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COLORECTAL CARCINOGENESIS: MOLECULAR AND IMMUNOLOGICAL EVENTS IN A PRECLINICAL MODEL

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Introduction

1. *Colorectal cancer.*

Colorectal cancer (CRC) is defined as a malignant neoplasm arising from the inner lining of the colonic epithelium, and is the third most common cancer worldwide. The incidence of colon cancer is higher in developed countries, where it is the second most common cancer (Wingo *et al*., 1998). It has a leading position in malignant cancer-related morbidity and mortality. The 5-years survival rate of CRC patients after diagnosis at an early and localized stage is 90%; however, when distant metastasis (the preferential sites are liver, lung and peritoneum) has occurred, the 5-years survival rate drops to 10%. The occurrence of colon cancer is strongly related to age, with 90% of the cases arising in people who are 50 years or older; until age 50, both men and women have equal risk for colon cancer, but in later life males predominate with this malignancy (American Cancer Society, 2008).

Colon cancer most commonly occurs in the large intestine. The predominant localization is rectum (50–60%) and sigmoid colon (15–25%) (**Figure 1**).

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Figure 1. Morphological and histological scheme of CRC lesion.

1.1. Cytological and histological characteristics of colon.

The principal functions of the large intestine are the recovery of water and salt and the propulsion of increasingly solid feces to the rectum before defecation. The colonic mucosa is folded in the non-distended state, but it doesn't exhibit distinct *plicae circulares*. The characteristic *muscularis mucosae* is fundamental for the rhythmic contractions showing a thick wall for peristaltic activity. The *muscularis propria* consists of inner circular and outer longitudinal layers but, except in the rectum, the longitudinal layer forms three separate longitudinal bands called *taenia coli*. Consistent with its functions, the mucosa consists of two types of cells: absorptive cells and mucus-secreting goblet cells arranged in closely packed straight tubular glands or crypts, which extend to the *muscularis mucosa*. Goblet cells predominate in the base of the glands, whereas the luminal surface is almost entirely lined by enterocytes, columnar absorptive cells, the most abundant cell type of intestine. Entero-endocrine cells are located throughout the crypt-villus axis and secrete intestinal hormones. Paneth cells are found at the bottom of crypts and release lysozyme and anti-microbial molecules (Crosnier *et al*., 2006) (**Figure 2a**). The *lamina propria* fills the space between the glands and contains numerous blood and lymphatic vessels and also collagen as well as lymphocytes and plasma cells. These form part of the defence mechanisms against invading pathogens with intra-epithelial lymphocytes and lymphoid aggregates, which are smaller than the Peyer's patches, found in the *lamina propria* and submucosa. The large intestine is inhabited by a variety of commensal bacteria, which further degrade food residues. Differentiated cells (enterocytes, entero-endocrine cells and goblet cells) occupy the crypts. With the exception of Paneth cells, terminally differentiated cells migrate along the crypt-villi axis and are shed into lumen after 5-7 days (Reya *et al*., 2005) (**Figure 2b**).

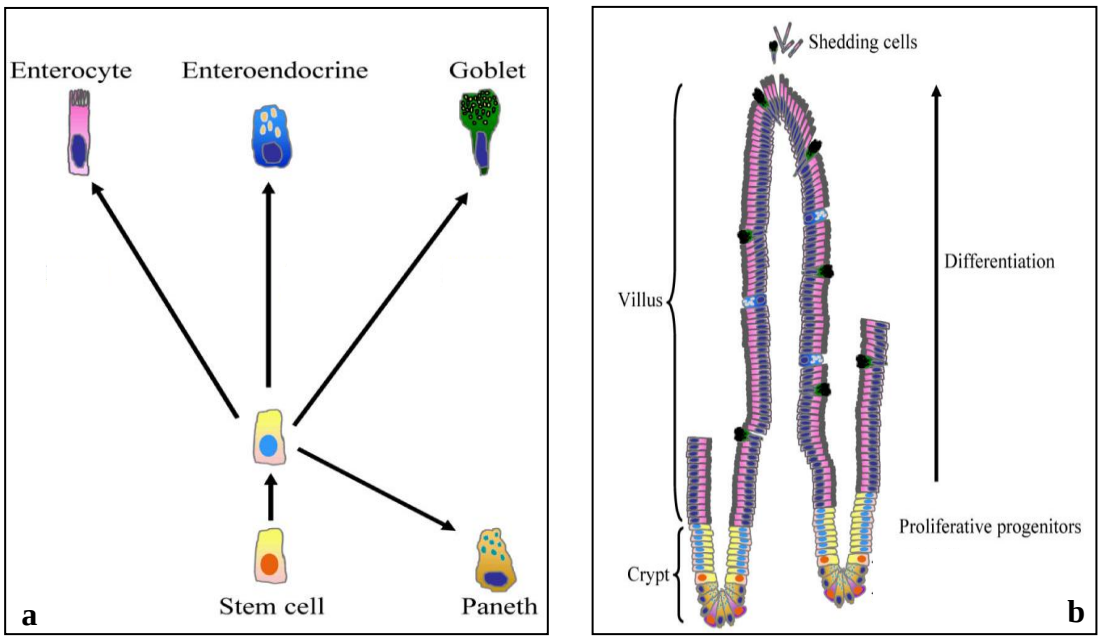

Figure 2. a) Main colon cellular types; b) Scheme of enterocyte differentiation

Stem cells reside near the bottom of the crypt and give rise to progenitor cells that are capable of differentiating toward all epithelial lineages. Stem cells self-renew to regenerate the epithelium after injury while progenitor cells arrest their cell cycle and differentiate, when they reach the tip of the crypt. Epithelial renewal occurs through a coordinated series of events such as proliferation, differentiation and migration (Clevers *et al*., 2006). In this way, the large number of cells produced by the crypt compartment is compensated by apoptosis at the tip of the crypt in a process that requires about 2–3 days. Recent studies suggest that a small subset of cells in tumours has stem cell like characteristics. It has been also reported the identification of a colorectal cancer initiating cell based on the surface marker CD133 (O'Brien *et al*., 2007). An abnormal pattern of cell replication has been detected in several clinical conditions associated with an increased risk for colorectal malignancies. The cells with damaged DNA do not cause apoptosis and reach the uppermost part in the crypt and, continuing proliferation process, generate a pre-cancerous change (Bird, 1995). There are two models for the development of adenomas from stem cells. In the '**top-down**' model, mutant

cells appear in the intra-cryptal zone between crypt openings (Shih *et al*., 2001). Therefore, whereas the stem cell, that is the likely oncogenic precursor, must have originated in the base or depths of the crypt, the lesion originates in the top or in the space between the crypts. In the "top-down" model, there may be the establishment of a new source of stem cells in the intra-cryptal zone; a mutated stem cell may migrate toward intra-cryptal area and, with a 'second hit' conferring growth potential, can expand from this location. In the more intuitive '**bottom-up**' model, a stem cell resident in the base of the crypt, with a mutational defect in growth control, normally proliferates; the cells pushed up to the intra-cryptal area, still retain this mutation. Thus, during the migration, they can accumulate other mutation and generate a neoplastic lesion (Preston *et al*., 2003).

1.2. Neoplastic manifestation in the colon.

Epidemiological studies have suggested that colon cancer can be a manifestation of a number of inherited cancer predisposition syndromes, including Familial Adenomatous Polyposis (FAP), Hereditary Non-polyposis Colorectal Cancer (HNCC), and personal or family history of colorectal cancer and/or polyps and inflammatory bowel disease (Rowley, 2004). Furthermore, other factors such as obesity, lack of exercise, smoking, alcohol consumption, diet rich in high fat, red and processed meats and inadequate intake of dietary fibres, fruits and vegetables are also associated with increased risk of sporadic colon cancer (Cappell *et al*., 2007) (**Figure 3**).

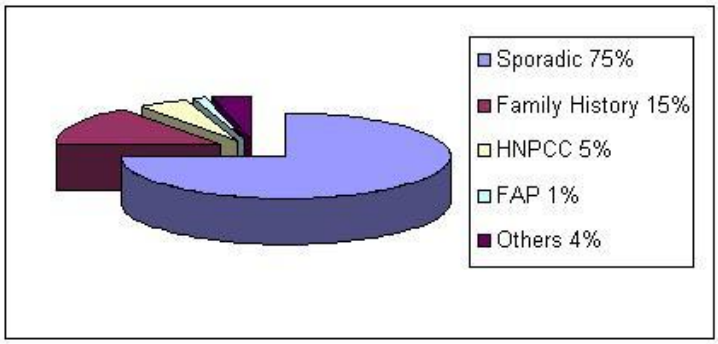

Figure 3. Incidence of colorectal cancer.

1.3. Mutations leading to colorectal cancer.

Colorectal cancer is a multi-factorial disease involving the interaction between a large number of genes and their environment. Vogelstein et al. in 1988 first described the time dependent accumulation of genetic mutations and sequential phenotypic correlation in the colonic epithelium. Genetic changes in colorectal cancer include an *Adenomatous Polyposis Coli* (*APC*, a tumour suppressor gene) mutation in 85% of all colorectal cancers (Takahashi *et al*., 2004). APC is a fundamental regulator of Wnt pathway; in general, the de-regulation of this pathway leads to colon cancer development. p16-INK4a is a tumour suppressor gene that inhibits the cell cycle in response to DNA damage (Liu *et a*l., 2009). Hypermethylation of p16-INK4a, which silences this tumour suppressor gene, is an early event in colorectal neoplasia, which can occur in adenomas and aberrant crypt foci (Kim *et al*., 2010).

Oncogenic K-*ras* apparently contributes to tumour progression relatively early, during the transition from moderate to late adenomas (Furukawa *et al*., 2002). Ras-guanosine 5'-triphosphate (GTP) binds cytoplasmic Raf-1 and translocates it to the plasma membrane, where Raf-1 becomes activated by poorly understood mechanisms. The signal is transmitted to the extracellular signal-regulated portion of mitogen-activated protein kinase which downstream activates and promotes cell proliferation and differentiation. Activating mutations in K-*ras* genes have been identified in a great variety of human cancer. The mutated forms are found to stimulate cell proliferation, transformation and differentiation. CRCs contain a K-*ras* mutation in exon 12 in about 40–50% of the cases; the most of the mutations are Guanosine → Thymidine transversions (Pretlow *et al*., 1993). It is assumed that the K-*ras* mutation occurs after the *APC* gene mutation in the CRC (Takahashi and Wakabayashi, 2004). The epigenetic inactivation of *Ras-associated factor* (*RASSF*) *1A* by hypermethylation of the promoter region is frequently detected in flat-type carcinoma. RASSF1A regulates a pro-apoptotic pathway through heterodimerization with the Ras effector NORE1 and interacts with pro-apoptotic

protein kinase MST1, which mediates the apoptotic effect of *ras*. Therefore, it is thought that the inactivation of *RASSF1A* causes an aberration in the *ras* signaling pathway without involving the K-*ras* gene mutation (Khokhlatchev *et al*., 2002).

More than 90% of the primary CRCs with Loss Of Heterozygosis (LOH) of chromosome 18q show a deletion in the *deleted colorectal carcinoma (DCC) gene* included in the region of allelic loss. Recent studies reported *DCC* functions as part of a receptor complex for netrin-1. Furthermore, in various cell lines, DCC, on netrin-1 binding, activates the *ERK* pathway and in the absence of netrin-1, induces apoptosis via caspase-9. The presence of netrin-1 blocks DCC induced apoptosis. A mutation of *DCC* that yields cell immortality is caused by continual transmission of the living signal in the absence of *netrin-1* (Mehlen *et al*., 1998).

The most important point that determines the borderline between the adenoma and the adenocarcinoma is a mutation of the *p53* gene. The *p53* gene is a typical tumour suppressor gene and its mutation has been detected in a variety of cancers and about 75% of CRCs. The p53 protein acts as a cellular stress sensor and a rise in p53 levels causes arrest in the G1 phase of cell cycle, cellular senescence or apoptosis by inducing various target genes. This mechanism limits the propagation of potentially oncogenic mutations. The p53-dependent apoptotic pathway is also induced by DNA damage in certain cell types as well as in cells undergoing inappropriate proliferation. The major players of the p53-induced cell cycle arrest are *p21* and *growth arrest and DNA damage inducible gene 45* (*GADD45*) (Vousden and Lu, 2002). The *p21* gene is a cyclin-dependent kinase inhibitor that can influence cell cycle progression from G1 to S phase, by controlling the activity of CDK. *GADD45* inhibits the cell progression from G0 to S phase and plays an important role in the maintenance of the stability of the chromosome. Other major players of the *p53*-induced apoptosis, are pro-apoptotic Bcl-2, protein Bax and BH-3-only proteins Noxa (Nakano *et al*., 2001). Thus, trans-activation of their promoters through p53 might induce caspases activation. Therefore, the loss of *p53* function as a transcription factor affects cellular malignant transformation (**Figure 4**).

In humans, others important mutations leading to CRC are localized in *TGF-β* gene. TGF-β signalling can inhibit the growth rate of epithelial cells but the response to TGF-β is often lost in cancers. TGF-β receptor type II mutations are relatively common in replication error–prone colorectal tumour cell lines (Pretlow *et al.*, 1994)Furthermore, a small but significant fraction of colorectal tumours show loss of the tumour suppressor *DPC4*, the gene encoding human Smad4 which co-transduces all TGF-β-like signals, and some harbour mutations in the TGF-β transducing Smad2 (Pretlow *et al.*, 1997). Consistently, in humans the earliest mutations in the TGF-β receptor are found at the late adenoma stage, apparently correlating with the transition from benign adenoma to malignant carcinoma (Paulsen *et al.*, 2005).

In sporadic colorectal tumours, that retain wild-type *APC*, mutations are frequently found in the β-catenin gene (*CTNNB1*) or Axin2.

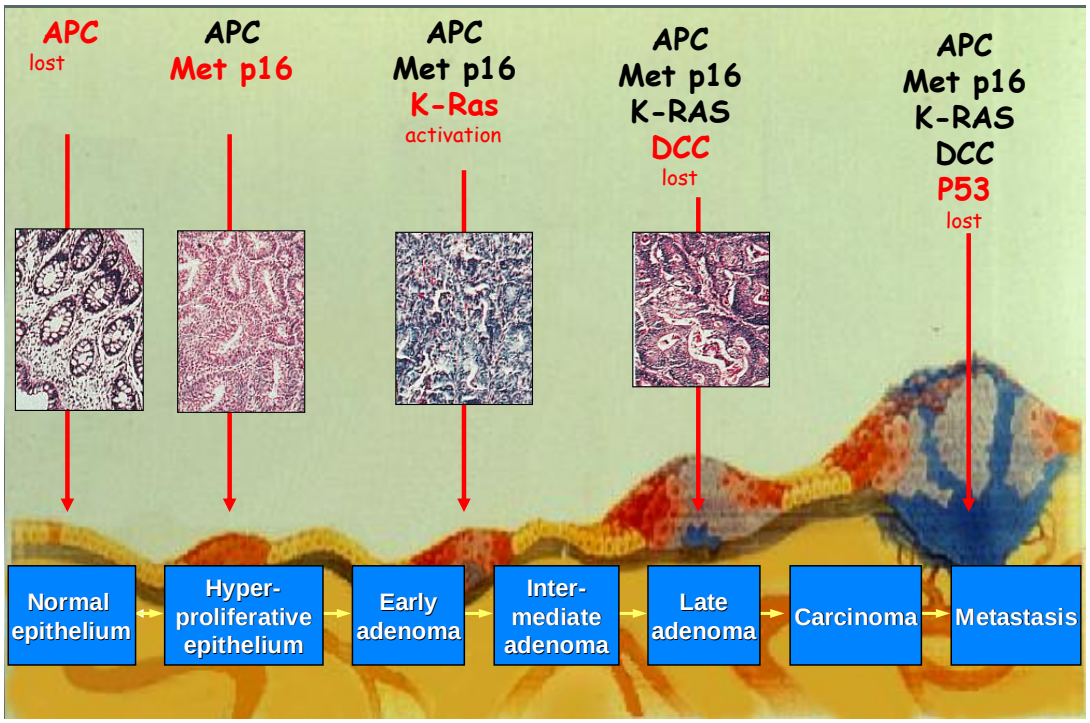

Figure 4. Accumulation of genetic mutations and sequential histological alteration in the colonic epithelium.

The relationship between a stability gene aberration and CRC is revealed by HNPCC, also termed Lynch syndrome. Instability of short tandem repeats, or microsatellites (MSI), is a characteristic of these tumours (Haydon and Jass, 2002). In most HNPCC CRCs, MSI has been shown to result from mutations in the DNA mismatch repair, *hMSH2*, *hMLH1*, *hPMS1*, *hPMS2* and *hMSH6* genes. Others gene may contain MSI within their coding regions such as *Transforming Growth Factor-β Receptor, insulin-like growth factor receptor,* regulators for cell cycle and regulators of apoptosis. The transformation to malignancy thus occurs when these target genes are mutated (Rampino *et al.,* 1997).

Several inflammation-related proteins, transcribed by Nuclear Factor-κB (NF-κB), such as cyclo-oxygenase (COX2), Inducible Nitric Oxide Synthase (iNOS), Interferon γ (IFN γ), Tumour Necrosis Factor-α (TNF-α) and Interleukin-1 (IL-1), are increased in inflamed mucosa and remain elevated in colonic neoplasms. NF-κB is also a central regulator of the transcriptional activation of a number of genes involved in cell adhesion, immune and pro-inflammatory responses, apoptosis, differentiation and growth. However, chronic activation of NF-κB induces promotion of epithelial cell turnover and generation of reactive oxygen and nitrogen species (ROS) causing DNA damages that drive the carcinogenesis processes (Tanaka, 2009).

There are several studies that demonstrate a role for DNA methylation very early in colorectal tumorigenesis involving *p16*, *MGMT*, *hMLH1*, *MINT31*, *MINT2*, and/or *MINT1* (Chan *et al.,* 2002). A high frequency of methylation was found for the newly described *SLC5A8* gene, a sodium transporter that is implicated in colon cancer (Li *et al.,* 2003). It was also showed hypermethylation of the cellular retinol-binding protein 1 (*CRBP1*), *MINT31*, or H-cadherin (*CDH13*) (Luo *et al.,* 2005). One of the most interesting findings is the frequent methylation of the secreted frizzle-related protein (*SFRP*) genes. This epigenetic inactivation allows constitutive Wnt signalling in some precursors lesions that usually lack *APC* mutations (Suzuki *et al.,* 2004).

1.4. Histological type of lesions.

Carcinogenesis is a multistep process involving the clonal selection and expansion of initiated preneoplastic cells. The clonally expanding cell population is generally termed as a preneoplastic lesion (Bird and Good, 2000).

Aberrant crypts are defined by several characteristics (McLellan and Bird, 1988): larger than the normal crypts, with increased pericryptal space, having a thicker layer of epithelial cells that often stain darker, and generally having oval rather than circular openings. The occurrence of colon cancer is mainly associated with the incidence of **aberrant crypt foci (ACF),** an earliest neoplastic lesion, which are clusters of mucosal cells with an enlarged and thicker layer of epithelia than the surrounding normal crypts that progress into polyps followed by adenomas and adenocarcinomas. Other early pre-neoplastic lesions are "β-catenin-accumulated crypts" (BCAC) and "mucin-depleted foci".

Polyp is a circumscribed mass of cells that project above the surface of the surrounding normal mucosa. Colorectal polyps can be defined as well demarcated, circumscribed lumps of epithelial dysplasia with uncontrolled crypt cell division. Most adenomas remain benign. However, a small fraction of these lesions may evolve into malignancy and there are evidences indicating that a large majority of colorectal carcinomas develop from adenomatous polyps. **Adenomas** can be classified into three major histological types: tubular, villous and tubulo-villous adenomas. An adenoma is pedunculated when it possesses a stalk. Sessile adenomas rise above the background mucosa without any stalk. They can show different grades of dysplasia (a structural and cytological alteration in the epithelium that predispose an organ to cancer development). There are also flat adenomas, difficult to detect but with high malignant potential. They can be completely flat or show a central area of depression (depressed adenoma) (Cappell, 2007).

Patients with **Ulcerative Colitis (UC)** and **Crohn's disease (CD)** have an augmented risk for colorectal malignancies, increasing with the duration of disease and the extent of colorectal involvement. In Inflammation Bowel Disease (such as UC and CD), elevated, sessile and reddish nodules, which are known as pseudo-polyps or inflammatory polyps, are often seen in the otherwise flat mucosa. These lesions are typically small and multiple and largely composed of granulation tissue, mixed with inflamed and hyperemic mucosa. Dysplasia may grow as a flat lesion or as a "dysplasia-associated lesion or mass" (DALM). Recently, several molecular alterations have been detected in long-standing UC. These include oncogene mutations, inactivation of tumour suppressor genes, LOH and chromosomal and microsatellite instability (Xie and Itzkowitz, 2008).

Adenocarcinoma is the typical tumoural lesion, able to invade the *muscolaris mucosae* and reach the tunica submucosa and so can generate metastases. It is characterized from different extents of peduncular infiltration, several degrees of differentiation and vascularisation.

1.5. Wnt pathway and colorectal cancer.

Wnt proteins constitute a large family of cysteine-rich, lipid-modified signaling proteins that control development in organisms ranging from nematode worms to mammals (Wodarz and Nusse, 1998). Wnt proteins control diverse developmental processes such as gastrulation, limb, and central nervous system development (Huelsken and Birchmeier, 2001). Recent studies have also shown that Wnt signaling may regulate the maintenance and differentiation of stem cells (Taipale and Beachy, 2001). The intracellular signaling pathway of Wnt is also conserved evolutionally and regulates cellular proliferation, morphology, motility, fate, axis formation, and organ development (Polakis, 2000). Wnt regulates at least three distinct pathways: the canonical β-catenin pathway, planar cell polarity pathway, and Ca^{2+} pathway (Veeman *et al*., 2003). Among these intracellular cascades, the canonical β-catenin pathway has been most

extensively studied. In humans it has been shown that abnormalities of this pathway lead to several human diseases, including tumour formation in specific tissues such as the intestine, liver, skin, and mammary gland and bone abnormalities. The NH_2 and COOH termini of β-catenin are unstructured regulatory regions that largely recruit essential co-factor for adhesion and signaling (Taipale and Beachy, 2001; Van Noort *et al*., 2002). According to the most widely accepted current model of the β-catenin pathway, in absence of Wnt signaling, Casein Kinase I (CKI) and Glycogen Synthase Kinase-3β (GSK-3β a serine threonine kinase) can phosphorylate β-catenin in the Axin complex for the presence of the "scaffold" protein APC (Kikuchi, 1999): APC binds to the RGS domain of Axin. In this way, GSK-3 β, β-catenin, and CKI interact with the different sites of the central region of Axin to form the cytoplasmatic complex. Dvl binds to the following C-terminal region of Axin including the DIX domain (Kishida *et al*., 2001). The amino acid sequence specifying the phosphorylation of β-catenin is 32SGXXSXXXTXXXS45. CKI–dependent phosphorylation of Ser45 proceeds and the phosphorylation induces subsequent GSK-3β-dependent phosphorylation of Thr41, Ser37, and Ser33. Asp32 and Gly34 are necessary for the interaction of phosphorylated β-catenin with *Fbw1*. Therefore, Fbw1 directly links the phosphorylation machinery to the ubiquitination apparatus. Phosphorylated β-catenin is ubiquitinated, resulting in the degradation of β-catenin by the proteasome. In general, the degradation of the proteins by the ubiquitin-proteasome pathway involves a ubiquitin-activation enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin-ligase (E3) (Matsuzawa and Reed, 2001). The ubiquitin ligase is generally thought to be directly involved in substrate recognition and consists of a multi-protein complex. As a result, the cytoplasmic β-catenin level is lowered. When Wnt acts on its cell-surface receptor consisting of Frizzled and Lipoprotein Receptor-related Protein 5/6 (LRP5/6), β-catenin escapes from degradation in the cytoplasmatic Axin complex. The accumulated β-catenin is translocated to the nucleus, where it binds to the transcription factor T cell factor (Tcf)/lymphoid enhancer factor (Lef) and thereby stimulates the expression of various genes

such as *C-myc*, *cyclin D1*, *MMP-7*, etc. (**Figure 5**). One of the strategies that a cell uses to combat the aberrant expression of β–catenin is to utilize additional regulatory pathways that can modulate its level. For instance, β–catenin induces its own negative regulators *Naked* and *Axin2* (Jho *et al*., 2002).

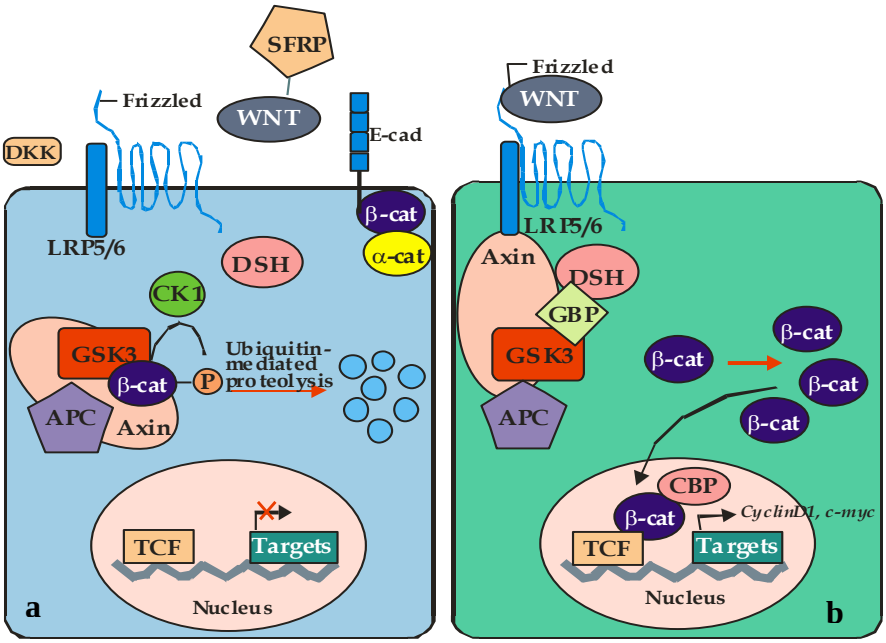

Figure 5. Wnt/ β-catenin pathway scheme in: a) normal cellular condition and b) in tumoural cellular condition.

APC acts as a critical component for β-catenin destruction. In colon cancers, mutations of APC correlate with high levels of β-catenin and transcriptionally active Tcf/β-catenin complexes. Expression of wild type APC in colorectal cancer cells reduces the β-catenin level, and the fragment of APC containing the 20-amino acids repeats is sufficient for this activity. However, an APC fragment with either a mutated β-catenin binding site or Axin-binding site fails to induce the degradation of β-catenin. Therefore, the interaction of APC with both Axin and β-catenin is required for the ability of APC to degrade β-catenin. In the complex, GSK-3β bound to Axin phosphorylates APC, enhancing the stability of β-catenin/APC complex, and leading to a more efficient β-catenin phosphorylation by GSK-3β (Hinoi *et al*., 2000). APC has also an important function as a shuttle protein between the nucleus and cytoplasm, and its

nuclear export may affect β-catenin localization and turnover. Mutant APC lacking a C-terminal NES is trapped in the nucleus. The ability of APC to exit from the nucleus may be important for its tumour suppressor function (Rosin-Arbesfeld *et al.*, 2003) **(Figure 6)**.

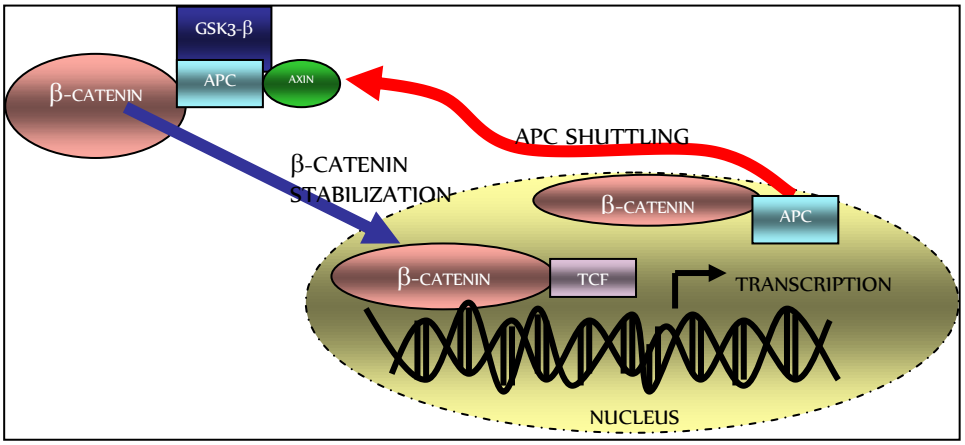

Figure 6. APC shuttle protein function between nucleus and cytoplasm.

APC is also involved in polarized cell migration and cell-cell adhesion. Early studies showed that APC is localized to the tips of plasma membrane projections in migrating cells in association with bundles of microtubules. More recently, this APC distribution has been linked to the activation of a signaling complex by integrin-based adhesion that orients the cell for polarized migration. Thus, APC is a multifunctional protein that provides further links between cell-cell adhesion and β-catenin stability and is involved in processes that are not linked directly to Wnt signaling but that contribute to cellular morphogenesis.

In addition to its function in the Wnt signaling pathway, β-catenin also plays a major role in the cell-cell adhesion function, binding tightly to the cytoplasmic domain of type I cadherins and playing an essential role in the structural organization and function of cadherins by linking E-cadherins to the actin cytoskeleton. Another catenin, p120, binds to the membrane proximal domain of cadherin and regulates the structural integrity and function of the cadherin complex (Brembeck *et al.*, 2006). Phosphorylation of p120 by Src or Fer results in loss of cadherin

complexes from the cell surface, perhaps as a consequence of simultaneous phosphorylation of β-catenin or because p120 is a binding site for several protein tyrosine phosphatases (PTPases) that antagonize the effects of these tyrosine kinases. In general, activation of tyrosine kinases results in a loss of cadherin mediated cell-cell adhesion and an increase in the level of cytoplasmic β-catenin, either by direct release of β-catenin into the cytoplasm or by activating cadherin endocytosis (Korinek *et al*., 1997).

In the carcinogenetic process, other protein are involved. E–cadherin is a cell surface protein involved in homophilic Ca^{2+}-dependent cell–cell interactions. Specific adhesive binding is conferred by the cadherin ecto-domain, which engages an identical molecule on the surface of an adjacent cell (Boller *et al*., 1985; Leckband *et al*., 2000), whereas the cadherin cytoplasmatic domain mediates the structural and signalling activity required for adhesion. In addition to the interaction with β-catenin, cadherin associates with two other catenin proteins, termed α and p120 catenin. A catenin looks as an armadillo domain and is, therefore, structurally unrelated to β-catenin. Although α-catenin and p120 are important regulators of cell-cell adhesion, β-catenin binding to cadherin remains a prerequisite for adhesion due to its role in protecting the cadherin cytoplasmatic domain from rapid degradation (Huber *et al*., 2001) enhancing the efficiency of protein transport from endoplasmic reticulum to cell surface (Chen *et al*., 1999) and recruiting α-catenin at cell-cell contacts (Drees *et al*., 2005; Yamada *et al*., 2005). Therefore, posttranslational modification, that regulate β-catenin/cadherin interaction, will have important consequence for cell–cell adhesion. In colon cancer, mutations of E–cadherin gene rarely occur but modifications of expression level and cellular localization have been reported. E-cadherin expression in colon carcinoma was found to be associated with tumour stage, lymphonodal metastases, and patient survival (Bellovin *et al*., 2005). In fact, loss of E-cadherin causes a decrease of cell-cell adhesion and increased invasion and motility (Thompson *et al*., 1994). Cell-cell adhesion mediated by E-cadherin is required for the maintenance of epithelial tissue architecture in adult organisms (Vleminckx *et al*., 1999). Down-modulation of E-cadherin is

observed during the later stages of tumorigenesis (Thiery, 2002). This down-regulation of E-cadherin is accompanied by a loss of epithelial characteristics and the acquisition of mesenchymal properties, a process known as epithelial to mesenchymal transition (EMT). During EMT, carcinoma cells become more motile and invasive acquiring characteristics similar to embryonic mesenchymal cells, thereby allowing penetration of the stroma surrounding the initial neoplastic focus (Guarino *et al*., 2007). CRC metastasis is a multi-hit, multistage process (Chiang and Massague, 2008). In addition to greater motility, cells must be able to invade the extracellular matrix, to survive at low density outside the tumour microenvironment, and to develop resistance to apoptosis triggered by loss of cell-matrix interaction (Tse and Kalluri, 2007).

It was been demonstrated the important contribution of oncogenic *Ras* to altered regulation of E-cadherin and β-catenin. The overactive mutated *Ras* promotes transformation of intestinal epithelial cells associated with altered regulation of E-cadherin (Schmidt *et al*., 2003).

1.6. Colorectal cancer in animal models (rodents).

There is good evidence demonstrating reduced morbidity and mortality associated with early detection of invasive lesions and precursor adenomatous polyps. However, most CRC in the world is diagnosed at an advanced stage. Therefore, most attention has focused on screening for targets for cancer chemoprevention to reduce the number of CRC patients. The identification/discovery of these biomarkers ranges from exposure assessment, risk assessment and management to clinical trials. Along with these, there is also a need to develop and validate molecular biomarkers reflective of exposure and risk from etiological factors. For these reasons it's very important to understand the mechanisms leading to neoplastic transformation and progression of human CRC, using an animal model mimicking the histological and molecular alterations observed in humans (Freedman, 2007).

Similar neoplastic human lesions could be obtained in animal models (rodents) in a short period of time with high doses of carcinogens that are not ordinary in the human environment. These animal models are chemically induced and genetically modified. More importantly, animal ACF provide the earliest identified lesions in the colon to investigate the changes that take place during the transformation of normal colonic epithelial cells to colorectal cancer. Many characteristics identified in ACF in rodents are also seen in the ACF from humans (Rosenberg *et al*., 2009).

One of the most frequent alterations (93%) in human ACF is the increased expression of carcinoembryonic antigen (CEA) (Pretlow *et al*., 1994). The expression of CEA was not associated with the degree of dysplasia but increase as a function of size of the ACF (Augenlicht, 1994). *P-cadherin* is not expressed in normal colonic epithelium, but it was expressed in 65% human ACF. The expression was independent of dysplasia. All of these ACF continued to have normal E-cadherin expression, a few had cytoplasmic expression of β-catenin (Hardy *et al*., 2002). The expression of hexosaminidase and α-naphthyl butyrate esterase activities is increased in human ACF compared to adjacent normal mucosa (Pretlow *et al*., 1991). While only a small proportion of ACF showed reduced expression of fragile histidine triad (*FHIT*) gene, its reduced expression was strongly associated with dysplasia (Hao *et al*., 2000) and may play a role in the progression of lesions in human colon tumorigenesis. hTERT can be detected at a low level in some normal cells including lymphocytes and at the base of colonic crypts (Hiyama *et al*., 2001; Pretlow *et al*., 2003). The expression of iNOS is strong cytoplasmatic in the normal colonic epithelial cells while 50% ACF and 56% carcinomas showed a marked reduction of iNOS expression (Hao *et al*., 2001). Sialyl Lewis^{x} (Le^{x}) and sialyl Tn antigens are overexpressed in 70-89% of colon cancers but are not detectable in normal colonic mucosa and are detected only rarely in hyperplastic polyps (Itzkowitz, *et al*., 1986). Overexpression of glutathione-S-transferase P1-1 (GSTP1-1) was observed 89% human ACF. All of these GSTP1-1 positive ACF also stained for p21$^{K\text{-}ras}$. The overexpression of GSTP1-1 appears to be induced by mutant *KRAS*

(Miyanishi *et al*., 2001). COX-2 was not expressed in these same ACF, and apoptosis was decreased compared to normal mucosa. It appears that GSTP1-1 may protect ACF from apoptosis and thus contribute to the progression of ACF to cancer (Nobuoka *et al*., 2004). The increased expression of $p16^{INK4a}$ correlates inversely with proliferation markers (Dai *et al*., 2000). Increased expression of c-*myc*, a target of β-catenin signalling, was seen in 34.9% ACF and in 55.6% dysplastic ACF (T.P. Pretlow and T.G. Pretlow, 2002). The increased expression of c-*myc* in ACF suggests an expansion of the immature colonocytes that normally express c-*myc* at higher levels than their mature counterparts (Mariadason *et al*., 2005). ACF in rats and mice express multiple phenotypic alterations:

i) reduced expression of hexosaminidase and α-napththyl butyrate esterase activities. The alteration of these two enzymes is more marked or frequent as that observed in the human ACF, and exhibit opposite changes in expression, decreased rather than increased;

ii) increased expression of periodic acid Schiff reactive material;

iii) increased expression of glutathione-S-transferase isoforms.

The expression of iNOS and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*] pyridine (PhIP) in rat are quite different from that in human, but not relevant. The first alteration identified in rodent ACF and tumours, induced with the chemical carcinogenetic, was mutation in *K-ras*, varied from 7% to 32 %. Generally, induced colon tumours had a higher frequency of *β-catenin* (Ctnnb1) gene mutations (75%) than *Adenomatous Polyposis Coli (APC)* mutations (25%). *P53* mutations were not detected in tumours induced with 1,2 dimethylhydrazine (DMH) (Theresa P. Pretlow and Thomas G. Pretlow, 2005).

1.7. Chemical Carcinogenesis.

The ability to induce colon tumours in animals has provided the opportunity to study various aspects of the carcinogenesis process. Oncogenesis studies using these models have also elucidated the role of genetic and environmental factors and other influences on the various aspects of this complex disease. The direct-acting carcinogens are compounds that do not require biological catalysis, such as the action of enzymes to form the ultimate reactive species that alters cellular macromolecules. These agents spontaneously break down in an aqueous environment to electrophilic species that react with nucleophilic centers on the DNA molecule. Indirect-acting carcinogens require enzymatic action (e.g. intestinal commensal bacteria) to be converted into the electrophilic species.

The carcinogen most used to obtained tumours in rodent is DMH. This substance induces colon cancer providing a useful model to study early carcinogenesis and sporadic cancer development mimicking many of the clinical, pathologic, and molecular features of human colon cancer (Whiteley and Klurfeld, 2000). DMH is a specific colon carcinogen that induced large bowel tumours in rodents. In the liver DMH is converted to its main active metabolite azoxymethane (AOM): the chemical structure changes, by oxidative steps, from CH3-NH-NH-CH3 to CH3-[O]N=N-CH3; then to methylazoxymethanol CH3-[O]NH-NH-CH2OH (MAM), which leads to CH3+ methylcarbonium ion, that seems to be the ultimate carcinogen which binds cellular DNA in the colon at the bottom of the crypts via the bloodstream or possibly via biliary secretion (Sunter *et al*., 1990). The exact nature of the mutations, caused by the carcinogen DMH, is still unknown. Pro-mutagenic lesions O^6-methylguanine has been detected in DNA from various rat and mouse tissues following exposure to DMH. DMH induces the formation of adducts with guanosine in the GTCCA sequences, especially in *Ctnnb1 gene*, resulting in point mutation or deletions in codons 32-49 causing removal of 4 codons codifying amino-acids target of phosphorylation. Moreover, like human tumours,

DMH induced tumours are often mutated in *K-ras* gene and show microsatellite instability. However, unlike human tumours, they are rarely mutated (15%) in *Adenomatous Polyposis Coli APC* gene, never mutated in *p53* gene. Nevertheless, even if *APC* is rarely mutated, rat tumours accumulate β-catenin in the nucleus like human tumours but this is due to *Ctnnb1* mutation. Thus, Wnt/B-catenin pathway plays a major role in carcinogen-induced rat tumours similarly to human tumours (Schwartz *et al*., 1995).

1.8. Immune Response in Colorectal Cancer.

Colorectal carcinoma, like most epithelial solid tumours, has been long considered poorly immunogenic and refractory to immunotherapy; these observations are supported from several epidemiological studies based on lack of spontaneous regression of cancer also if the host immune system should be able to react against a neoplastic cell. In effect, CRC spontaneous regression is only exceptionally observed, and does not appear to be associated with an immune response (Francis *et al*., 1997). *In vitro* studies performed on tumour infiltrating lymphocytes (TIL) cultures purified from colorectal carcinomas failed to demonstrate substantial lytic activity against autologous cancer cells (Rosenberg and Karnofsky 1992). Moreover, classical immunotherapeutic interventions known to be active against other type of tumour (e.g. melanoma), such as systemic administration of cytokines (IL-2, INF-α) or adoptive transfer of autologous lymphocyte effectors (LAK, lymphokine-activated killer cells, TIL), have proved ineffective in colorectal tumours (Wolmark *et al*., 1998).

Indeed, several evidences indicate that also colorectal cancer may express tumour associated antigens (TAA) recognised by T-cells, influencing patient prognosis and determining the tumour immunological profile (Dalerba *et al*., 2003). In general, histopathological studies have shown that spontaneously regressing tumours are heavily infiltrated by T-cells (Mackensen *et al*., 1993) and that T-cell infiltrates within the primary tumour are strong predictors of better prognosis (Clemente *et al*., 1996). Several studies addressed the prognostic role of intra-tumour

inflammatory infiltrates, but their results are contradictory, supporting a protective role of inflammatory infiltrates (Guidoboni *et al*., 2001), or not (Nielsen *et al*., 1999). In effect, the inflammatory infiltrates are biologically heterogeneous and can originate through different mechanisms, reflecting diversities in tumour biology and tumour-host interaction. From an immunologist's point of view, the type of immune effectors that can more consistently be considered as a sign of a systemic anti-tumour immune response are cytotoxic T lymphocytes (CTLs), classically $CD8^+$T-cells (Riddell and Greenberg, 1995). Distinction among different subsets of lymphocytes is important: for example the *lamina propria* of normal colorectal mucosa is rich in B-cells (Lee *et al*., 1988). $CD8^+$-Tumour Infiltrating Lymphocytes (TIL) in primary colorectal carcinomas are dividing in three groups (Guidoboni *et al*., 2001): 1) peri-tumoural, when distributed along the invasive margin of the tumour; 2) stromal, when infiltrating the tumour stroma; 3) intra-epithelial, when infiltrating within cancer cells and taking direct contact with tumour cells. TIL are characterized from the expression contemporary of the marker CD8 and CD25 (cell surface antigen that is expressed on T cells following activation) (Guidoboni *et al*., 2001).

Among the different immune cells involved in the control of human tumours, T-cells appear to have a correlation between the function of tumour-infiltrating lymphocytes ($CD8^+$-TIL and $CD4^+$-T regulatory, or T-Reg) and prognosis in different tumours (Alvaro *et al*., 2005). Type, density, and location of immune cells within human colorectal tumours predict clinical outcome (Jarnicki *et al*., 2006).

$CD4^+$ T-Reg activated express CD25 are dependent on the transcription factor Foxp3, for their development and function (Fontenot *et al*., 2003). $CD4^+CD25^+Foxp3^+$ T-Reg cells are present in the normal colonic *lamina propria*, suggesting a constitutive role in the prevention of aberrant responses to risk-free intestinal antigens (Uhlig *et al*., 2006). These cells have a protective role against autoimmune and other inflammatory diseases (including colitis) in several animal models (Holmen *et al*., 2006). It is possible to speculate that local suppression by T-Reg

lymphocytes in chronic inflammation may contribute to tumour growth by preventing effective early immune-surveillance. Conversely, an increasing wealth of studies suggests a direct role for T-Reg, not in the initiation of tumour growth, but in the prevention of immunity against established tumours (Zou, 2006). T-Reg inhibit classical cytotoxic cells such as TIL and NK cells (Ghiringhelli, *et al*., 2005) and contribute to tumour escape and poor survival. This would explain the inefficiency of the immune system to adequately attack primary tumours. More significantly, however, tumours seem to have evolved mechanisms to actively submerge and suppress potential anti-tumour immune responses (Zou, 2005).

Several studies have suggested a protective role of eosinophil infiltration in colorectal carcinoma (Fernandez-Acenero *et al*., 2000), but this effect frequently disappears when results are stratified and corrected for stage (Fisher *et al*., 1989). Moreover, eosinophils are normally well represented in the normal colonic mucosa (Lee *et al*., 1988), and their number can display significant geographical variations (Pascal *et al*., 1997). Indeed, most studies show that eosinophils are more abundant in early stage and adenomas than in invasive advanced carcinomas (Moezzi *et al*., 2000).

Similar considerations are true also for mast cells and for γ/δ T lymphocytes (Lachter *et al*., 1995).

Macrophages can produce angiogenic and immune-suppressive growth factors, such as TGF-β, and can promote tissue remodelling and metastatization process via secretion of matrix metalloproteinases. In colorectal carcinoma, the number of intra-tumour macrophages is higher than in normal colorectal mucosa, and progressively increases from early stage to advanced stage tumours, without any concomitant increase in T-cells (Hakansson *et al*., 1997). This suggest that infiltrating macrophages have a role in the promotion of neo-angiogenesis within the tumour tissue, either by direct production of pro-angiogenic factors like Platelet-Derived Endothelial Cell Growth Factor (PD-ECGF) (Takahashi *et al*., 1996), or by stimulating their production by tumour cells, such as in the case of angiogenin (Etoh *et al*., 2000). Interestingly, while the number of tumour-infiltrating macrophages increases with stage, the number of

dendritic cells (DC), a different subset of myeloid cells with a unique capability in antigen presentation and primary T cell activation, progressively decreases, being lower in tumours than in normal colorectal mucosa, and lower in primary than in metastatic tumours (Schwaab *et al*., 2001).

Aim of the study

The aim of this study is to validate a preclinical animal model to evaluate the carcinogenesis process. The model might reproduce all the histological steps of human colorectal cancer transformation and progression. Moreover, each step has to be correlated to associated molecular events. The study was made through immunohistological analysis of several proteins involved in carcinogenesis process, specifically in Wnt/β-catenin pathway well known to be modified in neoplastic human lesions. The preclinical animal model was also utilized to study the immunological response against the tumour and compared to the "immune-surveillance/immune-escape theory" identified in human colorectal cancer. This model could represent a useful experimental system to study differential biomarker expression and pathway activation during tumorigenesis processes. The biomarkers discovery was evaluated through a proteomic/peptidomic approach. The identified molecules could represent targets of new therapies. Thus, learning information about timing and sequencing of molecular events in our animal model is essential to control and prevent tumour growth and to identify the best therapeutic approach for the specific disease step in an individualized therapy.

Materials and Methods

1. *Animal Model.*

The animal model consist in the induction of colorectal carcinoma with a carcinogenic substance in the BDIX rat strain at age of 9 weeks; the carcinogen used was 1,2 Dymethylhydrazine (DMH). From literature data, DMH was administered subcutaneously once a week at dosage 18mg/Kg/rat for 5 weeks. The animals were sacrificed starting from to the 20^{th} the 30^{th} week after the first carcinogenic injection. At each time the rat colon was collected.

Table 1. Sample of first carcinogenesis experiment.

WEEK	ANIMAL	TISSUE COLLECTED
20	CTR2	Rat colon
28	CTR3	Rat colon
20	DMH4	Rat colon
20	DMH5	Rat colon
22	DMH9	Rat colon
22	DMH10	Rat colon
24	DMH13	Rat colon
24	DMH14	Rat colon
26	DMH19	Rat colon
28	DMH23	Rat colon
28	DMH24	Rat colon
30	DMH27	Rat colon
30	DMH29	Rat colon

To confirm the results obtained in the first experiment and to observe the connection between inflammatory disease and cancer, we repeated the same experiment sacrificing the animals starting from the 6^{th} week to the 35^{th} week after the first injection of the carcinogen**.**

Table 2. Sample of second carcinogenesis experiment.
The new weeks inserted respect to first experiment are marked in grey.

WEEK	ANIMAL	TISSUE COLLECTED
6	DMH2	Rat colon
6	DMH4	Rat colon
12	DMH6	Rat colon
12	DMH8	Rat colon
16	DMH10 (x2)	Rat colon
18	DMH12	Rat colon
18	DMH14 (x2)	Rat colon
19	DMH16 (x2)	Rat colon
20	DMH20(x2)	Rat colon
20	DMH22 (x2)	Rat colon
21	DMH24(x2)	Rat colon
22	DMH26(x2)	Rat colon
24	DMH27(x2)	Rat colon
24	DMH28(x2)	Rat colon
26	DMH30(x2)	Rat colon
26	DMH32(x2)	Rat colon
28	DMH33(x2)	Rat colon
28	DMH34(x2)	Rat colon
32	DMH37(x2)	Rat colon
35	DMH38(x2)	Rat colon
35	DMH39(x2)	Rat colon

2. *Tissue Samples Preparation.*

2.1 Procedure for O.C.T. embedding samples.

The tissue sample are been frozen as soon as possible using the embedding media O.C.T. (Sakura Finetech's Compound); this aqueous glycerol compound provide protection of the specimen during long-term, gives support to the tissue and aids in the cryo-sectioning process. The tissue was cut to a size no greater than one half the area of the cryomold (Sakura Finetech's), so that it will fit into the cryomold without touching the sides of the mold. For the standard cryomold, specimen samples didn't exceed 1cm in height or width, or a thickness of more than 0.5 cm. The frozen tissue were frozen at -80^0C and then cut in a cryostate (Leica) approximately 8 microns and affixed onto the LCM slide (Arcturus). Once mounted, the slides were frozen at -20°C until LCM processesment.

2.2 Procedure for paraffin embedding samples.

After collection, the tissue samples were washed with medium until the fixation procedure (necessary to preventing antigen elution or degradation and to preserve the position of the antigen, whether nuclear, cytoplasmic or membrane-bound). The tissues were fixed with 10% tamponate formalin (Bio-Optica) for 24h, washed in water, immersed in alcohol 70%, alcohol 95%, alcohol 100% (Fluka), histolemon (Bio-Optica) and finally embedded in liquid paraffin. After fixation, the tissue block was embedded in paraffin, then cut in a microtome (Leica) approximately 5 microns and affixed onto the positively charged slide (Carlo Erba). Once mounted, the slides were dried to remove any water and incubated at 60ºC for a few hours.

3. Histological techniques.

3.1 Haematoxylin Eosin staining.

Before proceeding with the staining protocol, the slides were deparaffinized and rehydrated following these passages:

- Bioclear (Bio-Optica): 30min
- 100% ethanol: 10 min
- 90% ethanol: 5min
- 80% ethanol: 5 min
- 70% ethanol: 5 min
- 50% ethanol: 5 min
- H_20: 10 min
- Staining with haematoxylin (Bio-Optica): 10 min
- H_20: 5 min
- Counterstaining with eosin 1% (Bio-Optica): 5 min

The tissue were dehydrated following these passages:

- 50% ethanol: 5 min
- 70% ethanol: 5 min
- 90% ethanol: 5min
- 100% ethanol: 10 min
- Bioclear: 30min.

In the final step, the slides was mounted using Fast drying mounting medium for cover slipping (Bio-Optica).

3.2 Immunohistochemistry.

Immunohistochemistry (or IHC) is a method used for demonstrating the presence and location of proteins in tissue sections. The antibody-antigen interaction is visualized using a chromogenic detection, in which an enzyme conjugated to the antibody cleaves a substrate to produce a coloured precipitate at the location of the protein. We used horseradish peroxidase (HRP) and its substrate peroxide/DAB for visible light microscopy (DAKO LSAB + System HRP).

Before proceeding with the staining protocol, the slides were deparaffinised and rehydrated following these passages:

- Bioclear: 30min
- 100% ethanol: 10 min
- 95% ethanol: 5min
- 75 % ethanol: 5 min
- 50 % ethanol: 5 min
- 1X Dulbecco's Phosphate Buffered Saline – PBS (Euroclone) 5 min x2

The formalin-fixed tissues required an antigen retrieval step before immunohistochemical staining to break the methylene bridges formed during fixation and expose the antigenic sites in order to allow the antibodies to bind. The method used was the "heat induced" antigen retrieval with Sodium citrate buffer (10 mM Sodium Citrate - SIGMA, 0.05% Tween 20 - Biorad, pH 6.0) or Tris-EDTA Buffer (10 mM Tris Base - SIGMA, 1 mM EDTA - SIGMA, 0.05% Tween 20, pH 9.0). After rinsing with PBS for 20', the activity of endogenous peroxidase was suppressed incubating the slides in 0.3% H_2O_2 (Fluka) added to methanol (Carlo Erba) for 20 min. From this point, all the steps were made in a humidified chamber to avoid drying of the tissue; a-specific binding sites were blocked with 1% BSA (SIGMA) in

PBS for 15 min at room temperature. It was applied primary antibody diluted in 1% BSA in PBS and incubated overnight at 4°C. A biotinylated secondary antibody was then bounded to the primary antibody, incubating the slides with a biotinylated link (DAKO) for 30 min at room temperature. In a separate reaction, a complex of avidin and biotinylated enzyme was formed by mixing the two in a ratio that leaves some of the binding sites on avidin unoccupied. This complex, called streptavidin-HRP (DAKO), was then incubated with the tissue section for 30 min at room temperature. The unoccupied biotin-binding sites on the complex bind to the biotinylated secondary antibody (DAKO). Finally the slides, were incubated with the substrate of HRP, 3,3'- Diaminobenzidine (DAB) (DAKO) for 9 min at room temperature and counterstained with haematoxylin for 5 minutes. The tissue were dehydrated following these passages:

- 50% ethanol: 5 min
- 75% ethanol: 5 min
- 95% ethanol: 5min
- 100% ethanol: 10 min
- Bioclear: 30min.

In the final step, the slides were mounted using Fast drying mounting medium for cover slipping.

Table 3. Primary antibodies used for Wnt/β-catenin pathaway detection.

Antibody	Manufacturers	Dilution	Specie
Beta-catenin	BD	1:150	Mouse monoclonal
E-cadherin	BD	1:200	Mouse monoclonal
GSK 3 beta	AbCam	1:100	Rabbit polyclonal
APC	AbCam	1:100	Rabbit polyclonal
c-myc	Santa Cruz	1:50	Mouse monoclonal
Cyclin D1	Santa Cruz	1:50	Mouse monoclonal
K-ras	AbCam	1:20	Mouse monoclonal

Table 4. Primary antibody for immunological analysis of infiltrated lymphocytes.

Antibody	Manufacturers	Dilution	Specie
CD4	Millipore	1:100	Mouse monoclonal
LAMP1	AbCam	1:200	Rabbit polyclonal
CD25	Thermo Scientific	1:80	Mouse monoclonal
FoxP3	Santa Cruz	1:150	Mouse monoclonal

4. *Laser Capture Microdissection (LCM).*

LCM incorporates an inverted light microscope and a near infrared laser to facilitate the procurement of desired cells. After direct visualization of the cells of interest, using a laser pulses to activate a thermoplastic polymer film that expands and impregnates the cells of interest that can then be lifted from the slide. The exact morphology as well as the DNA, RNA and proteins of the procured cells remain intact and bound to the film. Using LCM frozen tissues have been successfully dissected and recovered cells used for protein analysis. Normal epithelium, premalignant (dysplasia), in situ cancer, invasive cancer and metastatic cancer cells have been obtained from tissues. Frozen tissue has been cut at 8μm, mounted on plain, uncharged microscope slides and stained with haematoxylin protocol. Complete Mini protease inhibitor tablets (Roche Applied Science), were added to the 70% ethanol and haematoxylin staining solutions to reduce protein activation derangement.

Protocol for Staining Frozen Tissue

- 70% Ethanol: 5 seconds
- Deionised Water: 10 seconds
- Mayer's Haematoxylin: 15-30 seconds
- Deionised Water: 10 seconds
- Scott's Tap Water (Sigma): 10 seconds
- 70% Ethanol: 10 seconds
- 95% Ethanol: 10 seconds
- 95% Ethanol: 10 seconds
- 100% Ethanol : 1 minute
- 100% Ethanol: 1 minute
- Xylene: 1 minute
- Xylene: 1 minute

Laser capture microdissection was performed using a Pixcell II Laser Capture Microdissection system (Arcturus) to procure enriched tumour cell populations. A total of 25,000 cells, procured over several sections, were microdissected for each case and stored on microdissection caps (CapSure® Macro LCM Caps, Arcturus) at –80°C until lysed. It is possible to estimate the number of cells captured based upon the number of pulse fired during the collection of cells, using this formula:

# of laser shots X 5cells/spot = estimated number of captured cells.

5. Reverse Phase Protein Array.

Reverse Phase Protein Microarrays are a multiplexed proteomic platform used to evaluate cell signalling activity in many samples at once. Approximately 150 slides can be printed with 40µl of protein lysates and each slide is probed with a single antibody.

5.1 Tissue Lysates Preparation.

Cell lysis volume is calculated by assuming 1µl tissue extraction buffer/1,000 cells. The tissue lysis buffer (950µl Tris-Glycine SDS Sample Buffer 2X (Invitrogen), 50µl 2 β-mercaptoethanol (PIERCE), 1ml TPER Reagent (PIERCE)) was repetitively pipetted among on the LCM caps; the protein lysate was transferred to a screw cap tube and heated at 100˚C for 7 minutes. The lysated can be stored at -80˚C.

5.2 Reverse Phase Protein Microarray Printing.

The lysates were then spotted onto nitrocellulose-coated glass slides (Whatman) using a 2470 Arrayer (Aushon BioSystems), outfitted with 350-µm pins. Cases were printed in duplicate, in 5-point dilution curves, thus assuring that the linear detection range was encompassed for the chosen antibody concentration. As a high and low internal control for antibodies staining specificity, lysates derived from human cervical cancer HeLa cells and pervanadate treated HeLa cells, human immortalized T-cells Jurkat, calyculin treated Jurkat cells and etoposide treated Jurkat cells, were used and spotted onto every array along with the experimental samples. Slides were stored desiccated (Drierite anhydrous calcium sulfate) at –20°C until staining.

5.2.1 Slides pre-treatment.

All microarray slides, with the exception of the one probed with the chemoluminescent Sypro Ruby (Invitrogen) used to determine the total protein, should be blocked prior to staining procedure.

First, incubate the slides with 1X Mild Reblot™ Mild Antigen Stripping solution (Chemicon) in deionised water for 15 minutes on a shaker.

After washing PBS calcium and magnesium free solution (GIBCO) the slides were blocked with I-Block Protein Blocking Solution (Tropix) for a minimum of 60 minutes.

5.2.2 Total Protein amount determination: Sypro Ruby Staining.

Sypro Ruby staining is a fundamental step to quantify the amount of proteins that are present in the printed sample. Sypro Ruby procedure is based on fluorescent dye detection. For more accurate quantification of protein concentration, it was stained with Sypro Ruby 1 of every 25 slides. The slides were warmed to room temperature for 5-10 minutes, then incubated on a shaker for 15 minutes with fixative solution (3.5 ml acetic acid for a final dilution of 7% (Fisher) + 5 ml methanol for a final dilution of 10% (Fisher). After washing with deionised water the slides were incubated with Sypro Ruby solution (Molecular Probes) for 30 minutes and then rinsed with deionised water.

5.3 Microarray Immunostaining.

This method requires a single antibody-epitope interaction on the protein of interest. The number of slides to be stained was chosen in relation to the number of endpoints of interest. The Dako autostainer (Dako Cytomation) allows simultaneous staining of 48 slides. To quantify the unspecific background signal generated from the interaction between the secondary antibody and samples, it is essential in each staining run, that one slide is probed

only with each secondary antibody used. The subtraction of signal produced by the negative control from the primary antibody stained slides provides a more accurate intensity value for the protein of interest. Each antibody was validated to confirm specific interaction by Western blot analysis. Antibodies producing a single band in correspondence to the molecular weight of interest were considerate validated and eligible for immunostaining. It was used the Catalyzed Signal Amplification System kit according to the manufacturer's recommendation (CSA; Dako Cytomation). Development was completed using diaminobenzadine/hydrogen peroxide as chromogen/substrate. All protein values were normalized to total protein to account for differences in intensity due solely to starting lysate concentration variance. Stained slides were scanned individually on a UMAX PowerLook III scanner (UMAX) at 600 dpi (dots per inch) and saved as TIFF files in Photoshop 6.0 (Adobe). The TIFF images for antibody-stained slides and Sypro-stained slide were analyzed with array analysis software designed for protein microarray analysis: version 2.X00 (Vigene). The software performed spot finding, local background subtraction, replicate averaging, and total protein normalization, producing a single value for each sample at each endpoint.

6. *Western Blotting.*

Western Blotting has been used for validating the antibodies used in phospho proteomic analysis. Laser capture microdissected cells were lysed directly in SDS sample buffer and run on 4%-20% SDS-PAGE gels (Invitrogen), and subjected to Western transfer onto Immobilon PVDF membrane (Sigma-Aldrich).

Table 5. **List of used antibodies and manufacturers.**

Antibodies	Manufacturers
Akt	Cell Signaling
APC1 Ab-1	Lab Vision
E-cadherin	Cell Signaling
Catenin (beta)	Cell Signaling
CD44	Cell Signaling
CD133	Milteny
Cox2	Upstate
CiclinD1	Cell Signaling
EGFR	Cell Signaling
ErbB2/HER2	DAKO Cytomation
ErbB3/HER3	Cell Signaling
EGFR L858R Mutant	Cell Signaling
ERK1/2	Cell Signaling
c-Myc	Cell Signaling
Ras-GRF1	Cell Signaling
Smac/Diablo	Cell Signaling
Phospho-4E-BP1 (S65)	Cell Signaling
Phospho-4E-BP1 (T70)	Cell Signaling
Phospho-Acetyl-CoA Carboxylase (s79)	Cell Signaling
Phospho-Adducin (S662)	Upstate
Phospho-Akt (S473)	Cell Signaling
Phospho-Akt (T308)	Cell Signaling
Phospho-AMPKalpha1 (S485)	Cell Signaling
Phospho-AMPKbeta1 (S108)	Cell Signaling
Phospho-ASK1 (S83)	Cell Signaling
Phospho-BAD (S112)	Cell Signaling
Phospho-BAD (S136)	Cell Signaling
Phospho-Bcl-2 (S70)	Cell Signaling
Phospho-c-Abl (T735)	Cell Signaling
Phospho-c-Abl (Y245)	Cell Signaling
Phospho-Caspase 3, cleaved (D175)	Cell Signaling
Phospho-Caspase 6, cleaved (D162)	Cell Signaling
Phospho-Caspase 7, cleaved (D198)	Cell Signaling
Phospho-Caspase 9, cleaved (D315)	Cell Signaling
Phospho-Catenin (beta) (S33/37/T41)	Cell Signaling
Phospho-Catenin (beta) (T41/S45)	Cell Signaling
Phospho-Chk-2 (S33/35)	Cell Signaling
Phospho-CREB (S133)	Cell Signaling
Phospho-cofilin (S3)	Cell Signaling
Phospho-EGFR (Y845)	Cell Signaling

Phospho-EGFR (Y1068)	Cell Signaling
Phospho-EGFR (Y992)	Cell Signaling
Phospho-EGFR (Y1148)	Biosource
Phospho-EGFR (Y1173)	Biosource
Phospho-eIF4E (S209)	Cell Signaling
Phospho-eIF4G (S1108)	Cell Signaling
Phospho-eNOS (S1177)	Cell Signaling
Phospho-ErbB2/HER2 (Y1248)	Upstate
Phospho-ERK 1/2 (T202/Y204)	Cell Signaling
Phospho-FADD (S194)	Cell Signaling
Phospho-FAK (Y397)	BD
Phospho-FAK (Y576/577)	Cell Signaling
Phospho-FKHR (S256)	Cell Signaling
Phospho-FKHR (T24)/FKHRL1 (T32)	Cell Signaling
Phospho-GSK-3alpha/beta (S219)	Cell Signaling
Phospho-GSK-3alpha/beta (Y279/216)	Biosource
Phospho-GSK-3beta (S9)	Cell Signaling
Phospho-Histone H3 (S10)	Upstate
Phospho-IGF-1 Rec (Y1131/Insulin Rec (Y1146)	Cell Signaling
Phospho-IGF-1 Rec (Y1135/36IR (Y1150/51)	Cell Signaling
Phospho-IkappaB-alpha (S32)	Cell Signaling
Phospho-IkappaB-alpha (S32/36) (39A1431)	BD
Phospho-IRS-1 (S612)	Cell Signaling
Phospho-Jak1 (Y1022/1023)	Cell Signaling
Phospho-c-Kit (Y703)	Cell Signaling
Phospho-MARCKs (S152/156)	Cell Signaling
Phospho-Met (Y1234/1235)	Cell Signaling
Phospho-MSK1 (S360)	Cell Signaling
Phospho-mTOR (S2481)	Cell Signaling
Phospho-mTOR (S2448)	Cell Signaling
Phospho-NF-kappaB p65 (S536)	Cell Signaling
Phospho-p27 (T187/Y182)	Zymed
Phospho-p38 MAP Kinase (T180/Y182)	Cell Signaling
Phospho-p70 S6 Kinase (S371)	Cell Signaling
Phospho-p70 S6 Kinase (T389)	Cell Signaling
Phospho-p90RSK (S380)	Cell Signaling
Phospho-PAK1 (S199/204)/PAK2 (S192/197)	Cell Signaling
Phospho-PDGF Receptor beta (Y716)	Upstate
Phospho-PDGF Receptor beta (Y751)	Cell Signaling
Phospho-PKC alpha (S657)	Upstate
Phospho-PKC zeta/lambda (T410/403)	Cell Signaling
Phospho-PKCa/B II (T638/641)	Cell Signaling
Phospho-PKCtheta (T538)	Cell Signaling
Phospho-PRAS40 (T246)	Biosource
Phospho-PTEN (S380)	Cell Signaling

Phospho-Pyk2 (Y402)	Cell Signaling
Phospho-c-Raf (S338)	Cell Signaling
Phospho-S6 Ribosomal Protein (S235/236) (2F9)	Cell Signaling
Phospho-SAPK/JNK (T183/Y185)	Cell Signaling
Phospho-Shc (Y317)	Upstate
Phospho-SMAD2 (S465/467)	Cell Signaling
Phospho-Src (Y527)	Cell Signaling
Phospho-Stat1 (Y701)	Upstate
Phospho-Stat3 (S727)	Cell Signaling
Phospho-Stat3 (Y705)	Upstate
Phospho-Stat5 (Y694)	Cell Signaling
Phospho-VEGFR 2 (Y1175)	Cell Signaling
Phospho-VEGFR 2 (Y951)	Cell Signaling
Phospho-VEGFR 2 (Y996)	Cell Signaling

7. *Statistical Analysis.*

Statistical analyses were performed with SAS version 9 software (SAS Institute). The differences of endpoint intensities between 2 groups were assessed. Initially, the distribution of variables was checked. If 2 groups of the variable followed by the normal distribution, 2-sample *t* test was performed. If the variances of 2 groups were equal, 2-sample *t* test with a pooled variance procedure was used to compare the means of intensity between 2 groups. Otherwise, 2-sample *t* test without a pooled variance procedure was adopted. For non-normally distributed variables, Wilcoxon rank sum test was used. All significance levels were set at $P = 0.05$.

Results

1. *Colorectal carcinogenetic steps in the BDIX preclinical animal model.*

We set up an experimental model of colorectal carcinoma induced in BDIX rats by the administration of the carcinogen 1,2 Dymethylhydrazine (DMH). This model is characterized by a tumour biology strictly related to the clinical progression pattern of the CRC; moreover, the molecular pathways of carcinogenesis are extremely conserved.

In our model, we obtained all steps of colorectal carcinogenesis process observed in human neoplastic progression (**Figure 7**). We collected altogether rat colon tissues starting from the 6^{th} week to the week 35^{th} after the first carcinogenic injection.

Specifically, starting from the **6^{th} week** we observed characteristic modifications similar to that observed in human **inflammatory bowel disease (IBD)**: lesions were typically small and multiple, largely composed of granulation tissue, mixed with inflamed and hyperaemic mucosa.

Around the **16^{th} week**, we observed **low grade dysplasia (LD)** with structural and cytological alterations in the epithelium: crypts were larger than normal, with increased peri-cryptal space, had a thicker layer of epithelial cells that often stain darker, and generally had oval rather than circular openings.

Approximately to the **18^{th} week**, we observed **high grade dysplasia (HD)** with uncontrolled crypt cell division: it was characterized by ramification of the microscopic glands, loss of goblet cells, loss of basilar polarity of the nucleus, increased nuclear/cytoplasmatic ratio and loss of cytoplasmatic glycogen.

Starting at the **22^{th}week**, we detected **carcinoma in situ (IS)**: the high grade of dysplasia was associated with infiltration in the *tonaca propria* or in the *muscularis mucosae*; from the **24^{th} week**, the lesion can be defined **advanced carcinoma (K)**, because the neoplastic tissue infiltrates the *submucosa*.

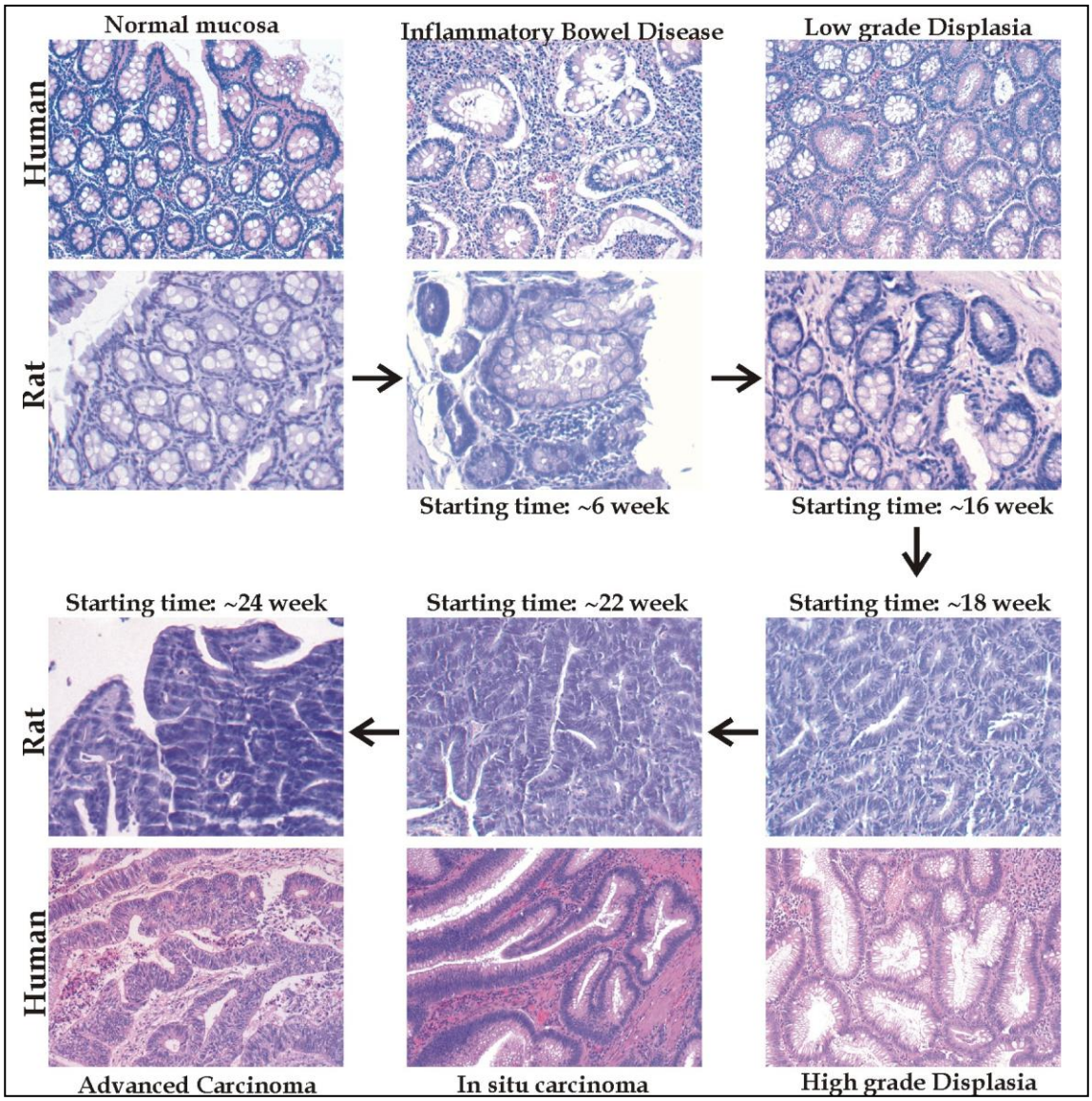

Figure 7. Comparative histological analysis of colorectal carcinogenesis steps in human and rat lesions.

2. *Immunohistochemical analysis of Wnt/β-catenin pathway.*

We analyzed some proteins involved in the Wnt/β-catenin pathway by immunohistochemical technique, to study their expression and localization changes during the carcinogenetic process in CRC. In particular we analysed:

1) Components of "destruction complex": **β-catenin, APC, GSK3β**
2) Wnt/β-catenin pathway target gene proteins: **E-cadherin**, **c-Myc**, **Cyclin-D1**, **k-Ras**

For each protein examined, a comparative study of expression and tissue distribution in human and rat colon lesions during the carcinogenesis process has been carried out.

2.1 β-catenin expression and tissue distribution.

In **normal mucosa**, **β-catenin** was mostly expressed at the cell membrane, mainly at the cell-cell junctions, and absent in the nucleus. In **IBD** tissue (6-16 weeks), the expression of β-catenin was augmented in the cytoplasm and at the cell-cell contacts, compared to normal mucosa; a low expression was observed also in the nucleus. During the **LD** stage (16-18 weeks) we observed an increased β-catenin expression in the cytoplasm and a low decrement at the cell-cell junction. In the **HD** stage (18-22 weeks), as compared to the **LD** stage, we observed an increased staining in the nucleus and in the cytoplasm, suggestive of β-catenin stabilization and Wnt signalling activation, characteristic of neoplastic colon cells. From the development of the **IS** until the **K** stages(22-28 weeks), the expression of β-catenin was maintained high in the cytosol, but associated with a slow decrease in the nucleus (**Figure 8**). Moreover, in **K** tissue the expression of β-catenin at the cell membrane seems to be increased compared to **IS** stage.

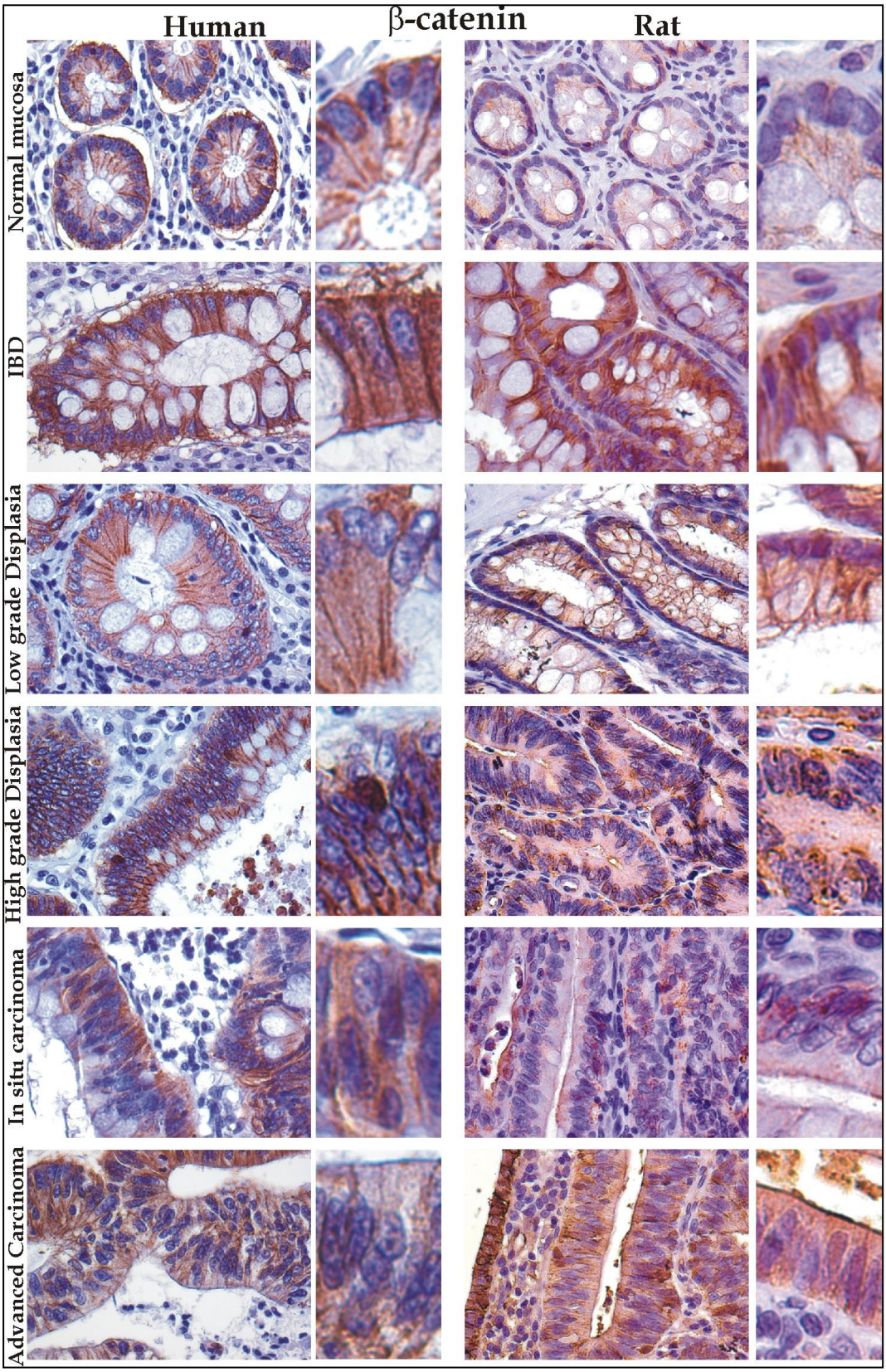

Figure8. Immunohistochemical analysis of β-catenin expression and tissue distribution in human and rat colorectal carcinogenetic process.

2.2 E-cadherin expression and tissue distribution.

The molecular adhesion protein **E-cadherin**, in **normal epithelial mucosa**, was expressed mainly at the cell-cell junctions, where it is an essential component for maintenance of cellular adhesion and architecture tissue. During the sequential steps of the carcinogenetic process (from the **IBD** to **IS** stages), we observed a progressive decrease of E-cadherin expression at the cell to cell contacts in accordance with the disaggregation of colon epithelium structure and acquisition of invasive and metastatic potential of neoplastic transformation (**Figure 9**). Interestingly, as observed for β-catenin, in **K** tissue the expression of E-cadherin at the cell membrane seems to be increased compared to **IS** stage.

2.3 APC expression and tissue distribution.

The onco-suppressor gene **APC**, in **normal mucosa** was expressed mainly in the cytoplasm and, at lesser extent, in the nucleus, coherently with its primary function of maintaining the β-catenin destruction complex in the cytoplasm, and with the secondary function of shuttle protein in the nucleus. In **IBD** stage as well as in **LD** stage, we observed a dramatic increased APC expression mainly localized in the cytoplasm. During the **HD, IS and K** stages the expression of APC was progressively decreased exhibiting a dot-like pattern distribution in the cytoplasm (**Figure 10**).

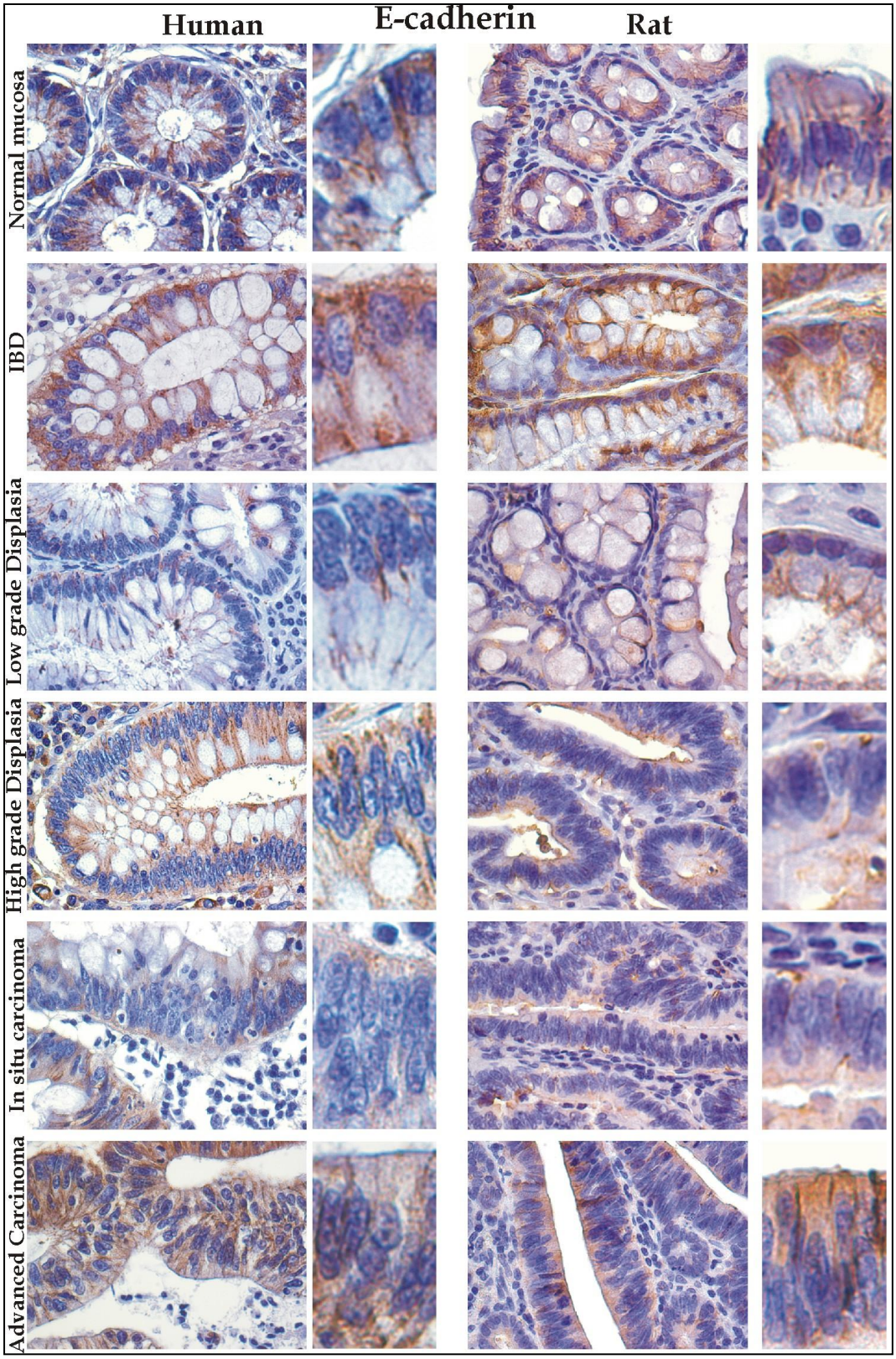

Figure 9. Immunohistochemical analysis of E-cadherin expression and tissue distribution in human and rat colorectal carcinogenetic process.

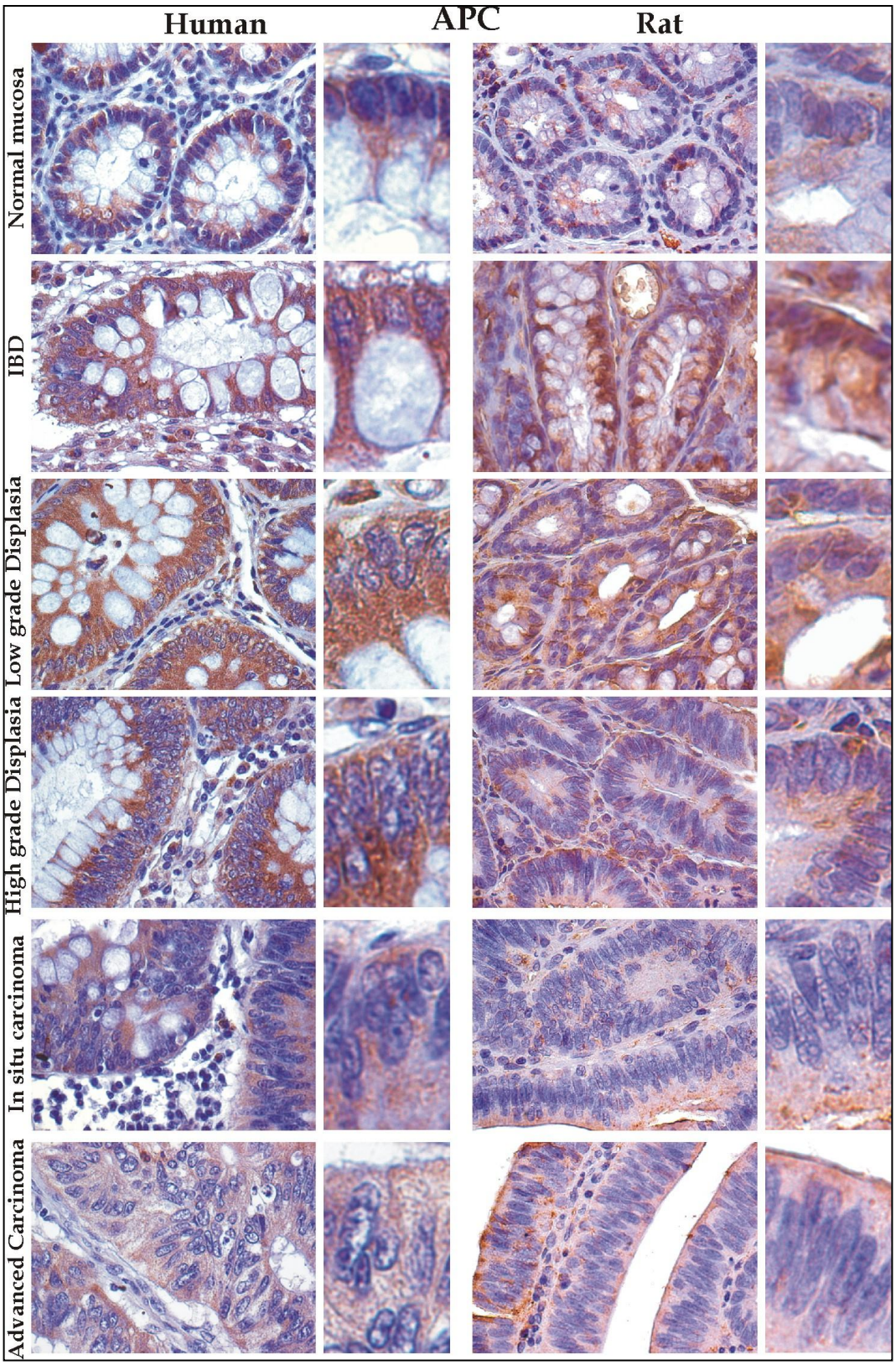

Figure 10: Immunohistochemical analysis of APC expression and tissue distribution in human and rat colorectal carcinogenetic process.

2.4 GSK3β expression and tissue distribution.

In **normal mucosa**, **GSK3β** was expressed mainly in the cytoplasm and, at lesser extent, in the nucleus. In the cytosol, GSK3β has a critical role participating to phosphorylation of several kinase substrates and in particular to β-catenin phosphorylation upstream its degradation at the proteosome, thus contributing to maintenance of cellular β-catenin homeostasis. In the nucleus GSK3β may also take part to c-Myc phosphorylation leading to its degradation at the proteosome, thus reducing cell proliferation. In **IBD** and **LD** stages, GSK3β expression appears to be decreased in the nucleus as compared to normal mucosa, while in **HD** stage a protein distribution at sub-membrane region of epithelioid cells became predominant. During the **HD, IS** and **K** stages the expression of GSK3β was progressively decreased, and in K stage it was almost completely negative (**Figure 11**).

2.5 C-myc expression and tissue distribution.

C-myc, one of the Wnt/β-catenin pathway target gene protein, contributes to cell proliferation in neoplastic cells. Consistently, in **normal mucosa** it was expressed at very low levels and was completely negative in the nucleus. Interestingly, in **IBD** stage, it appeared more expressed in both cytosolic and nuclear compartments, as compared to normal mucosa. During **LD**, **HD** and **IS** stages, C-myc expression was dramatically increased in both cytoplasm and nucleus (mainly in **LD** stage) coherently with the hypertropic/hyperproliferative features of dysplastic and neoplastic tissues. In **K** stage C-myc was almost completely negative (**Figure 12**).

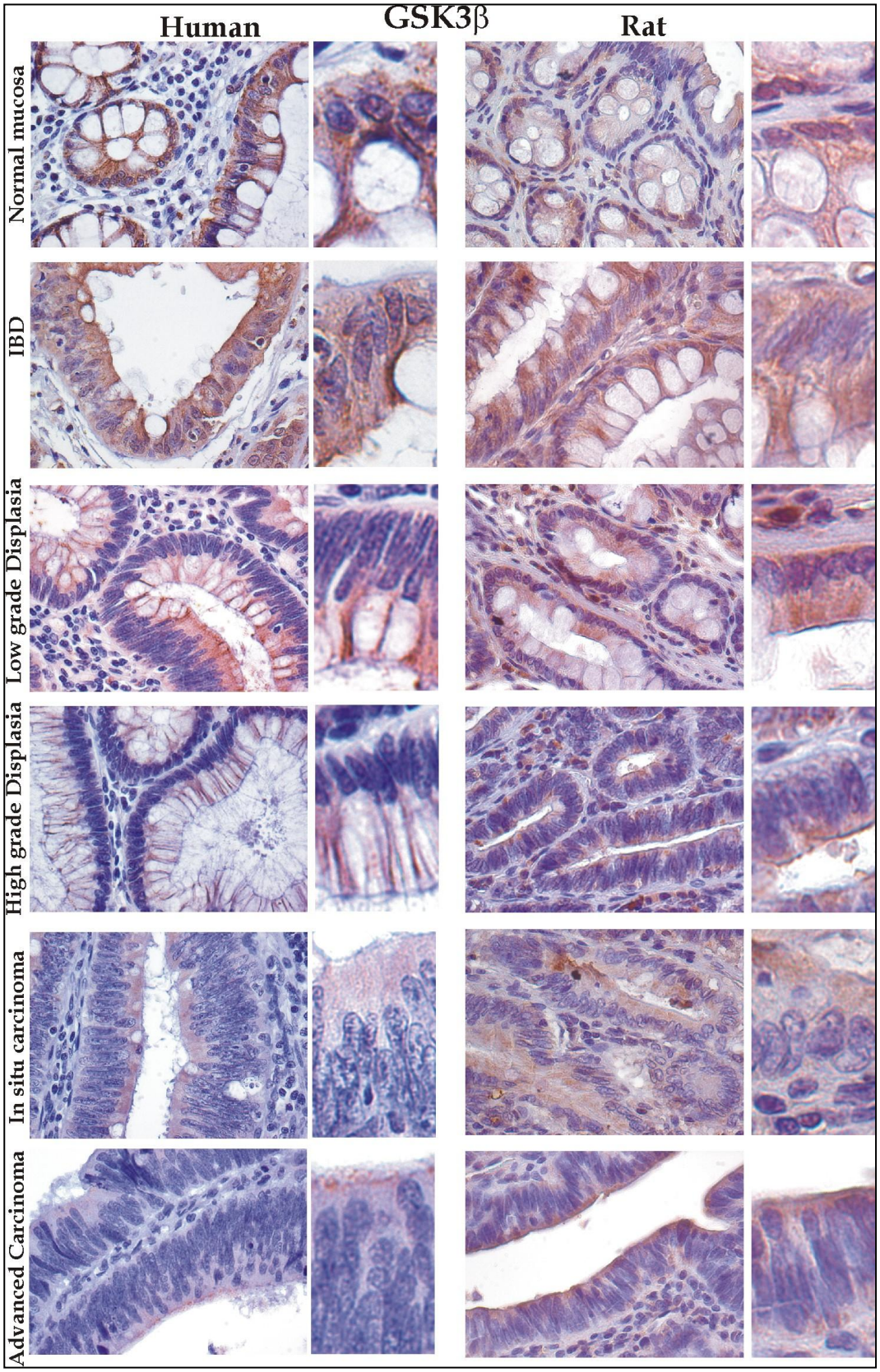

Figure 11. Immunohistochemical analysis of GSK3β expression and tissue distribution in human and rat colorectal carcinogenetic process.

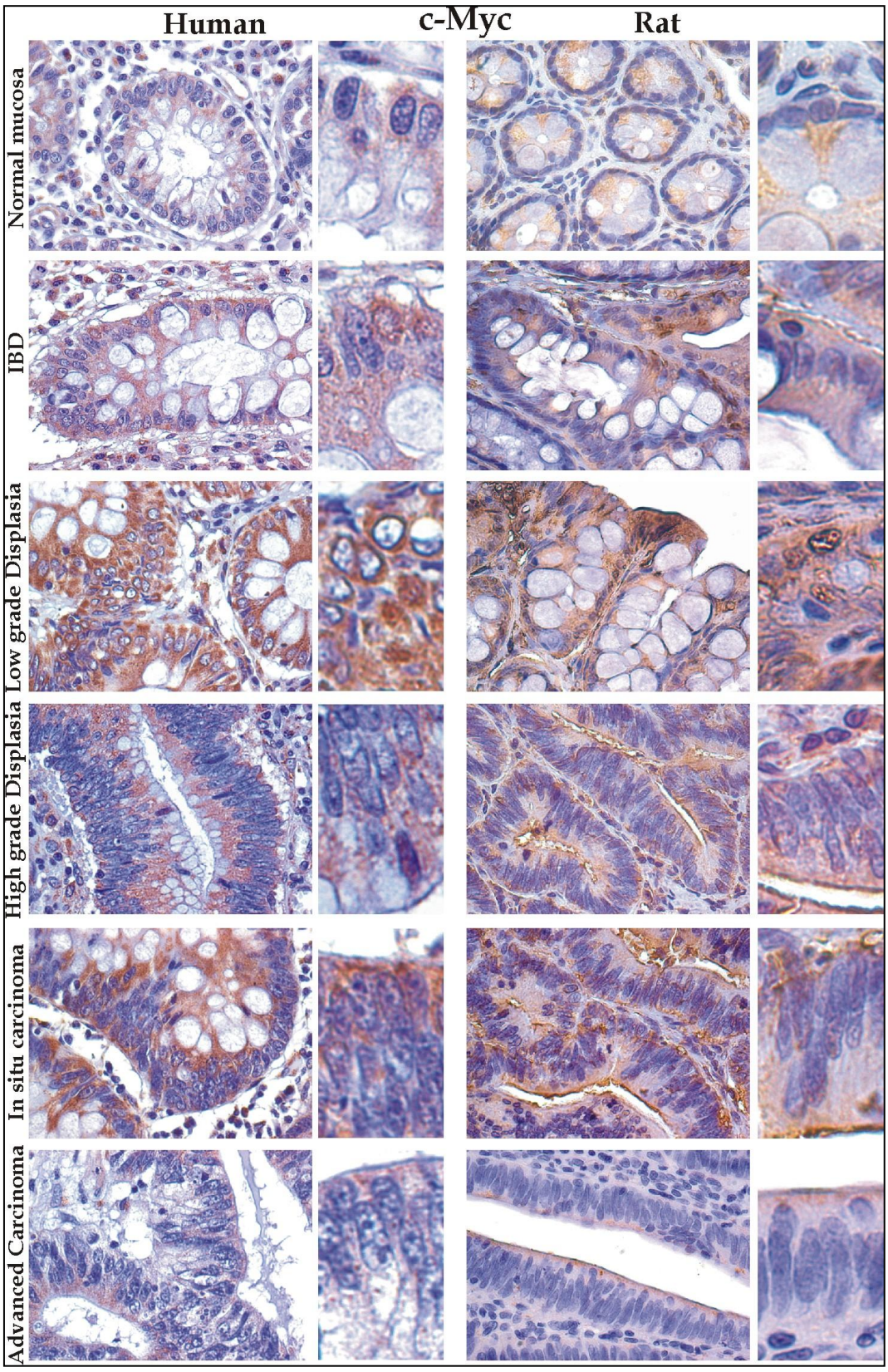

Figure 12. Immunohistochemical analysis of c-myc expression and tissue distribution in human and rat colorectal carcinogenetic process.

2.6 Cyclin-D1 expression and tissue distribution.

Cyclin-D1, another of the Wnt/β-catenin pathway target gene protein involved in the cell proliferation of tumour cells, was almost absent in **normal mucosa**. In **IBD** stage cyclin-D1 expression was dramatically increased in both cytosolic and nuclear compartments, as compared to normal mucosa. Interestingly, in **LD** stage the expression and tissue distribution is very similar to that observed in normal mucosa (quite negative), while during **HD, IS** and **K** stages the expression of cyclin-D1 was progressively increased (**Figure 13**).

2.7 K-Ras expression and tissue distribution.

The oncogenic protein **K-Ras,** contributes to tumour progression during the transition from moderate to late adenomas. It was almost absent in **normal mucosa** and in the **IBD** stage. A visible expression of K-Ras was observed in the **LD** stage and during **HD, IS** and **K** stages the expression of K-Ras was progressively increased (**Figure 14**).

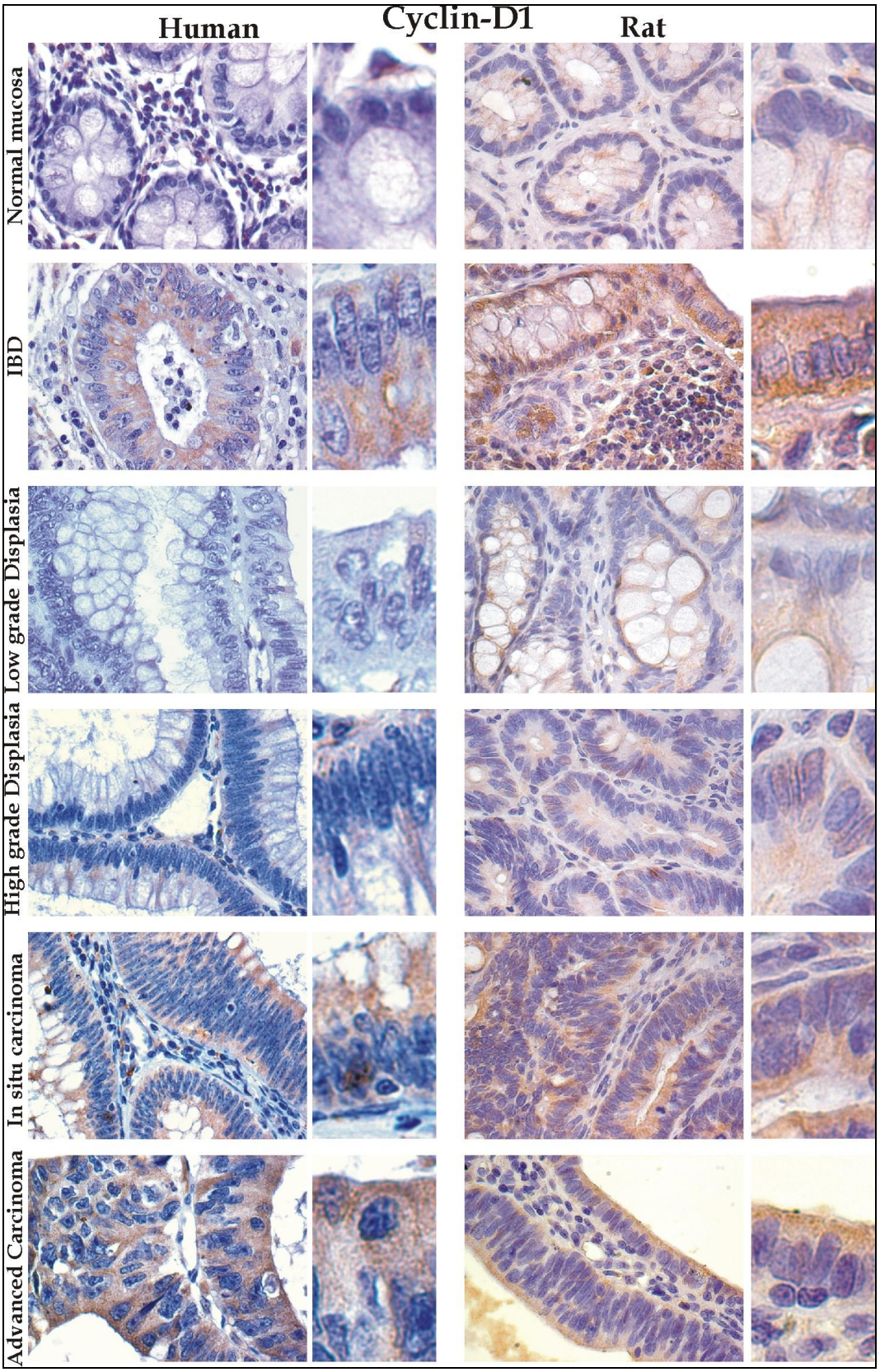

Figure 13. Immunohistochemical analysis of Cyclin D1 expression and tissue distribution in human and rat colorectal carcinogenetic process.

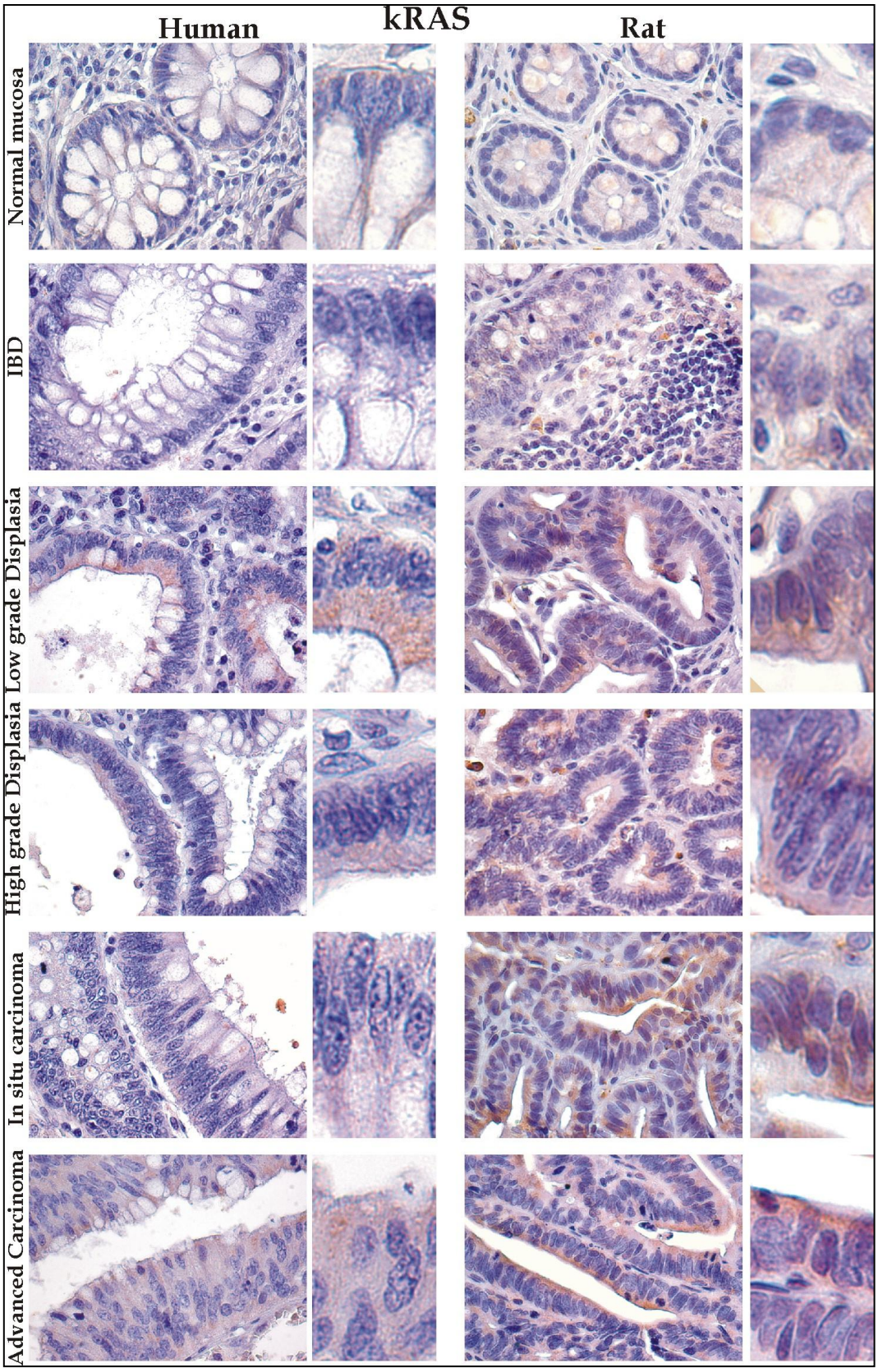

Figure 14. Immunohistochemical analysis of K-ras expression and tissue distribution in human and rat colorectal carcinogenetic process.

3. *Immunohistochemical analysis of immune-response markers.*

In our preclinical animal model, we analyzed the expression of several proteins involved in the host immune-response to tumour development. Specifically, we studied two kind of lymphocyte-T infiltrates in the normal/neoplastic tissues: the T-regulatory cells (**T-Reg**) and the Tumour infiltrate T-lymphocytes (**TIL**). T-Reg cells were analyzed for the concurrent expression of the membrane protein CD4, of the transcriptional factor FoxP3 and of the interleukin-2 receptor, CD25. TIL were analyzed for the simultaneous expression of the membrane protein CD8 and of the cell activation protein LAMP1.

3.1 T-regulatory cells (T-Reg) during tumour progression.

In **normal mucosa**, **T-Reg** cells were almost absent. At the beginning of the tumorigenesis process, such as in the **LD** stage, $CD4^+$ T-reg cells were very few and didn't show any localization around atypical cells. The used antibody showed a cross-reactivity with mucine present in the gland of colon mucosa. The concurrent expression of CD25 and FoxP3 were also very low. During **HD, IS** and **K** stages, the number of T-regulatory cells increased progressively around the neoplastic cells, as demonstrated by the higher expression of CD4 associated to FoxP3 and CD25 markers (**Figure 15**).

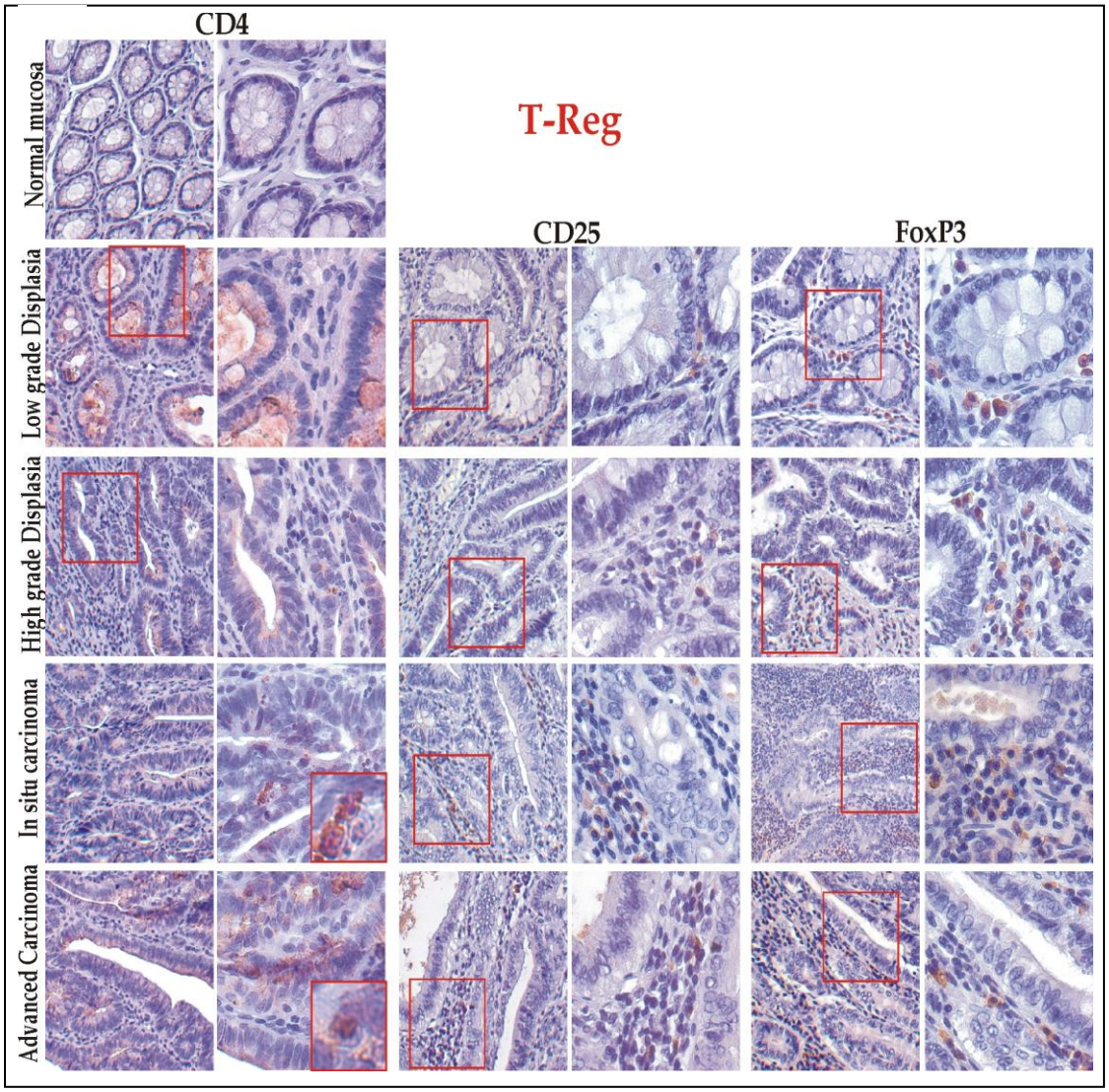

Figure 15. Immunohistochemical analysis of T-reg infiltrates during rat colorectal carcinogenesis process.

3.2 Tumour infiltrate T-lymphocytes (TIL) during tumour progression.

TIL infiltrates showed a time-distribution completely different from T-Reg. TIL are also nearly absent in **normal mucosa**, but in the early stages of carcinogenesis, such as **LD** stage and mainly **HD** stage, they appeared around the neoplastic crypts with strong and specific expression of CD8; these lymphocytes were activated, as demonstrated by a simultaneous expression of LAMP1. In **IS** and **K** stages, TIL decreased gradually as

showed by the low expression of CD8 and LAMP1. In **K** stage TILs were almost completely absent (**Figure 16**).

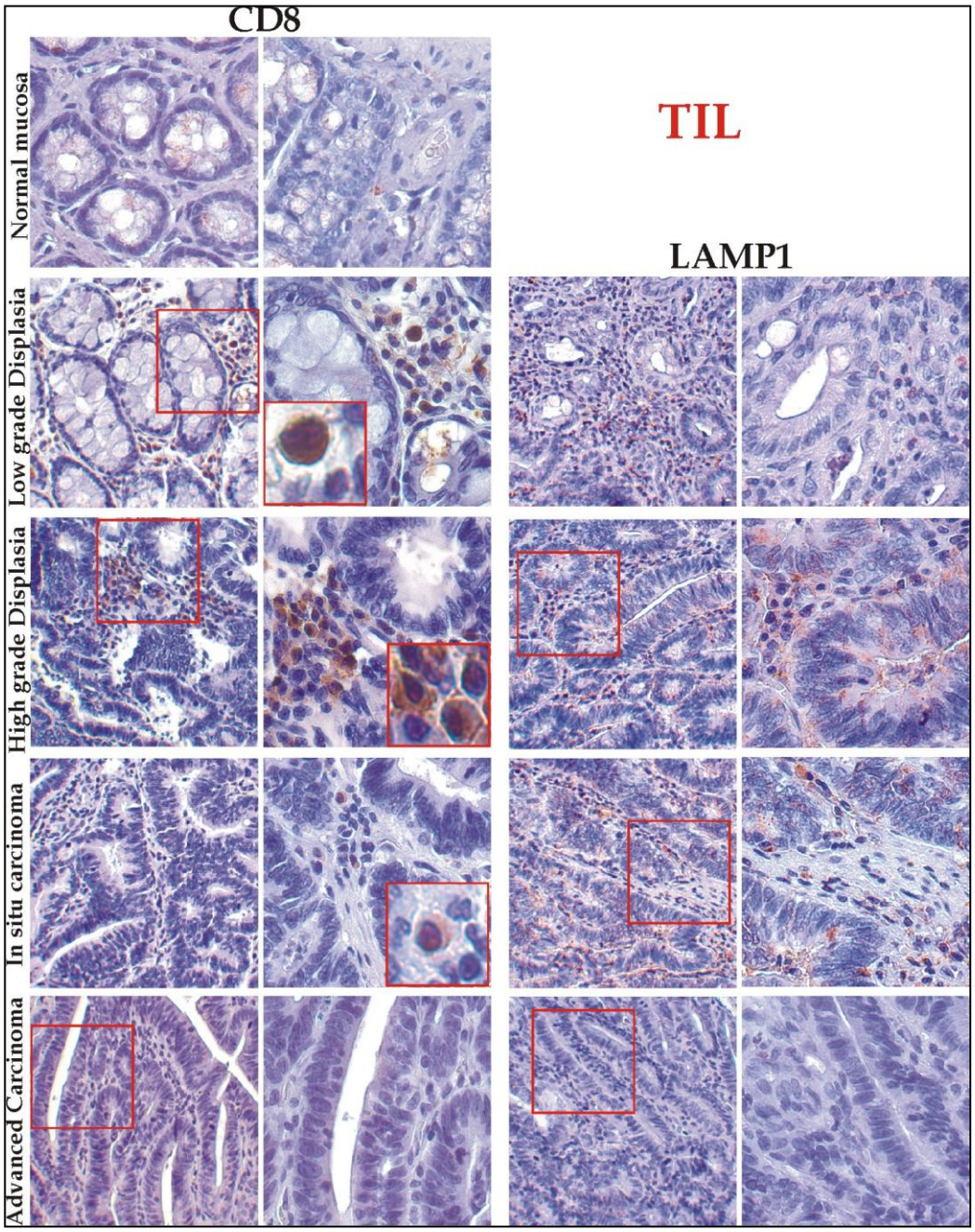

Figure 16. Immunohistochemical analysis of TIL infiltrates during rat colorectal carcinogenesis process.

4. *Proteomic analysis.*

All the tissues obtained in the first carcinogenesis experiment (20-30 weeks), have been microdissected to obtained nearly 25.000 pure cancer cells, 25.000 pure stroma surrounding cancer cells and 25.000 pure normal cells for each one. All the microdissected samples and the corresponding total tissues have been lysated; post-translational modifications and associated downstream proteins were quantitatively measured using RPPA technology. We analyzed the phosphorylation state of nearly 80 kinases substrates and the expression of nearly 20 total protein involved in cell growth and survival, apoptosis and invasion process. The specificity of all the used antibodies was confirmed by Western Blot experiments. We included in the protein panel, some end points of the Wnt/β-catenin pathways. The results of these experiments were reported by hierarchical clustering. In hierarchical clustering, the data are partitioned into clusters in a series of steps which combine the data into successively broader groupings. At each step, the distance between each cluster is calculated, and two clusters that are closest together are combined. This combining continues until all the points are in one final cluster. Two-way clustering method, clusters the data by both rows and columns. It is represented by a two dimensional diagram called "heat map" which illustrates the fusions or divisions made at each stage of analysis. The colour of the heat map plots the value of the data for each variable on a graduated colour scale: higher levels of protein phosphorylation are indicated in red, lowest relative levels in green and unchanged relative levels in black.

In the first heat map were included all the microdissected samples and all the relative total tissues, related to all the protein end point (**Figure 17**).

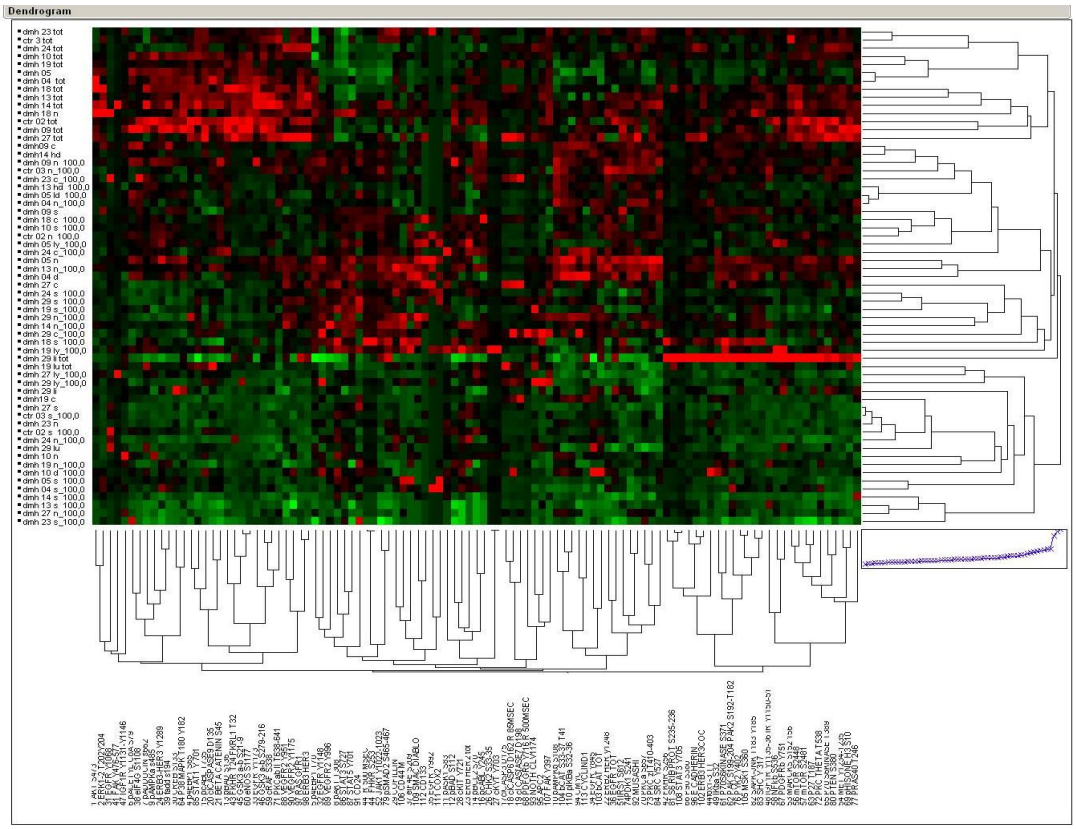

Figure 17. Each *row* (*horizontal axis*) represents a sample; each *column* (*vertical axis*) represents a cell signalling protein end point. In this heat map are reported all the samples and all the protein end point.

Unsupervised hierarchical clustering analysis revealed the presence of two major groups with whole tissue lysates clustered distinctly from the microdissected samples. This observation, indicate that microdissection of heterogeneous neoplastic tissues, it's essential for proteomics analysis; moreover there is an indication that LCM should be always used to better understand the signaling pathways driving the cancer progression in the context of tissue microenvironment.

In the second heat map, were included only the microdissected samples for all the neoplastic lesions obtained (excluding normal and stroma cells) related to all the protein end point (**Figure 18**).

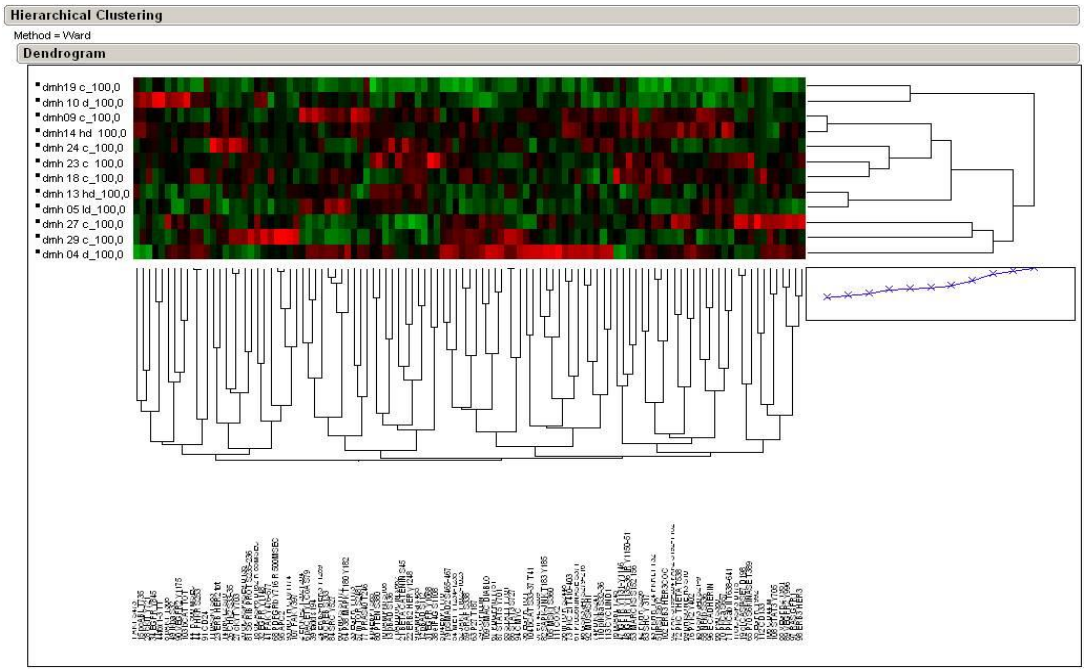

Figure 18. Each *row* (*horizontal axis*) represents a sample; each *column* (*vertical axis*) represents a cell signalling protein end point. In this heat map are reported all the samples and all the protein end point.

In this case, the unsupervised hierarchical clustering analysis didn't reveal the presence of interesting cluster. Probably it was caused from the relative small number on animals for each stage.

For the reason, after statistical analysis of the relative expression of all the samples, we did a hierarchical clusterization including just the microdissected cancer cells related to statistical significant protein end point. In particular, the statistically significant modifications as it concerns the activation state of the following end points: phospho-Acetyl CoA S79, phospho-SRC Y527, phospho-CREB S133, phospho-eNOS S1177, phospho-p38 T180/Y182, phospho-BCL2 S70, phospho-cABL Y245, phospho-P70 T389, phospho-GSKαβ S21/9, phospho-MARCKS S152/156 (**Figure 19**)

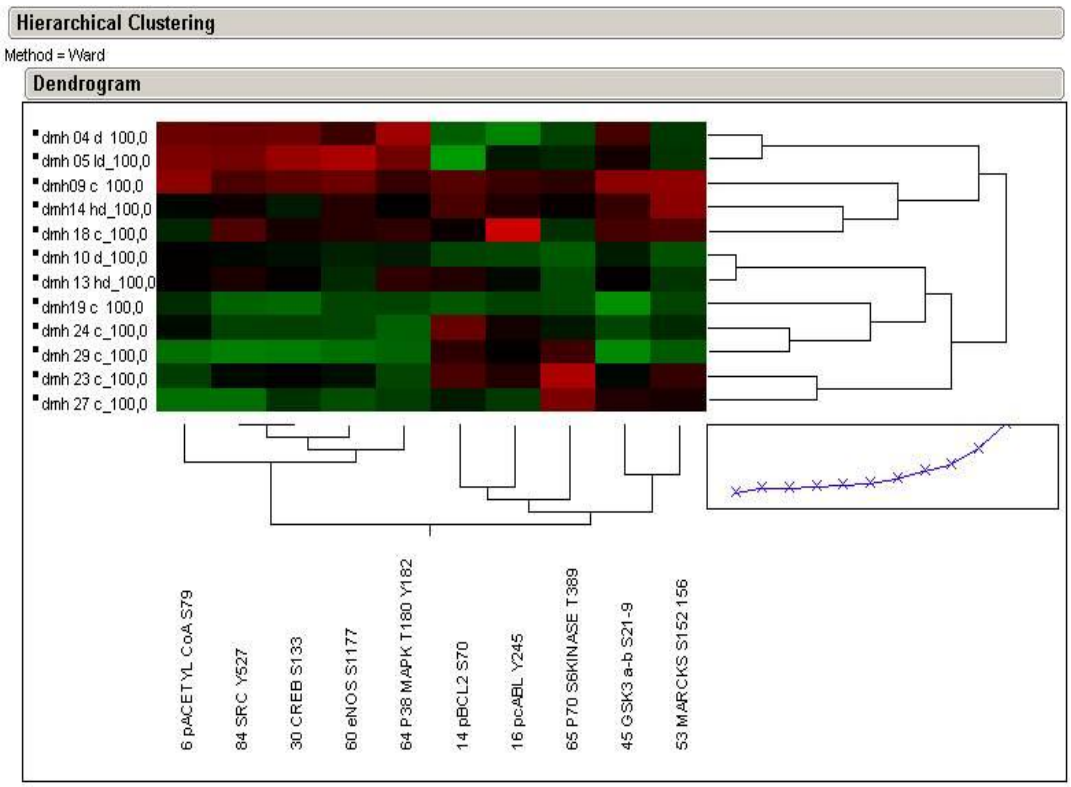

Figure19: Each *row* (*horizontal axis*) represents a sample; each *column* (*vertical axis*) represents a cell signalling protein end point. In this heat map are reported just the microdissected cells statistical significance protein end point.

The unsupervised hierarchical clustering analysis, revealed the presence of two clusters separating the early lesions from the late lesions, presupposing that the pathway activation state is characteristic in the different step of carcinogenetic process.

Confirmatory proteomic studies are in ongoing, focusing on the crucial steps of the carcinogenetic process, also including early times, to analyze the connection between the inflammatory disease and cancer. The number of animals for each timing has been enlarged, to obtain the most end point proteins with statistically significance; in this manner the hierarchical clustering analysis could show the presence of interesting groups of lesions related to the activation of specific time-dependent pathways.

Discussion

1. *Rationale of the use of pre-clinical models of colorectal cancer.*

The basis of colorectal cancer has been determined from clinical and pre-clinical studies. These revealed an abundance of pre-neoplastic and/or precancerous lesions. This is very important because these lesions in human colon can be found and removed quite easily. Unfortunately, the mortality related to CRC is still high due to the lack of relevant symptoms and to appropriate screening. CRC, thus, is escapable by interfering with the process of carcinogenesis that begins with an uncontrolled growth in the initiated cryptal cells, continues with the formation of an adenomatous polyp with or without dysplasia and, eventually, evolves into epithelial malignancy. The use of pre-clinical animal models in which carcinogens induce long-term changes in the intestinal environment leading to cancer insurgence and progression became very significant because it could be possible to develop early therapeutic intervention, essential for a favourable prognosis.

2. *Validation of pre-clinical animal model of colorectal cancer induced in BDIX rats.*

Cancer is the consequence of intra- and extracellular signalling network deregulation that derives from alteration in genetic (mutations in oncogenes, loss of tumour suppressor genes and abnormalities in genes involved in DNA repair) and proteomic cellular homeostasis. While genes and RNA encode information about cellular status, proteins are considered the engine of the cellular machine, as they are the effective elements that drive cellular functions such as proliferation, migration, differentiation, and apoptosis. Proteins have the ability to operate not as single molecule, but as an elaborate network

system regulating all cellular functions. The network of information inside cells is regulated by internal feedback processes that maintain cellular homeostasis. As a consequence, the study of the cellular expression, localization, signalling, and derangement of these proteins is a fundamental aspect for understanding and characterizing cellular activities in a variety of normal and/or disease processes.

Currently, it is well known the involvement of Wnt/β-catenin pathway in human colorectal carcinogenesis; in most of cases its deregulation is a key point for the tumour transformation and progression. The preclinical animal model of colorectal cancer in BDIX rats set up in this work, developed all the carcinogenesis steps observed in human: inflammatory bowel disease (IBD), early and late adenoma (LD and HD), carcinoma in situ (IS) and advanced carcinoma (K). The immunohistochemical study of modification of various proteins implicated in the Wnt/β-catenin pathway (β-catenin, E-cadherin, APC, GSK3β, C-myc and Cyclin-D1) showed a very similar expression and localization between human and animal tissues. Moreover, the oncogenic protein K-ras also contributes to tumour progression during the transition from moderate to late adenomas in both animal and human diseases: this pathway seems to be involved in the carcinogenesis process later than the Wnt/β-catenin pathway, as showed in our experiments. The results obtained in these study, showing similarities in the analysed molecular events during human and rat colorectal cancer insurgence and progression, firstly confirmed the validity of our pre-clinical model for studying the CRC carcinogenetic process in its early and late aspects, and for developing new preventive or therapeutic strategies targeting in particular the main actors of the Wnt signalling. Moreover, because of the immune-competence of animals used for setting our experimental model (BDIX rats), this model could be useful for studying the relationships between tumour and the host immune-response as well as the mechanisms by which cancer cells escape immune-surveillance.

3. *New technologies for developing innovative preventive or therapeutic strategies against colorectal cancer: the phospho-proteomic approach.*

Exploiting new technologies can represent a basis for the progress of novel therapeutic agents that intervene at different steps during the carcinogenesis process. In this work, we utilized a innovative proteomic approach, to study simultaneously the activation of several pathways involved in cell cycle, cell survival or apoptosis, proliferation and invasion in each different stage of carcinogenesis. To understand the activation state of different pathways and explain the functional cellular defects, is fundamental study some post-translational modifications, specifically phosphorylation. In fact, the intracellular balance is carefully conserved by constant rearrangements of proteins through the activity of a series of kinases and phosphatases. Therefore, knowledge of these key cellular signalling aspects will reveal information regarding the cellular processes driving a tumour's growth and response to treatment. A new phospho-proteomic technology, like Reverse Phase Protein Microarrays (RPPAs), can elucidate the working state of cellular signal pathways. One of the most common problems involved in the proteomic analysis of tissue samples arises from the tissue's heterogeneous nature and the need for a pure cell population for studies. Laser Capture Microdissection (LCM) was developed to provide a means of pure cells populations from heterogeneous tissue sections while maintaining the basic integrity of the RNA, DNA and proteins within the cells. For this reason, in our work all the tissues obtained in the experiments were processed by LCM and subsequently analyzed by RPPA associate to immunostaining of nearly 80 kinase's substrate proteins. The obtained results are very promising because they showed a clusterization between early and late stages of tumoural lesions; moreover some significant end point were individuated changing in a time depending manner: phospho-Acetyl CoA S79, phospho-SRC Y527,

phospho-CREB S133, phospho-eNOS S1177, phospho-p38 T180/Y182, phospho-BCL2 S70, phospho-cABL Y245, phospho-P70 T389, phospho-GSKαβ S21/9, phospho-MARCKS S152/156 showed a different activation state in the diverse carcinogenesis steps.

RPPAs provide the opportunity to generate a map of known cell signaling network or pathways for an individual patient. This protein network map aids in identifying critical nodes that may serve as drug target for individualized or combinatorial therapy. The studies of changes in specific posttranslational modifications such as phosphorylation, are now being used as translational research analyses to tailor therapeutic modalities for individualized or combinatorial therapy.

4. *The host immune-response to cancer: intervention of regulatory T cells (T-reg) and Tumour-Infiltrating T-Lymphocytes (TIL) during colorectal cancer development in the BDIX rat model.*

In general the malignant tumour such as CRC can escape the host immune-response also if the organism has an immune system able to react against the malignant cell or the whole tumour, like proposed in the immune-surveillance theory by Burnet in 1950. In effect, several observations indicate that human or experimental animal tumour are surrounded by infiltrates composed by T-lymphocyte, NK cells and macrophages. In melanoma for example, the presence of infiltrate is a favourable prognosis index. Unfortunately, in the most of the cases the host immune response is not able to prevent or destroy the tumour for several reasons. Genetic and epigenetic changes involved in carcinogenesis generate antigens that are recognized by T-lymphocytes but, unfortunately, tumour cells are very poorly immunogenic. Moreover, the escape mechanisms may result from loss of antigen or antigen presentation (by decreasing the

expression of MHC-I molecules) as well as from active biosynthesis of immunosuppressive molecules. These factors include TGF-β, VEGF, IL-8 and IL-10 which are known to cause significant inhibition of both innate and adaptive mechanisms of tumour immunity. The lymphocytes infiltration seems largely inefficient also for the poor expression of chemokines and vascular adhesion molecules in the malignant lesions.

The cellular type most representative in the tumour infiltrate is the T-lymphocyte. These cells can be classified functionally and phenotypically in two subsets. TIL (Tumour-Infiltrating T-Lymphocytes) are characterized by the membrane expression of CD8 and LAMP1 when activated; they are the true "effectors" of immune response, because they have the ability to destroy the recognized abnormal cell. Regulatory T cells (T-reg) are instead characterized by the membrane expression of CD4 and CD25, FoxP3 when activated. In a normal state T-reg have a lot of important functions in order to control and/or inhibit the immune response when it isn't necessary. The mechanisms involved in the balance between the two population are very complex, but they are essential to organize a correct and efficient immune response. The developing neoplasm can exploit and modify these mechanisms to own advantage. In effect, the lymphoid cells present in tumour stroma appear to be related more to the mechanisms of inhibition than to the activation of tumour immunity, suggesting a major role of T-reg (ineffective against the tumour formation) than TIL. T-reg can inhibit TIL activation and function *via* "T-T inhibition" or inhibition of antigen presenting cells; moreover a chronic exposure to high levels of antigen, such as the persistent tumour antigens, drives TIL to a state of non-responsiveness termed "exhaustion".

Human colorectal cancer have high numbers of peripherally circulating and tumoural T-lymphocytes. During the tumour progression T-reg are correlated in inverse proportion

with the presence of TIL; i.e. TIL are more prominent in the early stages and decrease in the advanced stages of tumour: this suggest that TIL have an anti-tumour activity as judged by their favourable effect on patients' survival, but they are not able to eradicate a formed tumour. T-reg became most important just in the advanced stages: this is an indirect evidence that T-reg are involved in the immune-pathogenesis of cancer.

In this work, we studied the balance between two classes of T-lymphocytes involved in immune responses in the set up animal model. We observed that the presence and distribution of T-reg and TIL around the neoplastic lesions, reflect very well the equilibrium changes correlating to timing carcinogenesis observed in human CRC.

The obtained results indicate that our preclinical animal model is also suitable for immune-response studies, in particular to set up new protocols of tumour immunotherapy. The tumour immunotherapy aims to improve the immune-response against the cancer or to supply to patients effective molecules or cells. This can be obtained "actively" through the vaccination with tumour cells or antigens or through the injection of tumour cells genetically modified (to express a high level of antigens or cytokines inducing modulation of T-lymphocytes). Immunotherapy can be realized "passively" by the injection of tumour-specific antibodies, immunotoxins (antibodies conjugated with toxic substances) and isolated, activated T or NK cell populations.

5. Conclusive remarks.

In this study we confirmed the validity of the pre-clinical model of colorectal cancer induced in immune-competent BDIX rats for studying the CRC carcinogenetic process in its early and late aspects, also using innovative proteomic approaches, and for developing new preventive or therapeutic strategies in a context of individualized medicine. Moreover, because of the immune-competence of animals used for setting our pre-clinical model (BDIX rats), this experimental system is also very useful for studying the relationships between tumour and the host immune-response as well as the mechanisms by which cancer cells escape immune-surveillance.

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