

Expression of β -Catenin, E-cadherin and APC in Canine Mammary Tumors

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Abstract. *Background: Mammary tumors are a very common neoplasm in the dog and show histological features and biological behaviour similar to human mammary carcinomas. Recently, a pathway named Wnt-1, involving β -catenin and APC protein, has emerged as an important player in many human tumor types, including mammary neoplasms. Materials and Methods: Thirty-five samples of canine mammary tumors (10 benign and 25 malignant) were studied in order to evaluate the co-expression of β -catenin, APC protein and E cadherin with confocal laser microscopical observation, by western blot analysis and by correlating data obtained with the histological grade of tumours. Results: A progressive decrease of E-cadherin together with disruption of β -catenin expression was observed in less differentiated malignant tumors. In addition, a loss of β -catenin membranous distribution and a cytoplasmic accumulation was often coexpressed with disrupted expression of APC protein. Western blot analysis showed a progressive increase of β -catenin in malignant tumors, which could be the expression of disrupted β -catenin catabolism leading to cytoplasmic accumulation. In some less differentiated malignant tumors, a marked β -catenin decrease was also observed. This feature could be linked to mutations in β -catenin gene coding for a truncated and lighter protein. Conclusion: These results may indicate the multifunctional role played by β -catenin in canine mammary oncogenesis.*

The catenins are a group of intracytoplasmic proteins, named α , β and γ catenin, involved in the cell adhesion mediated by E-cadherin, a transmembrane glycoprotein

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responsible for intercellular adhesion in epithelial tissue (1). The E-cadherin-catenin complex plays a crucial role in the organization and maintenance of epithelial tissue architecture (2). The decrease or loss of E-cadherin, together with alterations in catenin expression, mainly consisting in the cytoplasmic and sometimes nuclear localization of β -catenin from carcinomas is involved in the loss of cell differentiation and in the development of an invasive tumor phenotype (3). These features have been correlated to a high grade of malignancy and poor prognosis in many human (4) and animal neoplasms (5).

Alterations in E-cadherin and catenin expression were often linked to mutations in their coding genes (6-8). Alterations in β -catenin expression can also be linked to mutations of genes involved in its degradation, such as the adenomatous polyposis coli gene (APC) (9, 10) whose protein is involved in β -catenin turnover, as demonstrated in human colorectal tumors (11), malignant mesotheliomas (12), gastric (13) and mammary carcinomas (14). More precisely, in normal cells, free cytoplasmic β -catenin is entrapped by a complex containing axin, glycogen synthase kinase 3 β (GSK3 β) and APC (15). This complex facilitates the phosphorylation of β -catenin which can then be eliminated. When mutated, APC protein is not able to phosphorylate β -catenin which cannot then be degraded (16) and, as a consequence, it accumulates in the cytoplasm, functioning in signal transduction of the Wnt-1 signaling pathway (17), associated first with mammary oncogenesis in the mouse (18) and then in human carcinogenesis (19, 20).

To the best of our knowledge, little is known about the role played by APC and β -catenin in canine neoplasms.

The aims of the present paper were (i) to evaluate E cadherin, β -catenin and APC morphological co-expression in a series of canine mammary tumors with confocal laser scanning microscopy, and to correlate these expressions with the histological grade of the neoplasm; (ii) to evaluate E-cadherin, β -catenin and APC quantitative expression in neoplastic cells by Western blot analysis.

Materials and Methods

Thirty-five samples of canine mammary tumors (10 benign and 25 malignant) were cooled in isopentane in liquid nitrogen. The frozen sections were stained with hematoxylin and eosin and classified according to WHO criteria (21). Malignant tumors were graded by two observers as well, as moderately- or poorly-differentiated (Grade I, II and III) using the Misdorp parameters (22).

Immunofluorescent staining. Frozen sections were cut on a cryostat, fixed with acetone and washed in phosphate-buffered saline (PBS). The antibodies used were mouse monoclonal anti-human E-cadherin and β -catenin (Transduction Laboratories, Lexington, USA) and rabbit polyclonal anti-human APC (Santacruz Biotechnology, Santacruz, CA, USA). A two-color immunofluorescence method was applied coupling primary antibodies as follows: β -catenin/E-cadherin; β -catenin/APC at 1:20 dilutions. The method was the same used by Borzacchiello *et al.* (23). Briefly, of each combination, the first primary antibody (anti- β -catenin) diluted 1 in 20 in the antibody diluent (Dako, Copenhagen, Denmark) was applied overnight at 4°C. Slides were washed three times in PBS and incubated with a tetramethylrhodamineisothiocyanate (TRITC)-conjugated goat anti-mouse secondary antibody (Chemicon, Temecula, CA, USA), diluted 1 in 100 in PBS, for two hours at room temperature. After three washes in PBS, the second primary antibody of each combination, diluted 1 in 20, was applied and the sections were again incubated overnight at 4°C. Slides were washed three times in PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody diluted 1 in 100 in PBS (for anti-E-cadherin) and goat anti-rabbit secondary antibody diluted 1 in 50 in PBS (for anti-APC), for two hours at room temperature. Slides were rinsed with PBS and mounted under Fluorescent Mounting Medium (Dako, Copenhagen, Denmark). 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 detected with a 560 nm long-pass filter. Rabbit polyclonal anti-APC and mouse monoclonal anti-E-cadherin both bound to FITC were 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 individual 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. E-cadherin, β -catenin and APC protein expression was scored as + when the immunolabelling was strong, \pm when weak and - when absent.

The distribution of E cadherin and β -catenin were scored as: M (membranous) when the positivity was localized on the intercellular borders of all neoplastic cells; H (heterogeneous) when the immunolabelling was localized at the intercellular borders of some neoplastic cells intermingled with negative cells; C (cytoplasmic) when the immunolabelling was uniformly distributed through the cytoplasm. A semi-quantitative assessment of APC expression was performed by choosing 20 fields for each sample and counting at least 1000 neoplastic cells and expressing the results of positive cells as a percentage.

Western blot analysis. Samples were homogenized in 100 μ L of ice-cold hypotonic lysis buffer (10 mm HEPES, 1.5 mm $MgCl_2$, 10 mm KCl, 0.5 mm phenylmethylsulfonyl fluoride, 1.5 μ g/mL soybean trypsin inhibitor, pepstatin A 7 μ g/mL, leupeptin 5 μ g/mL, 0.1 mm benzamidine, 0.5 mm dithiothreitol (DTT)) and incubated in ice for 45 min, as previously described by Izzo *et al.* (24). After this time, the supernatant was isolated and protein quantification was performed using a BioRad assay according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA).

Immunoblotting analysis of APC, β -catenin and E-cadherin proteins were performed on cytosolic protein fractions isolated from the samples as previously described. Equal amounts of proteins (100 μ g) were mixed with gel loading buffer (50 mm Tris, 10% SDS, 10% glycerol 2-mercaptoethanol, 2 mg bromophenol/ml) at a ratio of 1:1, boiled for 5 min, then separated under reducing conditions in 6% (for APC) on 10% (for β -catenin and E-cadherin) SDS-polyacrylamide minigel. The proteins were transferred onto a nitrocellulose membrane according to the manufacturer's instructions (Bio-Rad Laboratories). The membranes were blocked by incubation at 4°C overnight in a high salt buffer (50 mm Trizma base, 500 mm NaCl, 0.05% Tween-20) containing 5% bovine serum albumin. Membranes were then incubated for 2 h at room temperature with anti- β -catenin (1:200 v:v) (BD Transduction Laboratories, San Diego, CA, USA), or anti-APC (1:200 v:v) (Santacruz Biotechnology), or anti-E-cadherin (1:200 v:v) (Chemicon, Temecula, CA, USA) followed by incubation with specific horseradish peroxidase (HRP)-conjugate secondary antibody (Dako). The immune complexes were developed using enhanced chemiluminescence detection reagents (Amersham, Cologno Monzese, Italy) according to the manufacturer's instructions and exposed to Kodak X-Omat film. The bands of proteins on X-ray film were scanned and densitometrically analysed with a GS-700 imaging densitometer (Bio-Rad Laboratories).

Results

Immunofluorescence staining

β -Catenin/E-cadherin coexpression. In benign and all grade I malignant tumors, a strong membranous coexpression of β -catenin and E-cadherin was observed in most neoplastic cells. Few cells showed an altered expression of these proteins. (+M/+M) (Figure 1A-C).

Among grade II malignant tumors, one papillary adenocarcinoma showed a strong expression with a membranous distribution of β -catenin and E-cadherin; in two tubular adenocarcinomas, both proteins were weakly expressed in only a few cells and the intensity was lower than in grade I tumors (\pm H/ \pm H) (Figure 1D-F), while in five samples (two solid carcinomas, two tubular and one papillary adenocarcinomas), a cytoplasmic distribution of E-cadherin was observed in several neoplastic cells (+C). In the same cells, β -catenin expression was weak and the distribution was heterogeneous (\pm H) (Figure 1G-I). Only in one out of the two tubular adenocarcinomas was β -catenin cytoplasmic (\pm C/+C).

Among grade III tumors, two tubular adenocarcinomas showed weak expression and heterogeneous distribution of

β -catenin and E-cadherin ($\pm H/\pm H$); four samples (one tubular adenocarcinoma and three solid carcinomas) showed a weak and heterogeneous distribution of β -catenin ($\pm H$), while E-cadherin was strongly expressed in the cytoplasm (+C); the remaining three solid carcinomas showed a cytoplasmic distribution of β -catenin and E-cadherin ($\pm C/+C$) in several neoplastic cells.

β -catenin/APC protein co-expression. In benign tumors, a strong and membranous β -catenin was coexpressed with strong expression of APC protein which was distributed in the cytoplasm of 90% neoplastic cells (Figure 1L-N).

In seven out of grade I malignant tumors, a coexpression of β -catenin and APC protein was observed (45% neoplastic cells), while in one tubular adenocarcinoma APC protein was expressed only by spindle cells localized at the borders of mammary ducts and ductules. β -Catenin was membranous; only in a few cells at the center of ducts did β -catenin lose its membranous distribution and appear diffused in the cytoplasm (Figure 1O-Q). In these samples, a co-localization APC/actin was performed in order to investigate the histogenesis of APC-positive peripheral cells, and a coexpression APC/actin was observed (not shown).

Among eight grade II malignant tumors, six samples showed a weak expression and heterogeneous distribution of β -catenin ($\pm H$) coexpressed with a weak expression of APC protein in 30% of tumour cells. In one tubular adenocarcinoma, a strong expression and membranous distribution of β -catenin was coexpressed with a strong APC protein expression; in one tubular adenocarcinoma showing cytoplasmic β -catenin APC was negative.

In all grade III malignant tumors, APC was weakly expressed only by a few spindle cells (20%) coexpressing actin, while β -catenin showed a heterogeneous distribution in six samples and a cytoplasmic distribution in three (Figure 1R-T).

Western blot analysis. Quantitative expression of E-cadherin, β -catenin and APC did not show marked changes in benign and malignant tumors. A slight decrease in E-cadherin was observed, while β -catenin and APC were raised. In five grade III carcinomas, a marked decrease of all proteins was observed (Figures 2 and 3).

Discussion

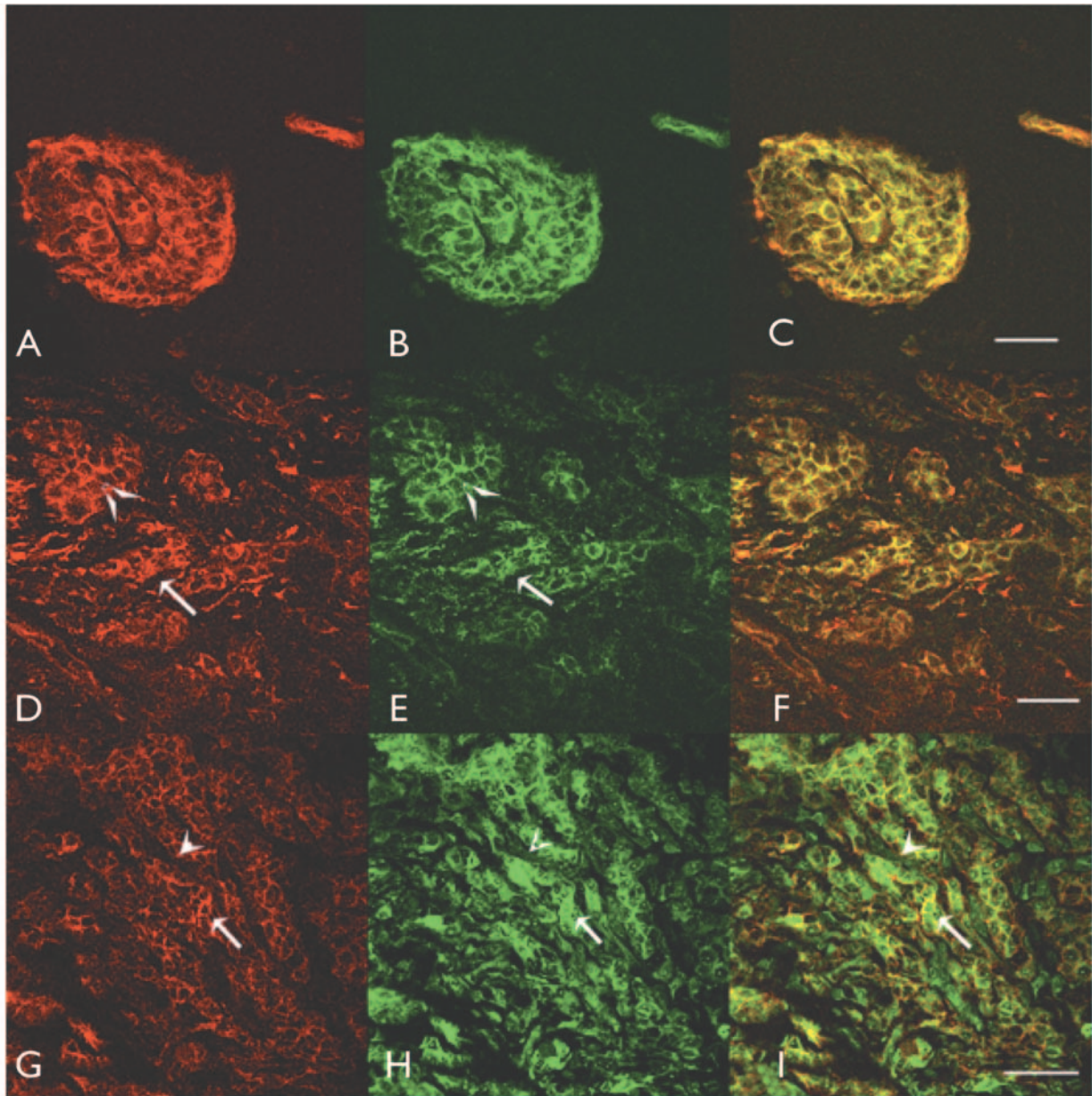
The ability of malignant cells to modulate their intercellular cohesiveness is considered to be an early and pivotal event in tumor progression. The loss of E-cadherin expression leads to the dissociation of cells, disrupting the tissue architecture determining the acquisition of less differentiated histological features, such solid proliferation (25), and allows tumour cell detachment, the first step of

metastasis (26). Some previous studies showed that E-cadherin expression and distribution were altered in malignant canine mammary tumors up to complete loss in less differentiated malignant phenotypes (27, 28).

In this study, a decrease or loss of E-cadherin expression was coexpressed with β -catenin alteration in malignant tumors. β -Catenin cytoplasmic positivity was often associated with E-cadherin cytoplasmic positivity in some malignant less-differentiated tumors. It has been demonstrated that β -catenin cytoplasmic accumulation may be linked to disruption of its catabolism controlled by APC protein, which complexes and degrades β -catenin (29). In colorectal tumors, mutations of APC result in truncated APC protein which can no longer degrade β -catenin and the net result is its cytoplasmic accumulation and, thereafter, its entrance into the nucleus (nuclear positivity) (30). At this level, β -catenin can activate a transcription factor, named TCF-4, which is involved in the first transformation of intestinal epithelial cells (31). Interestingly, mice lacking TCF-4 are characterized by a lack of intestinal stem cells (32), suggesting that TCF-4 may be important for the maintenance of stem cell characteristics, as well longevity (33). In addition, c-myc and cyclin D1 were identified as TCF/ β -catenin transcriptional targets in colon cancer (34). Hence, the alteration of β -catenin can play a crucial role both in neoplastic transformation, by activating TCF-4, and in neoplastic progression, preventing the linking with the E-cadherin cytoplasmic tail on which the correct orientation of E-cadherin on the cell membrane depends (35). This may explain the cytoplasmic distribution of both E-cadherin and β -catenin observed in malignant tumors.

Interestingly, Western blot analysis revealed two different and contrasting features in β -catenin expression in less differentiated malignant tumors: a high and low quantity of protein. This equivocal situation may be explained by the different oncogenic pathways involving β -catenin. The β -catenin quantitative increase could be the expression of the disrupted catabolism leading to cytoplasmic accumulation in malignant tumours. On the other hand, a β -catenin decrease could be linked to a mutated gene which could codify for a truncated and lighter protein.

In our study, a localization of APC protein in peripheral ductal cells was observed and the coexpression of APC and actin in these cells demonstrated a myoepithelial origin. This allows us to hypothesize a role for myoepithelial cells in retaining the mammary architecture also *via* APC/ β -catenin, confirming the myoepithelium as a natural tumor suppressor, by exerting paracrine effects on the glandular epithelium and regulating the progression of carcinomas (36-38). The impairment of APC protein is involved in the activation of the Wnt signal transduction pathway, which inhibits the activity of glycogen synthase kinase 3 β (GSK-3 β) causing β -catenin cytoplasmic accumulation and its nuclear

Figure 1. *continued*

translocation (33). In many tumor types, including mammary carcinomas, this oncogenic pathway is followed and this could determinate the tumoral phenotype (39). Studies of transgenic murine models demonstrate that Erb/Ras pathway tumors form solid nodules consisting of poorly differentiated cells without myoepithelial differentiation and characterized by poorer prognosis. In contrast, Wnt1 pathway carcinomas exhibit myoepithelial, acinar or glandular differentiation, forming caricatures of elongated and branched ductules in a well-developed stroma (40) and are characterized by a better clinical outcome. Transgenic mice expressing a β -catenin mutant form developed

mammary adenocarcinomas with an identical Wnt1 phenotype, confirming that β -catenin is a major downstream effector of the Wnt-mediated tumour pathway (41). Therefore, β -catenin can act as a multifunctional protein involved in two independent process: cell-cell adhesion and signal transduction in the Wnt-1 signaling pathway (42).

The presence of APC protein in myoepithelial cells together with β -catenin impairment in malignant carcinomas suggests that canine mammary tumors, most of which are characterized by the myoepithelial phenotype, may originate from a Wnt1 pathway and β -catenin may play two relevant roles both in neoplastic transformation and in malignant

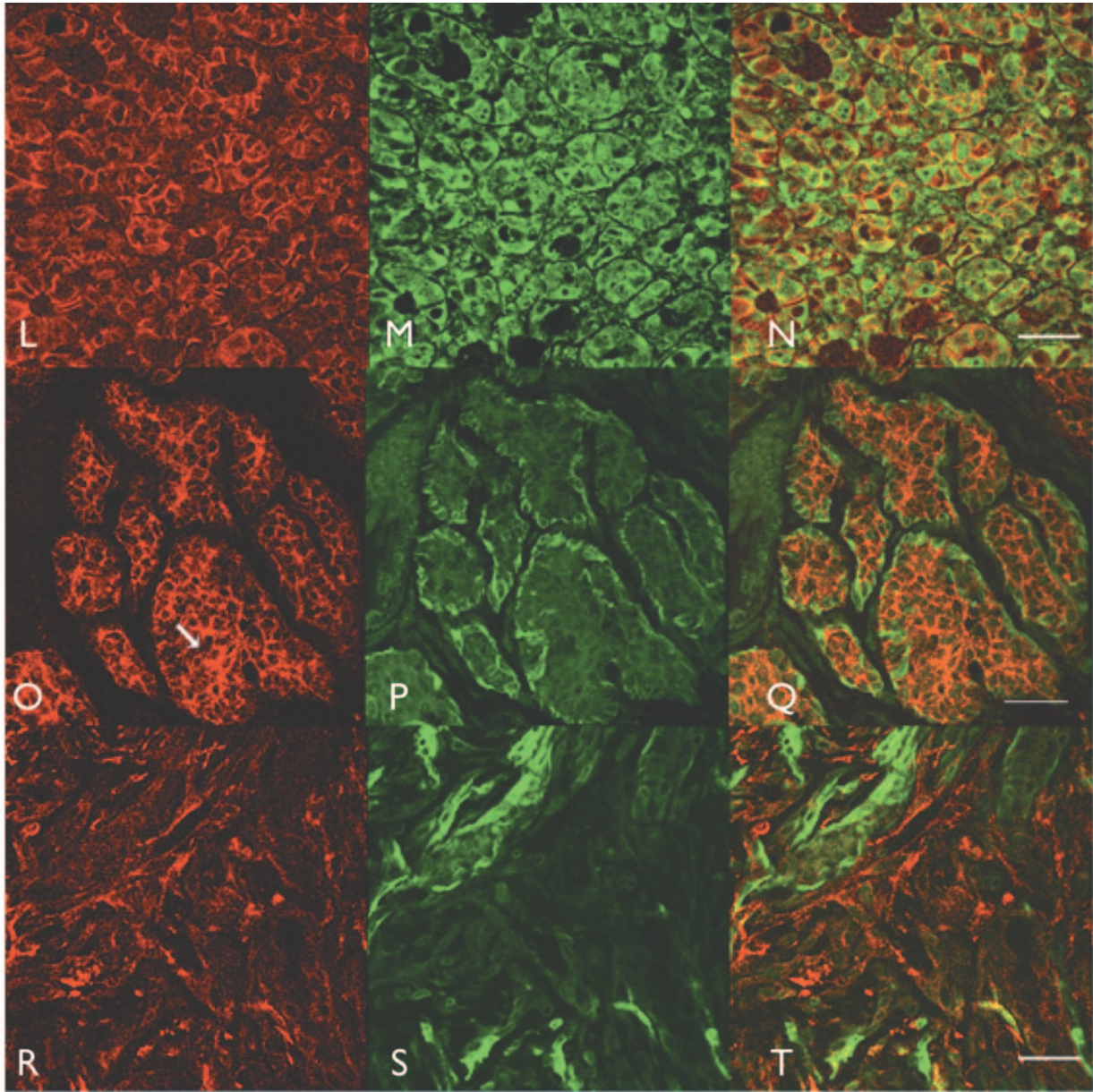


Figure 1. TRITC/FITC double immunofluorescence. A) Tubular adenoma; (+M/+M), intense membranous positivity for β -catenin (red TRITC immunofluorescence) in neoplastic cells of a mammary duct. B) Tubular adenoma (+M/+M), the same finding as in 1A (green FITC immunofluorescence). C) Tubular adenoma (+M/+M), coexpression of β -catenin and E-cadherin, (yellow fluorescence). D) Grade II tubular adenocarcinoma; ($\pm H/\pm H$), membranous (arrowhead) and cytoplasmic (arrow) distribution of β -catenin (red TRITC fluorescence). Some cells are negative or show a weak intensity; E) Grade II tubular adenocarcinoma; ($\pm H/\pm H$), membranous (arrowhead) and cytoplasmic (arrow) distribution of E-cadherin (green FITC immunofluorescence). F) Grade II tubular adenocarcinoma; ($\pm H/\pm H$), coexpression of β -catenin and E-cadherin. G) Grade II solid carcinoma; ($\pm H/+C$); Few cells expressing β -catenin (TRITC red immunofluorescence) with strong intensity and membranous distribution (arrow) are intermingled with cells weakly positive or negative (arrowhead). H) Grade II solid carcinoma; ($\pm H/+C$); strong expression of E cadherin (green FITC immunofluorescence) expressed in all neoplastic cells with membranous and cytoplasmic distribution (arrowhead). I) Grade II solid carcinoma; ($\pm H/+C$); coexpression of β -catenin and E-cadherin on the cell membrane (arrow). Most β -catenin-negative cells exhibit E-cadherin cytoplasmic distribution (arrowhead). L) Tubular adenoma; membranous distribution of β -catenin (red TRITC immunofluorescence) in all neoplastic cells. M) Tubular adenoma; strong expression of APC protein, (green FITC immunofluorescence) in all neoplastic cell cytoplasm. N) Tubular adenoma; no coexpression of red membranous β -catenin and green cytoplasmic APC. O) Grade I tubular adenocarcinoma; β -catenin membranous distribution. Few cells at the center of mammary duct show cytoplasmic distribution (arrow). P) Grade I tubular adenocarcinoma; APC protein (green FITC immunofluorescence) expressed only by spindle cells localized at the borders of mammary ducts. Q) Grade I tubular adenocarcinoma; no coexpression of red β -catenin and green APC protein. R) Grade III solid carcinoma; β -catenin cytoplasmic distribution in few cells intermingled with negative cells. S) Grade III solid carcinoma; APC protein expressed with strong intensity only in few spindle cells. T) Grade III solid carcinoma; coexpression of both red β -catenin and green APC protein. β -Catenin cytoplasmic distribution is evident in APC-negative cells.

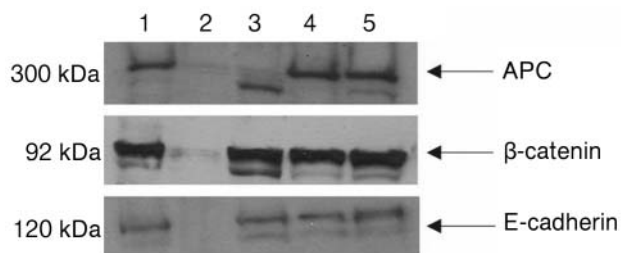


Figure 2. Mammary carcinomas. Western blot for APC, β -catenin and E-cadherin. Line 1: Benign tumor; Line 2: Grade III malignant tumor; Line 3: Grade III malignant tumor; Line 4: Grade I malignant tumor; Line 5: Grade II malignant tumor. A marked decrease of all proteins is evident in line 2 while a marked increase of β -catenin is evident in line 3.

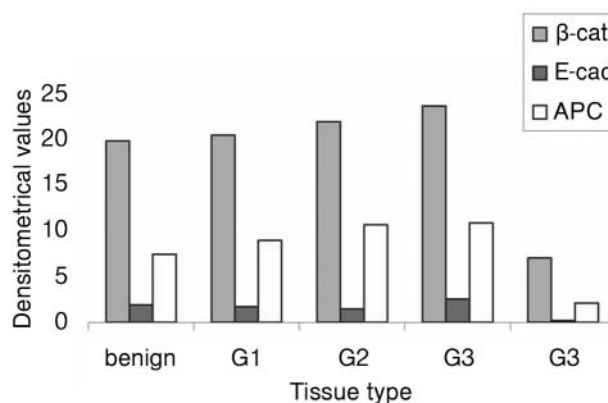


Figure 3. Densitometric values of β -catenin, E-cadherin and APC in benign tumors and in various histological grades of malignant canine mammary carcinomas demonstrate a decrease in E-cadherin expression and an increase in β -catenin and APC expression. Grade III carcinomas show two different and opposite expression of β -catenin and APC: a marked increase and a marked decrease.

progression. These hypotheses could also have important prognostic and therapeutic implications. In fact, a large number of drugs, such as NSAIDs, which are in clinical use for the treatment of many diseases other than cancer are inhibitors of the Wnt-1 signaling pathway and could also find use as anticancer agents in canine mammary carcinomas (43).

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