Immunohistochemical Expression of β-catenin, Ki67, CD3 and CD18 in Canine Colorectal Adenomas and Adenocarcinomas

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Abstract

Background Inflammation is believed to influence the human colorectal carcinogenesis and may have impact upon prognosis and survival. High presence of tumour-infiltrating CD3+ T-cells, is associated with a better outcome in humans with colorectal cancer. The mucosal immunophenotype in dogs with colorectal cancer is poorly described.

The aim of this study was to investigate whether the density, distribution and grade of tumour-infiltrating immune cells (TIIs) in canine colorectal tumours is associated with histologic indicators of malignancy and can be considered a prognostic factor in dogs.

This retrospective case-control study was performed on formalin-fixed, paraffin-embedded tissue samples from dogs with histologically confirmed colorectal adenoma (n=18) and adenocarcinoma (n=5) collected from archived samples. The samples had been collected by colonoscopy, surgery or during postmortem examination. Healthy colonic tissue obtained post mortem from dogs euthanized of reasons not involving the gastrointestinal tract, served as control tissue (n=9).

Results: The tumour samples had significantly lower numbers of CD3+ T- cells in the epithelium compartment (Wilcoxon test, p=0,0006), as well as significantly lower number of CD18+ cells in the lamina propria, compared to control samples (Wilcoxon test, p=0,001). The Ki67 positive cells showed a strong signal in adenomas and adenocarcinomas. There was no clear distinction with regards to expression levels of the markers for tumour progression (β-catenin, and Ki67) between adenomas and adenocarcinomas. Colonic samples from control dogs had uniform staining of β-catenin along the cell membrane of epithelial cells. When compared to normal colonic cells, the expression levels of cytoplasmic β-catenin were significantly higher in adenomas and adenocarcinomas (Wilcoxon test, p=0,0002). None of the control samples showed positive staining of β-catenin in the nucleus of colonic cells. In contrast, adenocarcinoma and adenoma showed moderate to strong staining of the cell nucleus.

Conclusions: β-catenin and Ki67 were not useful markers in distinguishing adenomas from adenocarcinomas. The lower presence of CD18- and CD3+ cells in tumours compared to controls, indicates a reduced presence of histiocytes and T-cells which may have implications for the defense against cancer progression.

Introduction

Colorectal cancer (CRC) made up by adenocarcinoma is one of the most common types of cancer in humans (1). In contrast, dogs are rarely diagnosed with colorectal cancer, albeit more frequently than other mammals (2, 3). This disease is associated with serious clinical signs and has a poor prognosis due to local recurrence and formation of metastases in both humans (4, 5) and dogs (6–8). Sporadic CRC in humans is believed to originate from adenomas through a process of multiple genetic and molecular events (9). Studies in dogs indicate that a similar process occurs in canine colorectal carcinogenesis (10–14).

Colonic stem cells are located at the bottom of the colonic crypt where they proliferate and migrate towards the top, resulting in mature cells without the capacity to divide. This proliferation process is tightly regulated through the wingless-related integration site (Wnt) signalling pathway and involves the protein β-catenin (15). Mutation of β-catenin is a key factor in the tumorigenesis, as described in humans (15) as well as in dogs (10, 16, 17). A failure in this mechanism results in colonic cells with increased proliferation capacity. The abnormal cellular proliferation along the tumorigenesis may be evaluated by Ki67 (18). The Ki67-protein is only present during active phases of the cell cycle, thus expression of this antigen indicates cell-growth (19).

Inflammatory cells have been proposed to play a role in the colorectal carcinogenesis in humans (20, 21), and chronic inflammation such as ulcerative colitis may progress into cancer (22). Colorectal adenomas and adenocarcinomas in humans are infiltrated by inflammatory cells, which may influence the capability of the tumour to proliferate and metastasize (23). The presence of tumour-infiltrating CD3 + T-lymphocytes in human CRC correlates with prognosis, as patients with high tumour infiltration have longer survival than those with poorly infiltrated tumours (24).

A study of Japanese miniature-dachshunds showed that the progression from inflammatory polyps into adenoma and carcinoma involves dysregulation of β-catenin (17). However, the inflammation and presence of T-lymphocytes associated with the canine colorectal tumour progression have not been described at the different disease stages. In this work, we characterized and quantified the infiltration of immune cells in canine colorectal adenoma, adenocarcinoma, and normal colonic tissue by using the antigens CD18 and CD3 labelling histiocytes and T-cells, respectively. Evaluation of tumor progression was performed using the antigens β-catenin and Ki67.

Materials And Methods

This retrospective case-control study was performed on archived formalin-fixed paraffin-embedded tissue samples submitted to NMBU during the period from 1998 to 2015. These samples had been collected for clinical purposes and leftover samples were archived. Owner consent for using leftover samples for research in cancer diseases was given by dog-owners. These samples had been taken for clinical purposes and leftover samples were archived and used for this study.

Inclusion criteria for this study were colorectal tissue from dogs with histologically confirmed colorectal adenoma or adenocarcinoma, and 18 adenoma and five adenocarcinomas were included in addition to nine control samples. Colonic tissue from control dogs were collected at necropsy from dogs euthanized for reasons that did not involve the gastrointestinal tract (n = 9).

Selection of cases and control dogs

For each case, information about the breed, gender, age, histopathological diagnosis, tumour localization, sampling technique and treatment were obtained from the clinical record (Table 1). The dogs with colorectal adenomas and adenocarcinomas were of various breeds represented by both genders and

were between 1 and 14 years old, with an average of 8 years. From these dogs, colorectal mucosal samples were collected during surgery (n = 9), colonoscopy (n = 8), or necropsy (n = 2), and in four cases by unknown procedure (Table 1).

Table 1
Overview of dogs and samples

Dog no.	Breed	Gender	Age (y)	Diagnosis*	Tumour location (C/R)	Method of sampling	Treatment
1	German shephard	M	9	Adeno-carcinoma	R	Surgery	Surgery
2	Irish Setter	F	10	Adeno-carcinoma	R	Colonoscopy	Surgery
3	Shetland sheepdog	M	14	Adeno-carcinoma	C	Post mortem	Meloxicam
4	English springer spaniel	M	8	Adeno-carcinoma	R	Colonoscopy	Piroxicam
5	Tibetanian spaniel	M/N	10	Adeno-carcinoma	R	Post mortem	Meloxicam
6	German shepherd	F	9	Adenoma	UN	UN	UN
7	Irish Setter	M	6	Adenoma	UN	UN	UN
8	English setter	M	8	Adenoma	UN	UN	UN
9	Mixed breed	M	10	Adenoma	R	Surgery	UN
10	German shephard	M	4	Adenoma	R	Surgery	Surgery
11	Staffordshire bullterrier	M	8	Adenoma	R	Surgery	Surgery
12	Papillon	M	10	Adenoma	R	Surgery	Surgery
13	Colli shorthaired	M	3	Adenoma	R	Surgery	Surgery
14	Norwegian lundehund	M	7	Adenoma	R	Colonoscopy	Surgery

*The diagnosis was not determined for all control dogs, thus symptoms/syndromes are described in some of the cases.

UN, unkown

NA, not applicable

RTA, road traffic accident

Dog no.	Breed	Gender	Age (y)	Diagnosis*	Tumour location (C/R)	Method of sampling	Treatment
15	Cocker spaniel	F	10	Adenoma	C	Colonoscopy	no
16	Golden retriever	M	2	Adenoma	R	Surgery	Surgery
17	Bichon havanais	M	5	Adenoma	R	Colonoscopy	Surgery
18	English setter	M	11	Adenoma	R	Surgery	Surgery
19	Gordon setter	F	10	Adenoma	R	Surgery	Surgery
20	Grand danois	M	10	Adenoma	C	Colonoscopy	Piroxicam
21	Cocker spaniel	M	12	Adenoma	C	Colonoscopy	no
22	Border collie	F	12	Adenoma	R	Colonoscopy	Surgery
23	English setter	F	8	Adenoma	UN	UN	UN
24	West highland white terrier	M	15	Respiratory distress	NA	Post mortem	NA
25	Miniatur pincher	F	12	Lung tumour	NA	Post mortem	NA
26	Staffordshire bullterrier	F	13	General weakness	NA	Post mortem	NA
27	French bulldog	M	3	Intervertebral disk hernia	NA	Post mortem	NA
28	Alaskan malamute	F	7	Polyneuropathy	NA	Post mortem	NA
39	French bullldog	F	3	Degenerative disk disease	NA	Post mortem	NA
30	Collie, longhair	M	UN	Epilepsy	NA	Post mortem	NA

*The diagnosis was not determined for all control dogs, thus symptoms/syndromes are described in some of the cases.

UN, unkown

NA, not applicable

RTA, road traffic accident

Dog no.	Breed	Gender	Age (y)	Diagnosis*	Tumour location (C/R)	Method of sampling	Treatment
31	Pug dog	M	5	Urolithiasis	NA	Post mortem	NA
32	Chihuahua	M/N	3	Multiple fractures, RTA	NA	Post mortem	NA
*The diagnosis was not determined for all control dogs, thus symptoms/syndromes are described in some of the cases.							
UN, unkown							
NA, not applicable							
RTA, road traffic accident							

The control dogs consisted of various breeds from both genders, and were between 3 and 15 years old, with an average age of 8 years (Table 1).

Tissue samples

Tissue specimens were fixed in 4% neutral buffered formalin, processed routinely, embedded in paraffin wax, cut into 4 µm thick sections and stained with haematoxylin and eosin (HE).

In total, 12 specimens from tumorous tissue were of full thickness, while in 11 specimens, the muscularis mucosae were lacking.

The histopathological diagnoses were evaluated by a board-certified veterinary pathologist (GG), according to the guidelines for classification of canine colorectal adenoma and adenocarcinomas (25). These guidelines suggest that tumours are classified as adenocarcinoma only if neoplastic cells invade muscularis mucosa. Although some of our tumours consisted of cellular features strongly indicating malignancy, they were still classified as adenoma if no invasion of basal lamina was found. (25).

Immunohistochemistry

The following antibodies were used; mouse anti-β-catenin (BD Biosciences, Franklin Lakes, New Jersey), rabbit-anti-CD3 (DAKO, A 0452 North America Inc, California), mouse-anti-dog-CD18 (Leucocyte Antigen Laboratory, California) and anti-Ki67 (Abcam, cat no. ab15580, Cambridge).

The sections were heat treated for antigen retrieval by autoclaving at 121 °C for 15 minutes in 0,01M citric acid pH 6.0 for CD3 and Ki67, and in the microwave in pH 6.6 Target Retrieval Solution (DAKO, Glostrup, Denmark) for CD18 and pH 9.1 tris-EDTA buffer for β-catenin.

Endogenous peroxidase activity was inhibited with blocking reagent for 10 minutes (DAKO Envision system-HRP AEC REF K 4009 for CD3 and 3.0% H2O2 in methanol for Ki67, β-catenin and CD18). Non-

specific antigenic sites were blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) for CD3, 1% normal goat serum (Vector/Bioteam) in 5% BSA/TBS for Ki67, 2% BSA in TBS for β-catenin and 10% normal goat serum in PBS for CD18. The sections were incubated at room temperature with the following primary antibodies, dilutions and incubation times: rabbit anti-CD3 (60 minutes), rabbit anti-Ki67 (1:1000 in 2,5% BSA/ TBS, 60 minutes), mouse anti-dog-CD18 (1:100 in 10% goat serum, 30 minutes) or mouse anti-ß-catenin (1:2500 in 1% BSA/TBS, 60 minutes). Sections were then incubated for 30 minutes with secondary antibody from the DAKO Envision-kit for CD3, CD18 and ß-catenin, and goat anti rabbit (DAKO, E 432) diluted 1:50 with 2% normal goat serum for Ki67. The Ki67- sections were then incubated for 30 minutes with Elite -ABC- kit (VECTASTAIN PK-6100) at diluted 1:50 in TBS. Colour was revealed for 10 to 15 minutes using DAKO Envision system-HRP AEC for CD3, CD18 and ß-catenin, and the substrate solution (IMMPACT AEC PEROXIDASE SUBSTRATE SK-4205) for Ki67. Between the various steps, the sections were rinsed thoroughly in TBS. Finally, the sections were counterstained with haematoxylin solution for 45 seconds and mounted. Negative control staining was performed by replacing the primary antibodies with non-immunized goat serum, and showed no staining.

Evaluation Of Immunohistochemistry

The sections were blinded and analyzed subjectively. Two pathologists evaluated the IHC score individually and agreed on the final score (CD18 and β-catenin; GG and RR, Ki67 and CD3; GG and ØK). For all sections, the IHC score was determined by evaluating the entire specimen.

For β-catenin, the scoring scheme included prevalence of cells with a positive staining nucleus, using the following grading system: 0: no cells have any positive staining nucleus, 1: <1/3 of the cells have positive staining nucleus, 2: 1/3–2/3 have positive staining nucleus and 3: > 2/3 of the cells have a positive staining nucleus. Furthermore, the intensity of the β-catenin staining in the cytoplasm and the nucleus were scored from 0–3 (no staining, weak staining, moderate staining and strong staining).

The Ki67 staining was considered as nuclear staining in cells, and absence of nuclear staining was considered negative for the antigen. The scoring of Ki67 was only evaluated in the intraepithelial compartment. None of the control samples expressed Ki67, thus the IHC scoring of Ki67 were only reported in tumour samples.

CD3 + cells were defined as clearly stained cytoplasm in the epithelial compartment and within cells in the lamina propria.

For CD3, Ki67 and CD18, a semi-quantitative scoring scheme based on the prevalence of positive staining cells using the following grading system was applied: 0: no staining, 1: few positive cells, 2: a moderate amount of positive cells, and 3: many positive cells throughout the examined tissue.

The scores of the two pathologists were averaged, resulting in one score for each variable. If the difference between the two pathologists deviated by more than one grade (8 out of 228 scores), the slides

were reviewed and discussed, resulting in a final score.

Statistical analysis

The IHC score between adenoma and adenocarcinoma were compared, but due to the low number of samples diagnosed as adenocarcinomas, the adenoma and adenocarcinoma were categorized as “tumour samples” when comparing the IHC score to controls. The difference in demographic factors and the IHC score between dogs with adenoma and adenocarcinoma (tumour samples) and control dogs were analysed using non-parametric tests (Wilcoxon test) JMP 14 (SAS, USA). A P-value < 0.05 was considered significant for all statistical tests.

Results

No significant difference in breed, age and gender were noted among dogs with colorectal adenoma, adenocarcinoma and control dogs (Wilcoxon test, p > 0,1).

The following breeds contributed with ≥ 3 samples: German Shepherd (n = 4), English Setter (n = 3) and Cocker Spaniel (n = 3). The control dogs consisted of various breeds, of which none contributed with more than one sample in the study material.

Only five out of 23 tumour samples were diagnosed as adenocarcinomas; the remaining were adenomas.

The tumours were located in rectum in 15 dogs, in colon in four, while the localization of the remaining four tumours were not specified (Table 1).

The number of CD3 + cells in the epithelial compartment was significantly lower in tumour samples compared to control samples (Wilcoxon test, p = 0,0006). No difference in the lamina propria CD3 + cell-number were detected between tumour and control samples (Wilcoxon test, p > 0,1), (Fig. 1A and B).

The tumour samples had lower expression of CD18 positive cells in the lamina propria, compared to control samples (Wilcoxon test, p = 0,001) (Fig. 1C and D).

The Ki67 positive cells showed a strong signal in adenomas and adenocarcinomas, but no significant difference in expression levels were detected between these two tumour stages (Wilcoxon p > 0,05) (Fig. 1E and F).

Colonic samples from control dogs had a uniform staining of β-catenin along the cell membrane of epithelial cells. When compared to normal colonic epithelial cells, the expression levels of cytoplasmic β-catenin were significantly higher in adenomas and adenocarcinomas (Wilcoxon test, p = 0,0002). (Fig. 1G and H).

None of the control samples showed any positive staining of β-catenin in the nucleus of colonic cells. In contrast, 14/23 of the tumour samples had moderate or strong staining of the nucleus. This difference in

β-catenin expression (signal strength and distribution) in nucleus of colonic cells between controls and tumours, was statistically significant (Wilcoxon test, p = 0,0002). No significant difference was detected for nuclear β-catenin expression (signal strength and distribution) between adenoma and adenocarcinoma (Wilcoxon test, p > 0,05)

Results of the IHC-scores are found in Additional file 1.

Discussion

Colorectal carcinoma develops from clonal expansion of genetically altered cells in humans (9) and in dogs (10–14). This process is also believed to be influenced by inflammation (26). Inflammatory cells may reduce the risk of metastasis and further improve the prognosis (21, 23). Low infiltrations of T-cells evaluated by CD3 in CRC was associated with reduced survival in humans (23, 24). In our work, epithelial CD3 + cells were reduced in colorectal adenomas and adenocarcinomas compared to healthy colonic tissue. Moreover, the amount of CD3 cell infiltration was not different in adenomas compared to adenocarcinomas. This may indicate that T-cells is not a major player in the colorectal carcinogenesis of dogs, or that a reduced number of T-cells may cause a reduced immune defence against tumour progression. However, an expression of different subpopulations of T-cells amongst tumour and controls samples and any significance of this, cannot be excluded.T-cells are divided in subpopulations depending on their functions (27, 28). CD8 + and CD4 + T-cells serve different functions, with cytotoxic and immunomodulating properties, respectively. In humans, the T-cells in the epithelial compartment consist mostly of CD8 + cells, whereas the CD4 + cells dominate the lamina propria, both in normal colonic mucosa and in CRC stroma (29). However, in the lamina propria of the colon, the number of CD4 + cells were lower than the CD8 + cells (30). In the healthy small intestine of dogs, more CD8 + cells are present in the epithelial compartment compared to the lamina propria, where the CD4 + cells predominate. Studies of dogs with inflammatory bowel disease have observed higher infiltrations of CD4 + cells in lamina propria in duodenal mucosa compared to control dogs (31). Another study of dogs with steroid-responsive diarrhea and food-responsive diarrhea that characterized CD4 + cells in duodenal and colonic mucosa observed a lower number of these cells. Furthermore, treatment with corticosteroids or hydrolyzed diet, did not change the magnitude of CD4 + cell infiltration (32). These studies indicate a lower presence of CD4 + cells in the lamina propria in colon compared with the duodenum, and that inflammation is present despite clinical response to treatment. Thus, identifying mucosal inflammation correlating with the clinical signs is challenging.

FoxP3 is a marker for T-regulatory cells, and the presence of these cells may inhibit an efficient immune defense against tumour development (33). Studies in humans with colorectal cancer have found an association between a low CD3+/FoxP3 ratio and shortened survival (34). Future studies should also aim to characterize the T-cell subpopulations including FoxP3, CD4 + and CD8 + cells in canine colorectal adenoma and adenocarcinomas.

In humans, inflammation dominated by lymphocytes and plasma cells is higher in CRC compared to normal colonic mucosa (29) and non-steroidal anti-inflammatory treatment seems to be effective (35). Despite our results with a reduced presence of T-cells and histiocytes in canine colorectal tumours, anti-inflammatory treatment is often effective. Dogs with rectal polyps of various malignancy, with minor infiltration of inflammatory cells treated with piroxicam rectally, improved with regards to clinical signs and size of tumours, indicating that suppressing inflammation in these cases may be advantageous (36).

Results from our study confirmed what is previously described, that β-catenin may be useful as a marker for tumour progression, as its expression is increased in cytoplasm and nucleus of cancerous cells compared to healthy colon (10, 16, 37). A study showed that the protein may correlate with the tumour stage (10). However, in the present study, the magnitude of nuclear location of β-catenin did not appear to correlate with the tumour stage, as our adenocarcinoma samples were not demonstrating a stronger staining of nuclear β-catenin than the adenomas. This has also earlier been observed(16). However, subtle changes may not be detected only by using light microscope. Other methods such as electron microscopy may be more sensitive for this purpose. Anyhow, the lack of consistency among studies may be explained by several factors, like other criteria for tumour classification, including World Health Organization classification of tumours in domestic animals (38).

The dogs with colorectal adenoma and adenocarcinoma consisted of various breeds, including German Shepherd and Cocker Spaniel, each represented by three individuals. These breeds are previously described as dominant in case-reports of dogs with colorectal adenoma and adenocarcinoma (8, 36, 39, 40). In the majority of dogs (15/19 dogs), tumours were present in the rectum, and only four dogs had tumours in the colon. Similarly, in a study of 78 dogs with colorectal adenocarcinomas, over 85% of dogs had rectal tumours (6).

As we used strict criteria for classifying a tumour as an adenocarcinoma, most tumours were diagnosed as adenomas and the low number of adenocarcinomas was a limitation of this study Thus, comparing the IHC score between adenomas and adenocarcinoma was challenging. Moreover, limitations by using archived samples include inadequate information of clinical data.

The control dogs represented a heterogeneous group, but were within similar age-groups as the dogs with tumours. Commonly, control dogs are healthy young laboratory dogs (16). As age affects the degree of gastrointestinal inflammation (41, 42), it may be more relevant to include older dogs as controls, as was done here.

The need for markers to distinguish adenomas and adenocarcinomas, and thus evaluate prognosis is necessary. In humans, identifying the density of CD3 + and CD8 + T-cells in the core and margin of tumours may aid this purpose (43). This methodology named “immunoscore” may also be useful to separate adenomas from adenocarcinomas in dogs, and future studies should aim to determine the types and magnitudes of immune cells infiltrating canine colorectal tumours.

The lower presence of CD18 and CD3 + cells in tumours compared to controls in this study indicates a reduced presence of T-cells, which may be of importance in future investigation and understanding of development and immunophenotype of canine colorectal cancer.

Declarations

Ethics approval and consent to participate

Owner consent for using leftover samples for research in cancer diseases was given by dog-owners. These samples had been taken for clinical purposes and leftover samples were archived and used for this study.

Competing interests

The authors declare no conflict of interest.

Consent for publication

Not applicable

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Authors' contributions

KH, ES and GG designed the study. LT and RR performed laboratory work. ØK, GG and RR performed the IHC scoring. KH performed statistical analysis. KH wrote the manuscript with contributions from all authors during manuscript preparation. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in this published article and its additional files.

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Figures

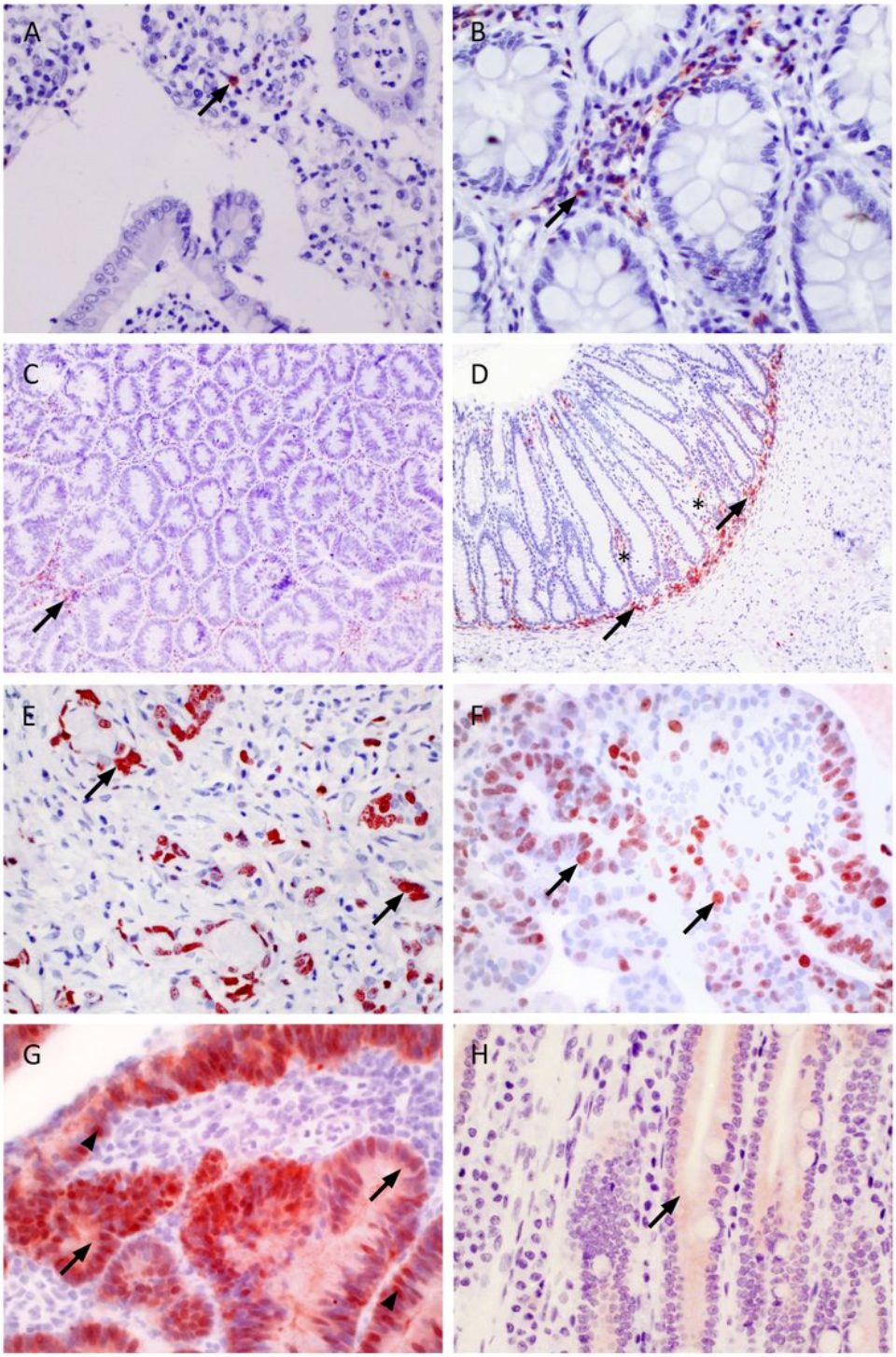

Figure 1

Immunohistochemistry. A. CD3, adenocarcinoma. A small number of positive cells were found in the lamina propria (arrow). 40x. B. CD3, normal colon. Scattered cells and clusters of cells were seen in the lamina propria (arrow) and the epithelium. 40x. C. CD18, adenocarcinoma. Fewer positive cells (arrow) and weaker signal than in the control. 10x. D. CD18, control. Characteristic distribution of positive cells, scattered in the lamina propria (*) and concentrated below the crypt epithelium (arrows). 10x. E. Ki-67,

adenocarcinoma. Numerous cells have strong, nuclear staining (arrows). 40x. F. Ki67, adenoma. Numerous cells have strong, nuclear staining (arrows). 40x. G. β-catenin, adenocarcinoma. Strong staining of neoplastic epithelial cells. Both cytoplasmic (arrows) and nuclear (arrowheads) staining are evident. 40x. H. β-catenin, control. Weak staining of epithelial cell cytoplasm only (arrow). 40x.

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