

## Immunohistochemical analyses of HNF-4 $\alpha$ and their controlled proteins in human ulcerative colitis and colorectal cancer tissues

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### Abstract

The gene coding the transcription factor HNF-4 $\alpha$  is located on chromosome 20q and is expressed in the liver, pancreas, kidneys, stomach and in the small and large intestine, where it controls important aspects related to morphogenesis and epithelial function.

Mucin type MUC4 and MUC2, and  $\beta$ -catenin are representative genes controlled by HNF-4 $\alpha$  and expressed in colonic tissue. We used in the present study immunohistochemical analyses to detect different levels of expression of the above mentioned proteins in colonic tissues of colorectal cancer and ulcerative colitis patients.

We demonstrate high expression levels of HNF-4 $\alpha$  in normal colon tissue, however, in adenoma, HNF-4 $\alpha$  levels of expression decreased and in carcinoma of the large intestine; the levels were very low or invisible. Similarly, in ulcerative colitis patients the expression HNF-4 $\alpha$  levels of the protein were significantly lower than the control group. Expression levels of MUC2 and MUC4 levels were significantly lower in the adenocarcinoma and ulcerative colitis groups than in the control group. In contrast, the expression level of  $\beta$ -catenin increased and changed from a membrane to a nuclear localization in the adenocarcinoma group only.

We surmise that HNF-4 $\alpha$  plays an important role in prevention of ulcerative colitis and colorectal cancer in humans. As a result, altered expression of the proteins MUC2 and MUC4 controlled by HNF-4 $\alpha$  indicate that the defensive role attributed to them is not properly performed. In contrast, the Wnt /  $\beta$ -catenin pathway controlled by HNF-4 $\alpha$  becomes active in samples from the stage of the adenoma and reached its peak in the carcinoma samples.

**Key words:** HNF-4 $\alpha$ , MUC2, MUC4,  $\beta$ -catenin, Wnt /  $\beta$ -catenin pathway, immunohistochemical, analyses.

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### **Introduction**

HNF-4 $\alpha$  is the first nuclear receptor identified from the HNF-4 family and was later associated with the NR2A1 ligand-dependent transcription factor family (1). The gene for HNF-4 $\alpha$  is located on chromosome 20, q13.1-13.2. The gene contains at least 12 exons and contains 2 promoters: P1 promoter (P1-HNF-4 $\alpha$ ) and P2 promoter (P2-HNF-4 $\alpha$ ). HNF-4 $\alpha$  binds to DNA as a homodimer only, to regions of AGGTCA X AGGTCA in the target genes (2). HNF-4 $\alpha$  is normally expressed in liver tissue, in proximal kidneys, in  $\beta$  cells in the pancreas and epithelial cells of the small and large intestine. In liver hepatocytes, HNF-4 $\alpha$  is expressed at the highest level (2). Silencing the gene for HNF-4 $\alpha$  in the mouse caused fetal death during the gastrulation phase (6.5E) due to a defect in the formation of the vesicular endoderm (3).

HNF-4 $\alpha$  is expressed in the nucleus of epithelial cells of the mucosa layer of the small and large intestine (4). It is expressed at a high level in the intestinal epithelium from duodenum to the colon, from crypt to villus (3, 5). HNF-4 $\alpha$  controls genes that give the intestine its unique physiologic characteristics. Among the genes controlled by HNF-4 $\alpha$  in the colonic tissue are Mucin 2 (MUC2), Mucin 4 (MUC4), and  $\beta$ -catenin (3, 5). In a study assessing the role of HNF-4 $\alpha$  in the intestine in mouse embryos, it was found that HNF-4 $\alpha$  is essential for normal bowel development (5). Inactivation of the HNF-4 $\alpha$  gene in the fetal colon epithelium, although allowing early stages of bowel development, caused an abnormality in the formation of crypts. Both, a small number of differentiated tubular cells and lower proliferation of epithelial cells accompanied

this phenomenon. HNF-4 $\alpha$  was also demonstrated to control the final differentiation of colon epithelial cells by controlling expression of additional genes (5). Inactivation of HNF-4 $\alpha$  in adult mouse tissue affects the line of intestinal cells and leads to an increase in the number of abnormal and atrophic cells. An additional effect was found in the level of cell stability and their location in the cell (3). The transcription activity itself is closely controlled also by epigenetic mechanisms (6).

The association between HNF-4 $\alpha$  and the Wnt /  $\beta$ -catenin pathway was demonstrated by Cattin et al (3). A greater amount of intracellular  $\beta$ -catenin was seen in crypts of mice lacking HNF-4 $\alpha$  in intestinal cells compared with the control group, whereas cytoplasmic  $\beta$ -catenin did not change, the amount of nuclear  $\beta$ -catenin increased by 50% in mice not expressing HNF-4 $\alpha$ .  $\beta$ -catenin is a multi-role protein, both in the intracellular interface and in the Wnt pathway, which is involved, inter alia, in the process of regeneration of epithelial cells in the intestine. This protein can also be expressed in the cell membrane, both in the cytoplasm and in the nucleus, depending on its control. When analyzing other components of the Wnt /  $\beta$ -catenin, such as Tcf-4, Axin 2, and c-Myc were significantly higher in mice that did not express HNF-4 $\alpha$  than control mice. It was also found that HNF-4 $\alpha$  in the colonic epithelium control cell proliferation by interacting with the Tcf-4 transcription factor and thereby interfering with the Wnt /  $\beta$ -catenin pathway (3).

The mucosal layer of the large intestine contains the cellular units called crypts (7), these crypts contain eight cell types that make up the intestinal epithelium, and each has structural characteristics that allow its

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morphological identification. Goblet cells are representative cells that function as secretory cells (8) that are developed from stem progenitor cells located at the bottom of the crypt and are under control of molecular pathways such as Notch and Wnt pathway during their differentiation (9). The shape of the goblet cells as a cup is aimed to concentrate a large number of granules containing mucin (8). Goblet cells synthesize and secrete various bioactive molecules that finally form the mucus secreted into the colonic epithelia in order to serve as a line of defense. The relative population of goblet cells in the different intestinal compartments increases from the duodenum (4%) to the colon (16%), similar to the number of bacteria present from the proximal intestine to the distant colon (10). The mucins secreted by the goblet cells and other epithelial cells are designed to allow the mucous layer along the digestive tract to deal with potentially damaging components to the epithelial cells (11), for example physical and chemical damage caused by digested food, bacteria (12). Mucins are large glycosylated glycoproteins with a protein-rich backbone structure and are associated with a wide range of oligosaccharides, which account for more than 70% of the weight of the molecule (13). MUC2 is synthesized and secreted from the goblet cells in the colon and is a specific phenotypic marker for the differentiation of these cells (14). MUC2 is the main source of the excretory secrete group synthesized and secreted from goblet cells in the colon and is a phenotypic specific marker for cell differentiation (15). The gene coding for MUC2 is located on chromosome 11 (12). The MUC2 monomer has more than 5,000 amino acids that contain regions of tandem repeats rich in proline, serine, and threonine. The MUC2 monomer after the glycosylation

process has a mass of 2.5 MDa and the polymer can reach above 100 MDa. MUC4 is a mucin expressed in epithelial cells of the respiratory system, gastrointestinal tract, and genital system in both stages of embryonic development and in adults (13) before and after cell differentiation and is mainly attributed to the intestine. One, as membrane-bound protein prior to differentiation, and the other as membrane-bound protein and excreted protein after cell differentiation (16). The gene for MUC4 is located on chromosome 3 (17). The gene contains two promoters, one close and the other distant. The expression of MUC4 is partly controlled by interferon- $\gamma$  retinoic acid and transforming factor- $\beta$  (17). Analysis of the gene sequence showed that there are many binding sites to HNF-4 $\alpha$  located in the distant promoter of MUC4. Our previous study (6); demonstrate that MUC4 is a target for HF-4 $\alpha$ . Inhibition of HNF-4 $\alpha$  transcription at the epigenetic level exerted a reduction in MUC4 gene transcription and other genes associated with proliferation such as PCNA. In patients with colorectal cancer, there are different expression levels of MUC4 ranging from lack of expression to high expression. Shanmugam et al (18) demonstrated that when MUC4 is highly expressed in colorectal tumors a poorer prognosis was evident.

Several studies have shown that nuclear receptors have a critical relationship to the pathogenesis of inflammatory bowel disease, including: Vitamin D receptor (19). In recent years, genetic studies have linked inflammatory bowel disease with several factors involved in epithelial regeneration, such as HNF-4 $\alpha$  (20). Genome-wide association and genotyping in patients with colitis compared to controls showed significant association with the site on the

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HNF-4 $\alpha$  encoding gene located on the chromosome 20 (q13). The relative risk of colorectal cancer in patients with persistent inflammatory bowel disease is 2-3 times greater than the general population (21). The link probably lies in the chronic inflammation of the intestinal mucosa rather than in a clear genetic predisposition (22).

In the current study, we demonstrate that HNF4 $\alpha$  expression was significantly downregulated in colonic sections of adenoma, carcinoma and IBD patients as compared to normal colonic tissue. Proteins under control of HNF4 $\alpha$  such as MUC2, MUC4 and  $\beta$ -catenin were significantly altered as well. These findings suggest that HNF4 $\alpha$  may have an important role in the etiology of colorectal cancer and ulcerative colitis.

### **Materials and Methods**

#### **Size of sample and participants' diagnosis and characteristics**

Intestinal tissue specimens were collected from 90 patients. The study protocol was approved by the Helsinki Committee at the Kaplan Medical Center (Rehovot, Israel) to conduct the study on human colon tissue. The Helsinki approval number was KMC-0151-11.

The average age of the participants was 60.9 $\pm$ 20.1. The distribution of patients was as follows: 30 patients for the adenoma group, 30 patients from the colon cancer group, and 30 patients from the ulcerative colitis group. From the 90 patients, 10 normal areas not showing any pathological changes, served as controls.

The adenoma group consisted of patients having sessile (broad base) or pedunculated adenomas, and according to the histological appearance were divided as tubular adenoma

or tubulovillous adenoma, with mild or severe dysplasia. The second group consisted of patients with a diagnosis of colorectal cancer (N = 30). The diagnosis was made on the basis of the extent of infiltration of malignant cells into the mucosa. Colorectal cancer was histologically rated as having high, medium or low differentiation. The third group consisted of patients with ulcerative colitis. The histological diagnosis was based on the extent of damage of the mucosa and granular and mesenchymal infiltration to the lamina propria.

Tissues obtained between 1990 and 2000, fixed in formalin and submerged in paraffin, were taken from the Archives of the Pathology Institute at Kaplan Medical Center. Pathological reports were reviewed by an expert pathologist. Slides stained with hematoxylin eosin were re-evaluated to confirm the diagnosis. The histologic ranking of the tumors was determined according to the criteria of the American Joint Commission on Cancer (<https://cancerstaging.org/Pages/default.aspx>).

#### *Immunohistochemical analyses:*

Four-micron slices of tissues fixed in formalin 4% and embedded in paraffin were sectioned in a microtome (Reichert-Jung 2030) and placed onto positive extra-adhesive slides. The sections underwent a process of deparaffinization in xylene and in absolute ethanol. Antigen retrieval was performed using pressure cooker treatment for 20 minutes in citrate buffer pH 6. To block endogenous peroxidase, sections were incubated for 15 minutes in 3% H<sub>2</sub>O<sub>2</sub> methanol solution and then washed with doubled distilled water (DDW). The sections were blocked for 20 min with 5% (v/v) horse serum and 1% (v/v) goat serum to prevent unspecific binding of the antibody.

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The sections were then incubated for one hour at room temperature with the following primary antibodies: rabbit polyclonal anti  $\beta$ -Catenin (ap15398pu-M) (Acris, Germany) used at 1:150 dilution; mouse monoclonal anti MUC2 (AM11177PU-M); (Acris, Germany) used at 1:200 dilution; rabbit polyclonal anti MUC4 (NBP1-86505); (NOVUS Biologicals, UK) used at 1:100 dilution. For nuclear antigens a nuclear decloaker pH 9 (Biocare Medical Pacheco, CA, USA) was used. The nuclear antibodies mouse monoclonal anti HNF-4 $\alpha$  (ab 41898) (Abcam, Cambridge UK) used at 1:200 dilution and rabbit monoclonal anti Ki67 (rm-9106-s) (Thermo scientific, Paisley, UK) used at 1:100 dilution.

After the antigen retrieval process, the sections were cooled for 20 minutes at room temperature, washed with distilled water, sealed and transferred to a semi-automatic immunohistochemical device (Biogenex i6000, CA, USA). Sections were washed three times in PBS and then incubated with corresponding HRP-conjugated anti-rabbit antibody (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA, USA). Immunodetection was performed with DAB substrate buffer (Zytomed systems, Anhaltinerstr, Berlin, Germany). For visualization of nuclei, the sections were counterstained with Mayer's haematoxylin (Pioneer research chemicals limited, Essex, UK). At the end of the immunohistochemical procedure to HNF-4 $\alpha$  staining some sections were counterstained with Periodic Acid Schiff (PAS) (Zytomed Systems, Anhaltinerstr, Berlin, Germany).

PAS is used to distinguish tissues and cells rich in carbohydrates, such as mucin, glycogen, and basal membrane. The sections were washed with distilled water and then exposed Periodic Acid for 10 minutes. Finally, the sections were washed in tap water. The sections were finally covered with an automatic covering device (Sakura, Tissue Tek-film, Japan).

#### **Scoring the immunohistochemical analysis –**

An expert pathologist assessed the stained slides. In each case, he examined in three different tissue fields, allowing for an average calculation of the three fields. The immunohistology staining score (ISS) is given for all sections as follows: One score was obtained following counting the total stained epithelial cells from all the crypt cells or tumor cells and expressed as percent. A second score was obtained for the intensity of the stain. Immunohistology staining score (ISS) was obtained by multiplying the score for percent of stained cells and the score for intensity of staining. For the evaluation of intensity and percent of HNF-4 $\alpha$  positive stained cells, we used the Image J program (National Institutes of Health, Bethesda, MD, USA). Blinded technicians manually performed the evaluation of the other stainings. Staining with the cellular proliferation-associated protein Ki67 served us only to localize active proliferating cells within the samples not for score purposes.

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Table 1. Score criteria for percent of stained epithelial cells from all crypt cells / tumor cells

Percent of stained epithelial cells from all crypt cells / tumor cells	Score
0-5%	0
6-30%	1
31-60%	2
61-100%	3

Table 2. Score criteria for staining intensity

Staining intensity	Score
Negative	0
Weak	1
Strong	2

**Statistical methods**

A preliminary Kolmogorov Seminarov test was conducted to examine the distribution of the main dimensions of the study, in order to ensure that the indices are normal and determine whether a parametric or a-parametric test is needed. The test resulted that an a-parametric test is needed, and therefore we used the Kruskal Valis test for comparison between more than two independent samples. The significance level for the various tests was at  $p < 0.05$ .

**Results**

Representative images of the normal mucosa group, the adenoma group and the carcinoma group that underwent immunohistochemical and histochemical analyses for the following proteins: MUC2, MUC4, HNF-4 $\alpha$ , PAS, Ki67 and  $\beta$ -catenin. Significant differences in the expression of proteins in controls versus adenoma and carcinoma cases are evident.

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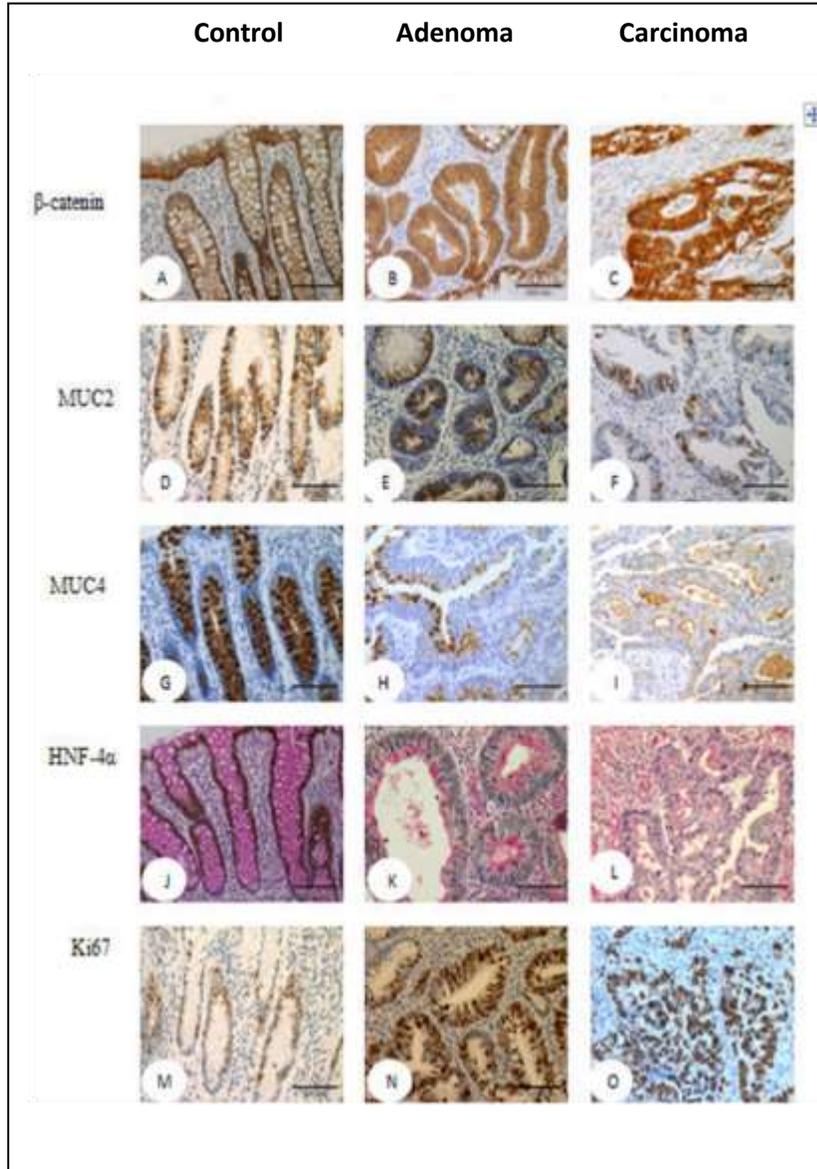


Figure 1 A-O: Representative photomicrographs are shown: A-C represent  $\beta$ -catenin expression in normal colonic tissue, adenoma and carcinoma of the colon respectively. D-F represent MUC2 expression in normal colonic tissue, adenoma and carcinoma of the colon respectively. G-I images represent MUC4 expression in normal colonic tissue, adenoma and carcinoma of the colon. M-O images represent Ki67 expression in normal mucosa, adenoma and carcinoma of the colon. Scale bar=100  $\mu$ m.

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$\beta$ -catenin staining is shown in Fig. 1 A-C. In normal mucosa (A)  $\beta$ -catenin staining appears to be expressed either at membrane or at cytoplasmic domains. In adenoma (B) a cytoplasmic and even slightly a nuclear domain  $\beta$ -catenin staining is evident. In carcinoma (C), the intensity of the staining is strong and is located in a large number of cell nuclei, indicating that Wnt /  $\beta$ -catenin pathway is activated. Fig. 1 D-F shows MUC2 staining. In normal mucosa (D), MUC2 expression is strong, in adenoma (E), the mucin expression is still strong however in carcinoma (F), a relatively small amount of cells are stained. MUC4 staining is shown in Fig. 1 G-I. In control colonic tissue, (G) MUC4 is expressed with great intensity. In adenoma (H), the expression is still relatively high, in large numbers of cells, however in carcinoma (I); the expression MUC4 is almost non-existent.

HNF-4 $\alpha$  expression and PAS staining are depicted in images Fig. 1 J-L. PAS staining is indicative of the amount of goblet cells in

normal mucosa, adenoma and carcinoma of the large intestine. In normal mucosa (J), HNF-4 $\alpha$  staining is expressed with strong intensity and a very large amount of Goblet cells can be identified by PAS staining. In adenoma (K), the expression HNF-4 $\alpha$  is very weak. PAS staining demonstrate negligible extent of goblet cells. In carcinoma (L), HNF-4 $\alpha$  is almost non-existent and no typical goblet cells are evident as indicated by PAS staining.

Ki67 expression is shown in Fig. 1 M-O images representing normal mucosa, adenoma and carcinoma of the colon. In normal mucosa (M), the Ki67 protein expression that is a marker of cell proliferation, is limited to the stem cells of the crypts. In adenoma (N), Ki67 is no longer confined only to the bottom of the crypt but it is also expressed in epithelial cells at the top of it, indicating a disarray in proliferation. In carcinoma (O), all tumor cells express the proliferation marker Ki67 marker very strongly.

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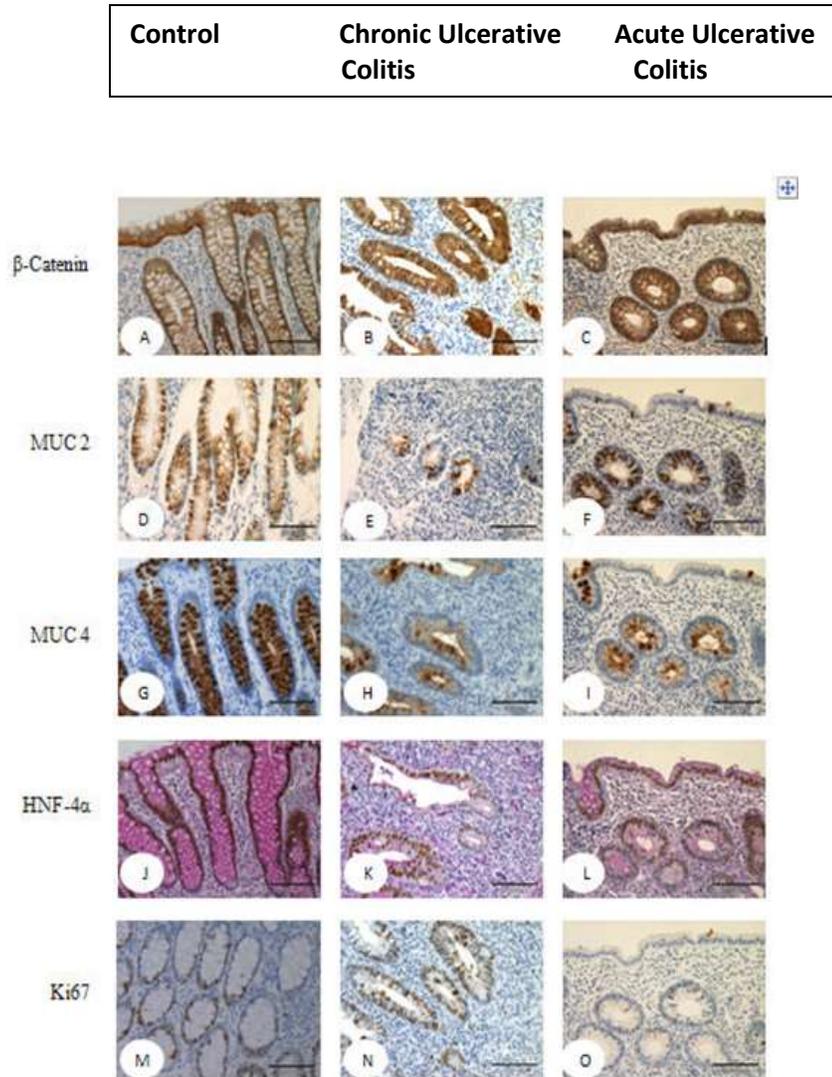


Figure 2 A-O: representative photomicrographs are shown. A-C are representative photomicrographs of  $\beta$ -catenin expression in normal (A) compared with chronic (B) and acute (C) ulcerative colitis. D-F are representative images of MUC2 expression in normal colonic mucosa (D) compared to chronic (E) and acute (F) ulcerative colitis. G-I are representative images of MUC4 expression in normal colonic mucosa (G) compared to chronic (H) and acute (I) ulcerative colitis. J-L are representative photomicrographs of HNF-4 $\alpha$  staining counterstained with PAS, indicating the amount of goblet cells (PAS staining) in normal mucosa, as compared to acute and chronic ulcerative colitis. M-O images represent Ki67 expression in normal colonic mucosa as compared to acute and chronic ulcerative colitis. Scale bar=100  $\mu$ m.

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Fig. 2 A-C shows  $\beta$ -catenin staining. In normal mucosa (A)  $\beta$ -catenin staining is low and appears to be associated with membrane and cytoplasm. In chronic (B) and acute ulcerative colitis (C), most of the cases were characterized by membrane and / or cytoplasmic staining that characterizes normal expression of  $\beta$ -catenin.

Fig. 2 D-F shows MUC2 expression in normal mucosa (D), in chronic ulcerative colitis (E) and acute ulcerative colitis (F). Fig. 2 G-I shows MUC4 expression in normal mucosa (G), in chronic ulcerative colitis (H) and acute ulcerative colitis (I). The staining pattern of MUC2 and MUC4 is very similar. D and G images represent normal MUC2 and MUC4 expression. In normal mucosa (D and G), these mucins are expressed with great intensity and in a large cell volume in mucosa layers. In chronic ulcerative colitis (E and H) this high expression pattern decreases, both in intensity and in the number of cells that secrete it.

Images in Fig. 2 J-L represent HNF-4 $\alpha$  expression and PAS counterstaining [indicative of the amount of goblet cells] in normal mucosa (J), chronic (K) and acute ulcerative colitis (L). In normal mucosa (J), HNF-4 $\alpha$  staining is expressed with strong intensity and a very large amount of goblet cells can be identified by PAS staining. In chronic ulcerative colitis (K), and acute ulcerative colitis (L) the expression HNF-4 $\alpha$  is severely downregulated and PAS staining demonstrate that the incidence of goblet cells is extremely low.

Photomicrographs in Fig. 2 M-O show Ki67 staining in normal mucosa (M), chronic ulcerative colitis (N) and acute ulcerative colitis (O). Ki67 protein expression, which is a marker of cell proliferation, is confined to the lower crypt compartments of highly proliferating intestinal stem cells. In chronic ulcerative colitis (N), we can see that Ki67 is no longer confined to the bottom of the crypt and is expressed in epithelial cells at the top. A similar pattern is observed for acute ulcerative colitis (O).

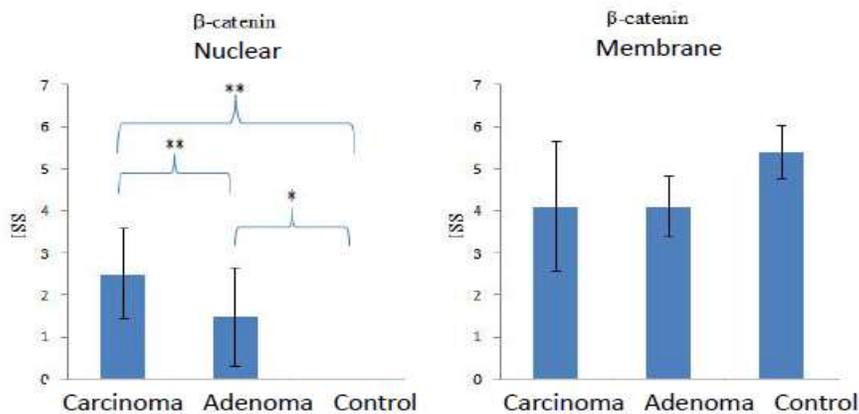


Figure 3. Differences in ISS scoring in the expression of  $\beta$ -catenin nuclear (nuclear) and  $\beta$ -catenin nuclear (membrane) tested between the control group, the adenoma group, and the carcinoma group. Substantial differences were obtained in the expression of  $\beta$ -catenin nuclear (nuclear). No difference was obtained for  $\beta$ -catenin membrane. \*  $p < 0.001$ ; \*\*  $p < 0.017$ .

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Nuclear  $\beta$ -catenin protein expression of adenoma was found to have a significant difference from the control group (\*  $p < 0.017$ ). Nuclear  $\beta$ -catenin protein expression of the carcinoma group differed also significantly from the control (\*\*  $p < 0.001$ ) and from the adenoma (\*\*  $p$

$< 0.001$ ). It can be noticed that nuclear  $\beta$ -catenin levels starts to increase in the adenoma tissue and reach its peak in tumor tissue. In contrast, no significant difference was found between membrane  $\beta$ -catenin expression ( $p = 0.02$ ).

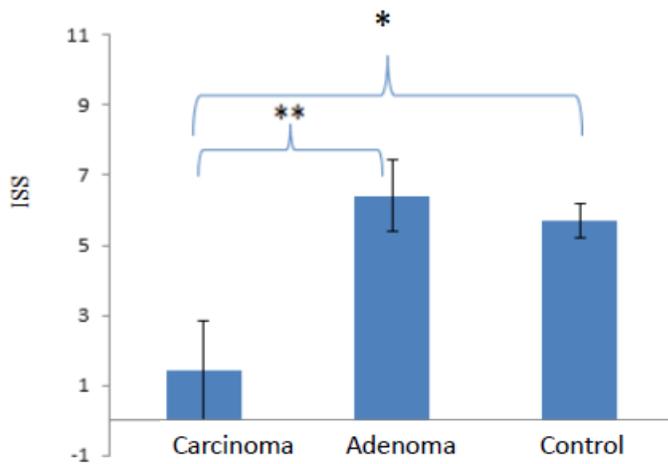


Figure 4: Significant differences in ISS representing MUC2 expression was found between the adenoma and carcinoma groups and between the adenoma and control groups (\*\*  $p < 0.017$ ) and between the control and carcinoma groups (\*  $p < 0.01$ ).

Figure 4 shows significant difference in the expression of MUC2 between the three groups. The significant difference of MUC2 expression between the carcinoma group and the control group and between the

carcinoma group and the adenoma group indicate that during the progress of malignancy, a significant decrease in the expression of MUC2 is accomplished.

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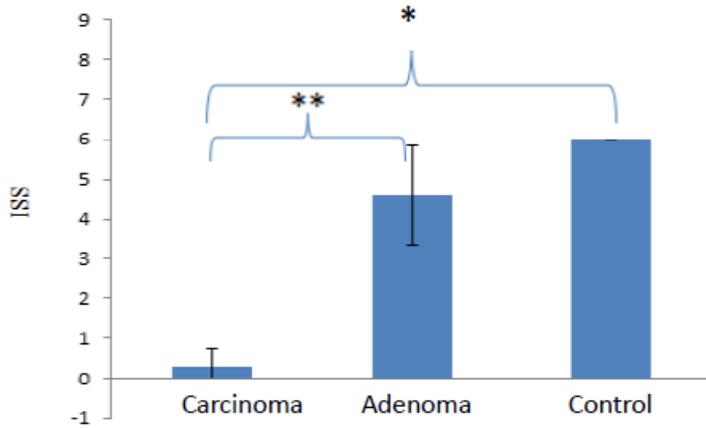


Figure 5. Significant differences in ISS representing MUC4 expression was found between the adenoma and carcinoma groups and between the adenoma and control groups (\*\*  $p < 0.017$ ) and between the control and carcinoma groups (\*  $p < 0.01$ ).

Figure 5 shows significant differences in the expression of MUC4 between the three groups. The significant difference of MUC4 expression between the carcinoma group and the control group and between the

carcinoma group and the adenoma group indicate that during the progress of malignancy, MUC4 expression is consistently downregulated.

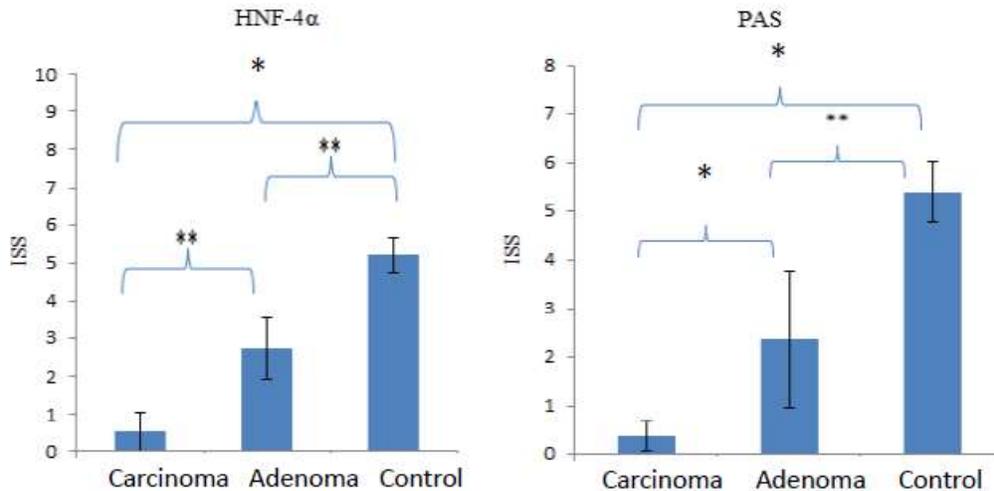


Figure 6. Comparison of PAS staining and HNF-4 $\alpha$  expression between control group, adenoma group and colon carcinoma group. Significant differences in ISS representing HNF-4 $\alpha$  expression was found between the adenoma and carcinoma groups and between the adenoma and control groups (\*\*  $p < 0.017$ ) and between the control and carcinoma groups (\*  $p < 0.01$ ). For PAS staining significant differences in ISS was found between the adenoma and carcinoma groups and between the control and carcinoma groups (\*  $p < 0.01$ ) and between the adenoma and control groups (\*\*  $p < 0.017$ ).

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There is a significant difference in PAS staining between the study groups. This difference is indicative for variation in cells able to synthesize mucins. It means that the number of goblet and columnar crypt cells populating the colonic epithelium decreases steeply from adenoma to carcinoma and both are lower than the control group. When HNF-4 $\alpha$  protein was tested, significant differences were also found between the groups. We can summarize that the expression of the HNF-4 $\alpha$  protein decreased as the process of progression towards malignancy take place and in carcinoma the level of HNF-4 $\alpha$  was the lowest.

We proceed with scoring analyses in control versus chronic ulcerative colitis and acute ulcerative colitis groups. Since no significant differences were measured between the ulcerative colitis groups (chronic and acute) for the process of scoring we unified this two groups. As a whole, significant differences are discernable in the expression of PAS staining, MUC2, MUC4, and HNF-4 $\alpha$  expression between control group, and ulcerative colitis group. However, no significant differences were observed for the expression of membrane and nuclear  $\beta$ -catenin expression (see Fig. 7).

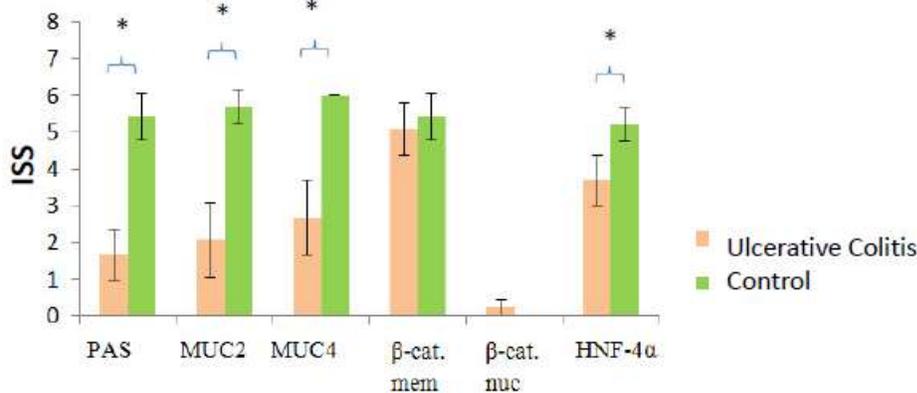


Figure 7. Comparison of ISS for PAS staining, MUC2, MUC4, membrane and nuclear  $\beta$ -catenin expression and HNF-4 $\alpha$  expression between control group, and ulcerative colitis group. Significant differences in ISS representing PAS staining, MUC2 and MUC4 expression and HNF-4 $\alpha$  expression was found between the ulcerative colitis patients and control groups (\*  $p < 0.01$ ). No significant differences were found for membrane and nuclear  $\beta$ -catenin expression.

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### **DISCUSSION**

HNF4- $\alpha$  is a transcription factor belonging to a large family of ligand-dependent nuclear receptors (1). HNF-4 $\alpha$  is unique in that it binds to DNA as a homodimer, to regions in the promoter containing sequences (AGGTCA X AGGTCA). HNF-4 $\alpha$  active binding sites have been identified in more than 140 genes, including those involved in glucose, lipid, amino acid and drug metabolism, but in the past few years, about 240 direct HNF-4 $\alpha$  sites have been identified for other activities such as signal transmission, immune response, DNA repair, cell structure, programmed cell death and others (23). Nine different isoforms of HNF-4 $\alpha$  have been identified and being these isoforms the result of the activity of two promoters P1 and P2. The expression of these promoters varies in different tissues; only the small and large intestine tissues express these two types of promoters (24). It is known that HNF-4 $\alpha$  play an important role in the development and normal functioning of the intestine, at the level of epithelial morphology and differentiation (25), cell junction assembly and adhesion (26) and cell proliferation (27). There are also evidences for the association between HNF-4 $\alpha$  and colorectal cancer (24, 27).

The aim of our study was to examine the extent of expression of HNF-4 $\alpha$  and proteins controlled by HNF-4 $\alpha$  in the intestinal tissue of colorectal cancer and ulcerative colitis patients in order to understand their putative role in the development of colorectal cancer and ulcerative colitis in humans. A previous study conducted in our laboratory demonstrated that HNF-4 $\alpha$  is highly expressed in malignant colon cells. Silencing HNF-4 $\alpha$  gene expression in HT29 malignant cells by siHNF-4 $\alpha$  inhibited the proliferation of these malignant cells. (27). Our previous results were supported by

results obtained from a proteomic analysis of colorectal and rectal tumors (28), in which they identified that HNF-4 $\alpha$  was closely related to changes in both mRNA and protein levels.

The immunohistochemical analyses performed in the present study allowed us to compare the extent of expression, or change in protein cell positioning in the different study groups (i.e. patients at various stages of colorectal cancer and ulcerative colitis). Interestingly, our present study demonstrate that the expression levels of HNF-4 $\alpha$  are significantly reduced with the progression of the pathological conditions of human colon neoplasia. In normal bowel tissue, a high expression of the HNF-4 $\alpha$  protein is detected and most epithelial cells in the intestinal tissue it is expressed very strongly. At the stage of the adenoma we revealed a significant decrease in its expression. Fewer cells expressed the protein and at weaker intensity. Interestingly, in colorectal carcinoma tissue, there was a further significant reduction in expression, and in proper tumor cells, HNF-4 $\alpha$  was not expressed at all. Similarly Lazarevich et al. demonstrated that in hepatocellular carcinoma (HCC) HNF-4 $\alpha$  expression was significantly reduced or completely disappeared in 70% of hepatic tumors, leading to the conclusion that the lack of control of the HNF-4 $\alpha$  gene was associated with progression of HCC (29). Additionally, Tanaka et al. reported a decrease in the expression of HNF-4 $\alpha$  protein in carcinoma of the large intestine (30) due to loss of P1 promoter. Similarly, Sehgal et al. demonstrated that the expression of the HNF-4 $\alpha$  gene was altered in the early stages of this type of cancer (31).

The reasons for the controversial results regarding the role of HNF-4 $\alpha$  in carcinoma of the large intestine whether is an oncogene

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or is a tumor suppressor gene are unknown. One reason to this discrepancy may be due to the expression of different isomers of HNF-4 $\alpha$  (24) expressed at different amplification levels (28).

We demonstrate herein that the Wnt /  $\beta$ -catenin pathway is significantly and highly expressed already from the stage of the adenoma and reaches a peak in the carcinoma stage. The association between HNF-4 $\alpha$  and the Wnt /  $\beta$ -catenin pathway was previously demonstrated by Cattin et al (3). They demonstrated that inactivation of the HNF-4 $\alpha$  gene in the small intestine of an adult mouse resulted in an increased expression of several genes controlled by Wnt /  $\beta$ -catenin system associated with the accumulation of  $\beta$ -catenin in the cell cytoplasm and in the nucleus of the cell is the result of an active Wnt /  $\beta$ -catenin pathway. Similarly, our present study demonstrate an inverse correlation between HNF-4 $\alpha$  and nuclear  $\beta$ -catenin, which is a marker of active Wnt pathway. As the expression of HNF-4 $\alpha$  decreases,  $\beta$ -catenin expression shifts from a membrane and / or cytoplasmic position to expression at nuclear position. We conclude that as the control of HNF-4 $\alpha$  and the Wnt /  $\beta$ -catenin pathway decreases, it enables increased proliferation processes from the stage of the adenoma to carcinoma.

The role of MUC2 in the development of colorectal cancer was also examined by us and found to be significantly reduced in the malignant tumor stage, similarly to the expression pattern found for HNF-4 $\alpha$ . Mucin molecules have been shown to exert a critical role in the function of the gastrointestinal barrier; mucin molecules prevent bacterial colonization, increase bacterial clearance, and serve to neutralize the acidic environment of the luminal colon. MUC2 expression level generally decreases

in colonic adenocarcinomas, but is maintained in the mucosal colonic tumor (32, 33). Similarly, Hanski et al. (34) found that expression of MUC2 in the normal tissues was higher than in metastatic tissues and adenocarcinoma. These findings suits our results and suggest that MUC2 plays a role in inhibiting development of colorectal cancer.

The gene for MUC4 is a target for the transcription factor HNF-4 $\alpha$ , as we previously demonstrated (6). We previously demonstrated that inhibiting HNF-4 $\alpha$  transcription at the epigenetic level resulted in a reduction in gene transcription to MUC4 and other genes associated with PCNA proliferation. We demonstrate in our present study that a low expression of MUC4 appears to be associated with low expression of HNF-4 $\alpha$  and the expression pattern from adenoma to carcinoma is similar to MUC2.

In addition to the association of HNF-4 $\alpha$  with colorectal cancer, we tested herein the association of the expression of this transcription factor and proteins controlled by it in patients suffering from different stages of ulcerative colitis. Similarly as for colon adenoma and carcinoma patients, in ulcerative colitis patients, we also demonstrate a significant decrease in the levels of protein expression relative to control patients, results supporting the view of HF-4 $\alpha$  protective role in this pathological condition.

There is ample evidence for the association between HNF-4 $\alpha$  and ulcerative colitis (20). They conducted genetic studies aimed at demonstrating the role of several transcription factors involved in epithelial regeneration that control crypt cell proliferation and intestinal epithelial cell (IEC) differentiation on IBD. They found that spontaneous colitis did not occur in all

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animal in which IEC-specific deletion of HNF-4 $\alpha$ , suggesting that further environmental triggers are required for the development of IBD disease. Additionally, Ahn et al, (35) demonstrated that silencing the HNF-4 $\alpha$  gene in the intestinal cells followed by the exposure of mice to dextran sodium sulfate (DSS) induced worsened changes in the clinical and pathological symptoms associated with inflammatory bowel disease such as enhanced weight loss, reduced colon length, and altered histological morphology. Their conclusion was that HNF-4 $\alpha$  protects against ulcerative colitis caused by exposure to DSS (35).

Regarding  $\beta$ -catenin expression we found that nor the extent level nor the distribution of  $\beta$ -catenin expression in the mucosa cells in ulcerative colitis patients was significantly different from the expression in the normal tissue.

Regarding the expression levels of MUC2 and MUC4 in patients with ulcerative colitis, the levels of these two mucin molecules were lower in all ulcerative colitis patients than in control. The mucosal expression level of MUC2 decreases significantly in the ulcerative colitis group compared to the control group, which is supported by the fact that PAS staining is significantly lower, i.e., the number of mature goblet cells decreases significantly. In this regards, it was previously demonstrated that production and excretion of MUC2 decreases primarily in the active stage of the disease (36), so that the epithelial barrier loses its protective function. In addition, it was found that in the active stage of ulcerative colitis, MUC2 rarely undergoes a sulfate binding process and only MUC2 undergoing this process is electively excreted. This type of MUC2 is less resistant to bacterial stress and less resistant to enzymatic degradation (36). The

importance of MUC2 was demonstrated in the work of Van der Sluis et al. (37) who demonstrated that mice lacking MUC2 developed an intestinal inflammation that contributed to the initiation and maintenance of ulcerative ulcerative colitis. They also demonstrated that in MUC2 deficient mice there was a direct physical relationship between bacteria and the epithelial layer. Similarly, Johansson et al. (38) demonstrated that MUC2 deficient mice are surrounded by bacterial species in direct contact with the epithelial cells located in lower compartments of the crypts, a phenomenon that can be associated with enhanced inflammation and cancer development observed in these animals. These findings show that the mucin MUC2 can build a mucus barrier that separates bacteria from the colon epithelia and suggest that defects in this mucus can cause colon inflammation.

A similar trend was also observed for expression of MUC4 in the ulcerative colitis group. A significant reduction was seen in the ulcerative colitis group compared with the control group, so it is clear that the protective layer of the intestinal mucosa is severely impaired. As previously mentioned, PAS staining was also decreased in ulcerative colitis group compared with the control group. Similarly, Dorofeyev et al. (39) demonstrated that the number of goblet cells decreased as the disease worsened.

In summary, our study demonstrates that the expression level of HNF-4 $\alpha$  protein and the proteins under its control in various pathological conditions that impair the functioning of the human colon are also in disarray. HNF-4 $\alpha$  appears to play a major role, since in the early stages of these diseases the protein expression was damaged.

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