak expression in many cells and negativity in the cells with cytoplasmic β-catenin distribution (arrows). (Two-colour immunofluorescence. Bar=40 μm); J, Well-differentiated tubular adenocarcinoma. Coexpression of both β-catenin and APC protein confirms the cytoplasmic distribution of β-catenin in APC-negative cells (arrows). (Two-colour immunofluorescence. Bar=40 μm); K, Poorly differentiated tubular adenocarcinoma. β-Catenin immunostaining, corresponding to red TRITC immunofluorescence, with cytoplasmic distribution in many neoplastic cells (arrows). (Two-colour immunofluorescence. Bar=40 μm); L, Poorly differentiated tubular adenocarcinoma. APC protein immunostaining, corresponding to green FITC imunofluorescence, shows the absence of protein in many neoplastic cells (arrows). (Two-colour immunofluorescence. Bar=40 μm); M, Poorly differentiated tubular adenocarcinoma. Coexpression of both β-catenin and APC protein confirms the cytoplasmic distribution of β-catenin in APC-negative cells (arrows). (Two-colour immunofluorescence. Bar=40 μm);*

Discussion

Intestinal epithelium undergoes constant turnover and the maintenance of glandular and villous structures requires sophisticated control mechanisms which are partly mediated through adhesion molecules (14). A failure of these control mechanisms may result in disorganization of the tissue and in abnormal tissue growth (15, 16). In this regard, the E-cadherin/β-catenin complex plays a key role. In fact, the progressive loss of E-cadherin causes a dissociation of epithelial cells which can detach from the primary mass and infiltrate both the surrounding tissues and the vascular structure. In addition, disruption of β-catenin, which can also be linked to *APC* mutation, interferes with E-cadherin metabolism, compromising its correct location on the cell membrane, which is indispensable for intercellular adhesion. In this study, a dysregulation of β-catenin with cytoplasmic distribution associated with a progressive decrease and loss of APC and E-cadherin expression was found, suggesting a possible role of the E-cadherin/APC/β-catenin complex also in canine colorectal tumours.

A marked decrease in APC expression was observed between non-neoplastic and neoplastic samples ($p<0.0001$), as well as between benign and well- and poorly differentiated malignant tumours, demonstrating that APC impairment is crucial for neoplastic transformation in canine colonic epithelial cells, and also for neoplastic progression.

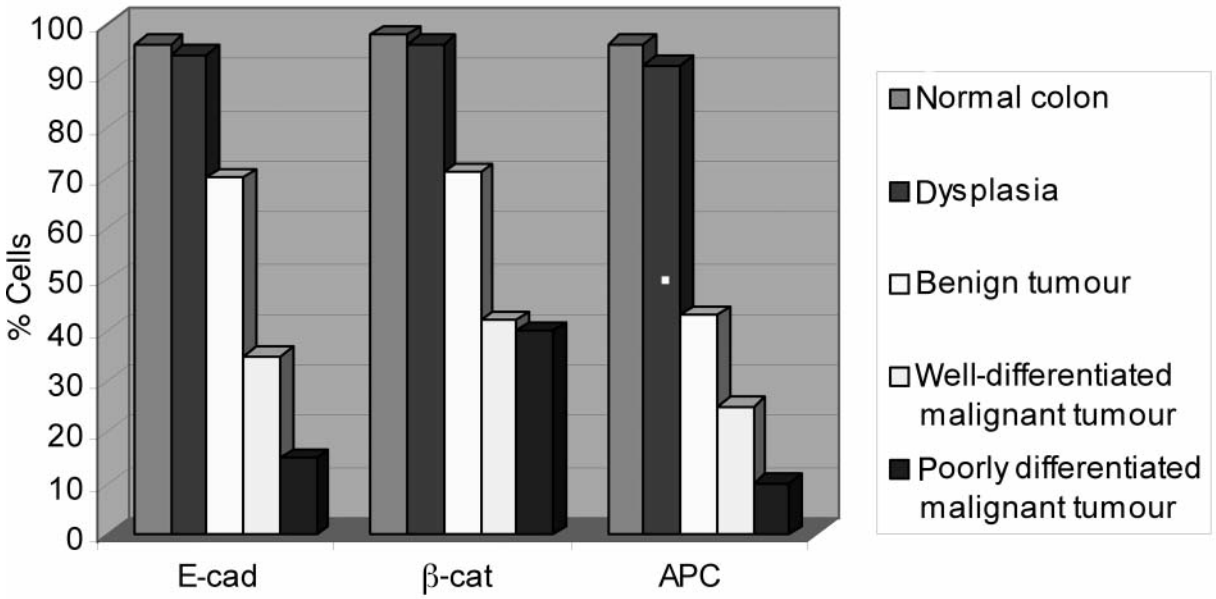

Figure 2. *Comparison of the percentage of cells immunolabelled with E-cadherin, β-catenin and APC in normal, dysplastic and neoplastic samples of canine colon.*

β-Catenin expression was lower in benign and malignant samples ($p<0.0001$), but no statistical difference was observed between well- and poorly differentiated carcinomas ($p=0.96$) (Figure 2), while a loss of membranous distribution was evident in less-differentiated tumours.

Confocal laser microscopical observation showed a cytoplasmic β-catenin distribution solely in APC-negative cells, suggesting that the lack of APC could be responsible for β-catenin cytoplasmic accumulation.

As regards E-cadherin expression, it decreased progressively until it was completely lost in malignant poorly differentiated carcinomas. A statistical correlation was observed between its expression in benign and malignant tumors ($p<0.0001$) and especially in well- and poorly differentiated malignant tumours ($p<0.0001$). These results could suggest that E-cadherin may be more involved in malignant progression than in early neoplastic transformation, confirming its role as an invasion suppressor (17, 18). A lower E-cadherin staining, significantly associated with β-catenin disruption, has already been evidenced in canine intestinal tumours by Mc Entee and Brenneman (19), suggesting that β-catenin dysregulation precedes E-cadherin alterations.

In canine colorectal carcinogenesis, therefore, two points can be distinguished at which APC/β-catenin and E-cadherin play different roles. Although the binding sites for APC and E-cadherin are different, they cannot link β-catenin simultaneously (20), so that in normal cells, they stay in mutual equilibrium; hence, β-catenin is partly bound to E-cadherin to maintain intercellular adhesion and partly degraded *via* APC. When mutations occur in the *APC* gene and APC protein is not able to link β-catenin, the equilibrium between APC and E-cadherin is disrupted and the cytoplasmic β-catenin level increases. This could constitute a factor in the early development of colorectal tumours (7). Cytoplasmic accumulation of β-catenin then prevents the linking with E-cadherin, hence disrupting intercellular adhesion. The impairing of E-cadherin, also linked to mutations in its gene, could act later, in malignant progression, allowing the development of an invasive phenotype (21).

In conclusion, deranged control mechanisms of cellular adhesion, rather than the acquisition of autonomous growth, may constitute important events both in the early stage of colorectal tumour development (22) and in malignant progression.

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