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# HSP60 IS THE MOST PREDICTIVE HEAT SHOCK PROTEIN DURING LARGE BOWEL CARCINOGENESIS

SSD BIO/16 Anatomia Umana

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# INTRODUCTION

# 1. ANATOMY OF THE LARGE INTESTINE

## 1.1 GENERAL FEATURES

The large intestine extends from the ileocaecal valve to the anus and is approximately 1.5 m long in adults, although there is considerable variation in its length. In the abdominal cavity it forms a border around the loops of small intestine that are located centrally within the abdomen. The large intestine begins in the right iliac fossa as the caecum which becomes the ascending colon that passes upwards in the right lumbar region and hypochondrium to the inferior face of the liver where it bends to the left constituting the hepatic flexure (right colic flexure) and becomes the transverse colon. This part of intestine across the abdomen until it reaches the left hypochondrium, where it curves inferiorly to form the splenic flexure (left colic flexure) and becomes the descending colon that proceeds through the left lumbar and iliac regions to constitute the sigmoid colon in the left iliac fossa. The sigmoid colon descends deep into the pelvis and becomes the rectum which ends in the anal canal at the level of the pelvic floor (Fig 1). The wall of the large intestine is relatively thinner than the small intestine. Along its entire length, on the outer surface, the large intestine shows sacculations (haustrations), which may partly be due to the presence of the taeniae coli that are three longitudinal bands of longitudinal muscle layer (1).

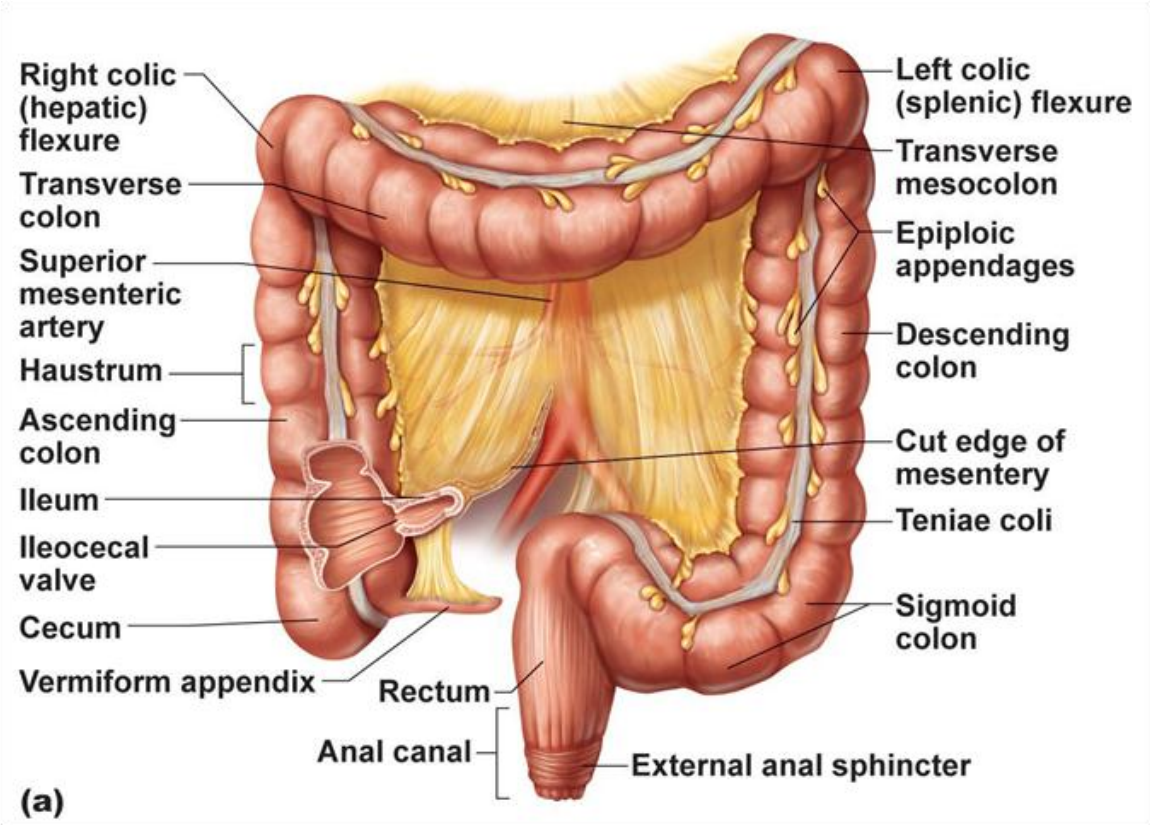


**Fig 1** Schematic vision of large bowel.
http://apbrwww5.apsu.edu/thompsonj/Anatomy%20&%20Physiology/2020/2020%20Exam%20Reviews/Exam%203/colon%20diagram.jpg

## 1.2 MICROSCOPIC FEATURES

In analogy to other portions of the alimentary canal, the intestinal wall is constituted by four tunics that are distinguished from the inside outwards in tunica mucosa, tunica submucosa, muscularis and tunica serosa or adventitia at the points where it is not covered by the peritoneum (Fig. 2). The mucosa is pale, smooth, and, in the colon, raised into numerous crescent-shaped folds between the sacculi (in the rectum it is thicker, darker, more vascular). It is constituted from a lining epithelium, from the underlying lamina propria and muscularis mucosae.

The normal homeostasis of the colonic mucosa requires a balance between proliferative processes at the base of the crypt glandular (crypt is the lower portion of the gland) and apoptotic processes in the most superficial part of the gland. The apoptosis is a fundamental process for the physiological turnover of senescent cells. Several studies have shown that a reduction of the apoptotic processes can increase the risk of cancer of the colon and rectum (2).

The cell types that constitute the glandular epithelium of the colon are (1):

1. **Columnar (absorptive) cells**: are the most numerous of the epithelial cell types. They are responsible for ion exchange, water resorption and other transepithelial transport. They all bear apical microvilli, which are shorter and less regular than those on enterocytes of the small intestine. These cells have typical junctional complexes around their apices, which limit extracellular diffusion from the lumen across the intestine wall.
2. **The goblet cells**: maintain secretory granules in the apical portion of the cytoplasm, the secretion of these cells is predominantly composed of mucin, it is also rich in antibodies of the IgA type.
3. **Microfold (M) cells**: as in the small intestine, are cells with long microvilli located in the epithelium overlying the lymphoid follicles. Their function is related to the transport and presentation of antigens.
4. **Stem cells:** they give rise to all the above mentioned types of epithelial cells, are found in the bottom of the intestinal glands and periodically undergo mitotic divisions. These cells are long-lived, able to self-renew and to differentiate into different cell types. Their mechanisms of proliferation and differentiation are

regulated by the so-called "niche" stem cell which consists of a complex stromal microenvironment that surrounds the stem cells themselves, and is constituted by the extracellular matrix and by different cell types, (neural cells, lymphocytes, macrophages, endothelial cells, fibroblasts and myofibroblasts). Each of these cell types regulates the activity of stem cells through the production of growth factors and cytokines.

5. **Neuroendocrine cells:** sit at the base of the glands and release their secretions in the vessels of the lamina propria.

The lamina propria of the tunica mucosa is composed of fibrillar and reticular connective tissue that provides support to the epithelium. In its interior it has capillaries lymphocytes, plasma cells, eosinophils and macrophages.

The **muscularis mucosa** is composed of two thin layers of smooth muscle, one circular and one longitudinal. It separates the tunica mucosa from the submucosa.

The **submucosa** consists of dense connective tissue containing several cell types such as lymphocytes, plasma cells, macrophages, blood vessels and lymphatics. Along its inner edge, the submucosa contains a network of nerve fibers that form the submucosal plexus of Meissner which contains sensory neurons, parasympathetic ganglia and postganglionic sympathetic fibers. The submucosa is raised to form the axis of the circular folds together with the mucous membrane and allows the implementation of the considerable variations in size, in the transverse and longitudinal directions, which accompany the peristaltic movements of the gut tube, giving at the same time an adequate support, elasticity and resistance.

The **muscle layer** is constituted by two layers of smooth muscle: an external longitudinal and internal circular one. The longitudinal bundles do not form a continuous layer, but thicken to form longitudinal strips, known as taeniae coli.

Between the two layers of smooth muscle is located the myenteric plexus or Auerbach's plexus, consisting of parasympathetic ganglia and post-ganglionic fibers synapse, which is fundamental for the coordination of the movements of the intestine. In the rectum the

taeniae disappear. The circular muscle bundles form a thin layer in the cecum and colon, and rectum form a thick layer in the anal canal and give rise to the internal anal sphincter consists of smooth muscle involuntary; the external anal sphincter is constituted by a ring of striated muscle that contracts and relaxes under the control of the will.

The tunica serosa consists of visceral peritoneum in intraperitoneal tracts while consists of adventitia on retroperitoneal tracts of intestine. Along the colon the peritoneum forms small fat-filled appendices epiploicae which are most numerous on the sigmoid and transverse colon but generally absent from the rectum. A connective tissue attaches the peritoneum to the muscularis externa. The adventitia is dense reticular connective.

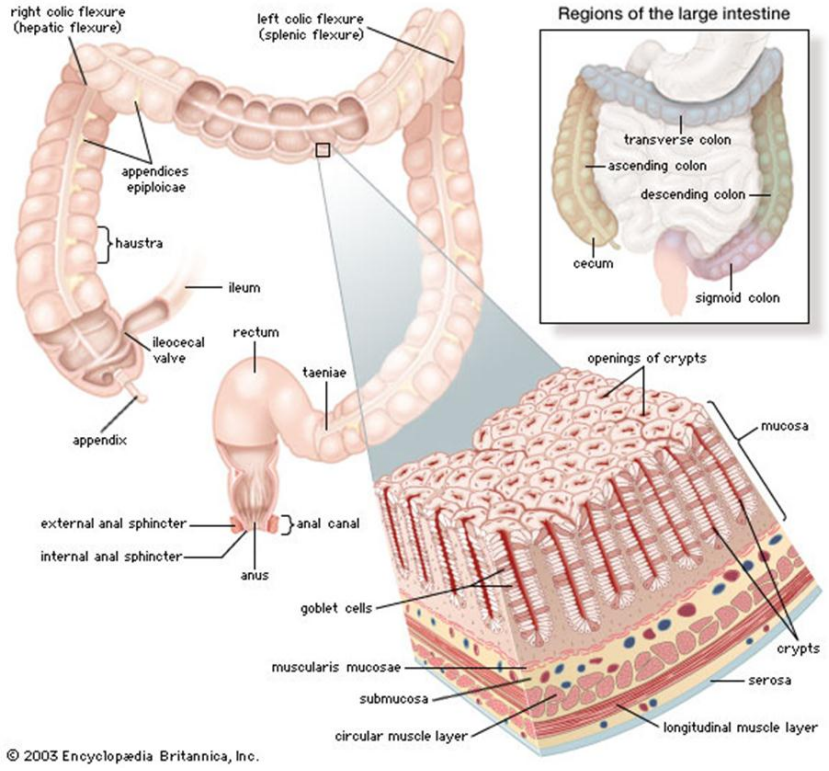


**Fig 2** Schematic vision of the wall of large bowel. http://media-2.web.britannica.com/eb-media/19/74319-004-68DBB5D6.jpg

## 1.3 MACROSCOPIC ASPECTS OF THE INDIVIDUAL INTESTINAL TRACTS

### Cecum

The cecum is the first portion of the large intestine, long on average 6 cm wide and about 7.5 cm, it has the shape of a bag to the bottom end, and is placed in the right iliac fossa, in continuity, at the proximal with the terminal ileum and distal to the ascending colon. From the posteromedial surface of the cecum, approximately 2-3 cm orifice ileocecal originates vermiform appendix, a tubular diverticulum of variable length from 2 to 15cm.

The cecum is located in the lodge cecal and is bordered posteriorly by the fascia of the psoas muscle that is covered by the lumbo-iliac fascia and the parietal peritoneum, by the right ureter, the external iliac vessels, the gonadal vessels and nerves genito and femoral-cutaneous covered by parietal peritoneum; postero-laterally by the fascia of the iliacus muscle, medially by the latest loops of small bowel; anteriorly by the anterior abdominal wall. The cecum is usually entirely covered by peritoneum but sometimes in its postero-superior portion cannot be covered by peritoneum and lie directly on the connective fascia iliaca (1).

The cecum is vascularized by the anterior and posterior caecal branch of ileocolic artery that arises from the right side of the superior mesenteric artery. Venous blood from the wall of the caecum drains into mesenteric arcades and subsequently into segmental veins that converge into the superior mesenteric vein a branch of portal vein.

### Colon

The colon is the middle part of the large intestine, is on average 1.30 meters long and is divided into the ascending colon, transverse colon, descending colon and sigmoid colon. Its diameter ranges from about 5 cm in ascending colon to about 3 cm in sigmoid colon. The ascending colon is about 15 cm long, starts at the upper edge of the cecum to ascend along the right side of the rear wall of the abdominal cavity until the lower surface of the right lobe of the liver on which leaves an imprint and, then turns abruptly forwards and to the left, at the hepatic flexure (2,3). It is a retroperitoneal structure covered anteriorly and on both sides by peritoneum. Its posterior surface is separated by loose connective tissue from the iliac fascia, the iliolumbar ligament, quadratus

lumborum, the aponeurosis of transversus abdominis, and the anterior peri-renal fascia inferolateral to the right kidney. Anteriorly it is in contact with loops of ileum, the omentum and, partly, the anterior abdominal wall. The transverse colon is approximately 50 cm long, and extends from the hepatic flexure in the right lumbar region across into the left hypochondriac region, where it curves posteroinferiorly as the splenic flexure. Its location and its length are highly variable. The transverse colon is intraperitoneal and is suspended from the anterior border of the body of the pancreas by the transverse mesocolon which is attached from the inferior part of the right kidney, across the second part of the duodenum and pancreas, to the inferior pole of the left kidney. The transverse colon superiorly adjoins with the liver and gallbladder, with the greater curvature of the stomach and with the rear end of the spleen; inferiorly adjoins with the small intestine, anteriorly with the greater omentum and posteriorly with the descending part of the duodenum-jejunum and some loops of the jejunum and ileum. The descending colon is approximately 25 cm long. It descends through the left hypochondrium and lumbar region following the lateral border of the lower pole of the left kidney, then descending in the angle between psoas major and quadratus lumborum to the iliac crest and, curves inferomedially to become the sigmoid colon. The descending colon is smaller in caliber, and is retroperitoneal though sometimes is covered posteriorly by peritoneum.

The sigmoid colon begins below the pelvic inlet and ends at the rectum. Characteristically it forms a mobile loop which normally lies in the lesser pelvis, but its length (about 40 cm) and form are the most variable of the total colon. It is usually completely invested in peritoneum. The sigmoid colon initially descends over the iliac crest into the pelvis and remains in contact with the peritoneum overlying iliacus muscle, and finally curve backward and reaches the median plane at the level of the third sacral vertebra and then fold down to terminate in the rectum. However its position is extremely variable.

The arterial vascularization of the colon is quite variable but all involved arteries are branches arising by superior and inferior mesenteric arteries. In particular the ascending colon and the first portion of transverse colon are vascularized by the right colon artery which anastomoses with the ileo-colic artery and the middle colic artery, a branch of superior mesenteric artery; the proximal two thirds of the transverse colon are vascularized by middle colic artery, while the distal third by colic artery of the left, a

branch of inferior mesenteric artery; the descending colon is perfused by the left colic branch of the inferior mesenteric artery which anastomoses with the sigmoid arteries; the sigmoid colon is perfused by the sigmoid arteries that anastomose with the superior rectal artery, the last branch of the inferior mesenteric artery (1) (Fig.3).

**Rectum**

The rectum is 15 cm long, follows the sigmoid colon from the level of the third sacral vertebra and ends at the upper end of the anal canal. The rectum is divided in pelvic tract and perineal tract. The pelvic tract, in its descent from the third sacral vertebra, follows the concavity of the sacrum and coccyx; its upper part has the same diameter of the sigmoid colon, while its lower part is dilated to form the rectal ampulla. The rectum crosses the pelvic floor muscle membrane of the perineum. The rectum pelvic, in its upper portion is covered by the peritoneum on the anterior and lateral faces, while in its middle portion is covered by peritoneum only on the front face (1). The rectum differs from the sigmoid colon in having no sacculations or appendices epiploicae. The taeniae blend approximately 5 cm above the rectosigmoid junction, forming two wide muscular bands which descend anteriorly and posteriorly in the rectal wall. The rectum anteriorly is related with the loops of the sigmoid colon and sometimes with the loops of the ileum, as well as the base of the bladder (in males) or the neck and body of the uterus (in females); inferiorly it is related with the bladder and, in males, with seminal vesicles, deferens duct, terminal tract of ureters and prostatic gland, while, in females, the rectum is related with the inferior tract of the vagina canal (1).

The upper two-thirds of the rectum are vascularized by superior rectal artery, branch terminal of inferior mesenteric artery; the middle third is supplied by the middle rectal arteries, branches of the internal iliac artery, or from the inferior vesical artery (vaginal artery in females); the distal third is vascularized by ascending branches of the inferior rectal arteries, terminal branches of the internal pudendal arteries. The veins are arranged in a network anastomotic, the hemorrhoidal venous plexus, which superiorly is tributary of the portal venous system through the hemorrhoidal branch of the inferior mesenteric vein, while inferiorly is a tributary of the internal iliac vein through the middle and inferior hemorrhoidal veins. In particular, the veins form the venous plexus which are distinguished in the internal hemorrhoid plexus or submucosal plexus and external hemorrhoidal plexus outside the muscularis tunic. Therefore, at rectal level, there is an anastomosis between the portal venous system and the systemic venous

system (Fig.3). The rectum is continuous with the anal canal at a point where its muscle layer merges with the voluntary muscle (striated) of the pelvic floor, giving rise to the anal sphincter.

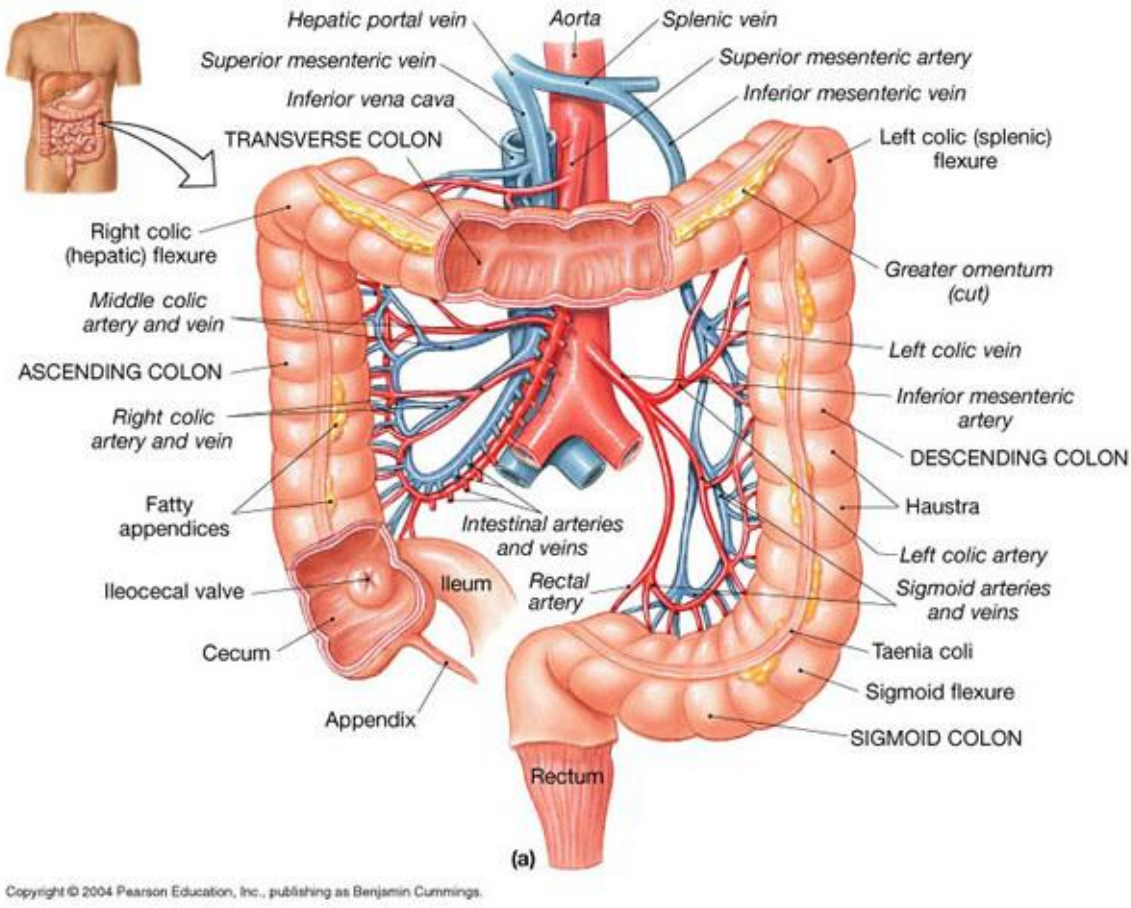


**Fig 3**: Vascularization of large bowel.

http://www.as.miami.edu/chemistry/2086/chap%2024/Chapter%2024-newPART2_files/image017.jpg

# 2. LARGE BOWEL CARCINOMA

## 2.1 EPIDEMIOLOGY AND ETIOLOGY

Colorectal cancer is a malignancy of great relevance social and health care. This neoplasia is common in Northwest Europe, North America, and other Anglo-Saxon areas. Its incidence is instead low in Africa, Asia, and some regions of South America (5). Globally, Colorectal cancer is the third most commonly diagnosed cancer in males and the second in females, with over 1.2 million new cases and 608,700 deaths estimated each year. In the United States, it is 12% of malignant tumors with 150000 new cases each year and, approximately 50,830 deaths. In Italy, the carcinoma of the colon and rectum is the second cause of death from malignancy in males (after lung cancer) and in women (after breast cancer), with approximately 35,000 new cases a year with 16,000 deaths. It is more frequent in males (52.2/100000) than in females (37.5 / 100,000). The mean age of incidence is 62 years. Only 1 to 2% of cases occur before age 40. The causes and pathogenesis of colorectal carcinoma are related to both environmental and genetic factors (6) Diet is implicated in the process of carcinogenesis of colorectal cancer. The reduced consumption of vegetables, and fibers and the increased consumption of fats and animal proteins are risk factors for the disease. The dietary factors are related to their influence on the intestinal microflora and on the chemical composition of the nutrients (7-9). Among the environmental factors will also recognize occupational factors (10). The genetic factors are related to the presence of familial syndrome such as Familial Polyposis, Gardner Syndrome, and Lynch Syndrome. Moreover, the presence of two or more first-degree relatives with colorectal cancer, in a family increases threefold the risk of development of this tumor (11).

## 2.2 EPITHELIAL POLYPS AND CARCINOGENESIS

Most tumors of the colon-rectum are derived from malignant transformation of polyps. This lesion is a clinical terms of any circumscribed tumor or growth that project above the surrounding mucosa. Polyps may be hamatomatous, hyperplastic, or adenomatous in nature. The hamatomatous polyps have no malignant potential. The hyperplastc polyps are benign epithelial proliferations and, are not considered to be premalignant. The adenomatous polyps, instead, are premalignant lesions. Adenomas are very common in the large intestine and are of clinical importance because they may develop into cancer. The incidence of adenomas parallels the incidence of large bowel carcinoma. Grossly, adenomas may be sessile or pedunculated and single or multiple. Microscopically the epithelium is adenomatous with an increase in the number of glands and cells compared to the normal mucosa. By definition, adenomas consist of dysplastic epithelium. The cells are enlarged, elongated, with hyper-chromatic nuclei that are stratified within the cell. Mucus production is significantly reduced or absent. Mitotic figures are prominent. Adenomas are traditionally divided into three types: tubular, tubule-villous, and villous according to the growth pattern. Tubular adenomas show gland and tubular structures that are separated from each other by normal lamina propria. In the villous adenomas there are leaf-like projections lined by dysplastic glandular epithelium comprise more than 80% of the luminal surface. These villi are covered by adenomatous epithelium. The tubulovillous adenomas show histological aspects of both tubular and villous adenomas. The dysplasia of adenomas may be mild, moderate and severe according to the architectural and cytological aspects.

The carcinogenetic model (proposed by Volgestein and his colleagues in the 1988) (12) of large bowel is an example of multistep process in which the transformation of normal large bowel mucosa in invasive tumor involves the accumulation of different genetic alterations. In fact the process of malignant transformation of epithelial tissue has been made possible by the demonstration that the morphologic progression from normal epithelium to hyper-proliferative lesions, and then to adenomatous lesions, and finally to carcinoma, is accompanied and caused by a series of molecular alterations (Fig.4). These molecular alterations include genetic mutations that active oncogenes and, instead, inactive tumor-suppressor genes. The most important of these genes are APC, mismatch repair genes, p53, K-ras, and DCC (13,14).

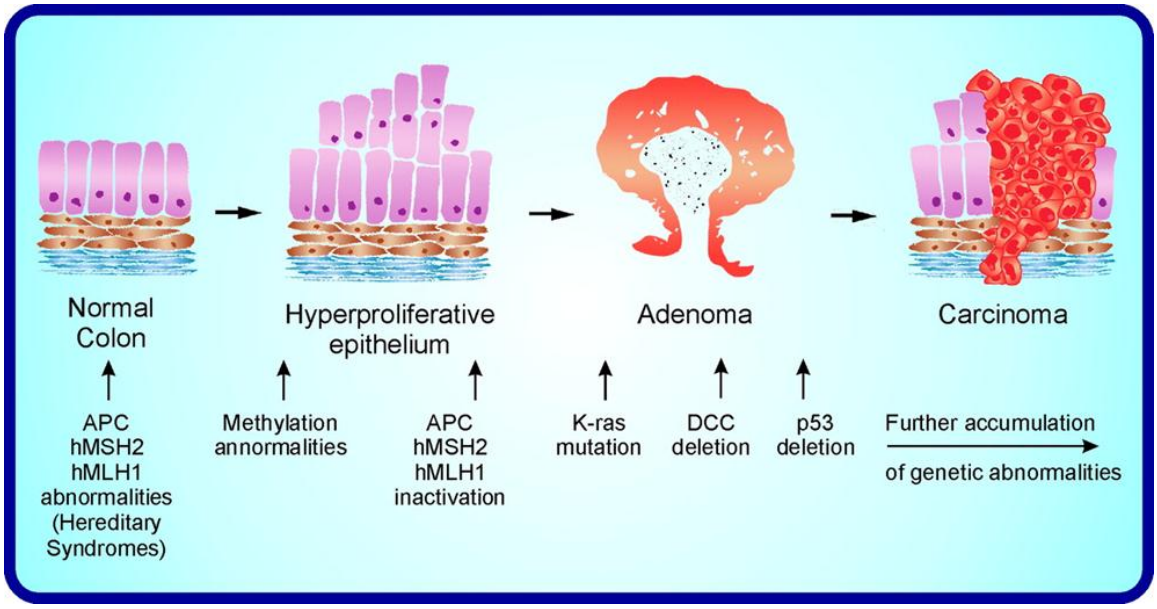


**Fig.4** Schematic representation of morphologic and molecular events in colorectal tumorigenesis. The loss of APC gene (located on chromosome 5) would be responsible for an increased cell proliferation (hyper-proliferative epithelium) and subsequent mutation of ras oncogene (located on chromosome 12), involved in the regulation of cell growth, would be responsible for the formation of a dysplastic polyp (adenoma); the loss of DCC gene (Deleted in Colon Cancer, located on chromosome 18) and the mutation of p53 gene (located on chromosome 17) confer to the cells a selective advantage of growth and invasive and metastatic ability.
http://4.bp.blogspot.com/RqRsi5m3E_s/UQJks7jG_BI/AAAAAAAAAm8/JDN3cyESfIc/s400/F1.large.jpg

## 2.3 MORPHOLOGICAL ASPECTS OF LARGE BOWEL CARCINOMA

Approximately 50% of all carcinomas occur in the recto sigmoid area, 30% occur in the cecum and ascending colon tract and 10% in the transverse colon. There is evidence of changing distribution in recent years, with an increasing proportion of more proximal carcinomas (15).

The macroscopic features are influenced by the phase in the natural history of tumors at the time of discovery. Grossly, most colorectal cancer are either polypoid or exophytic with predominantly intraluminal growth, ulcerative/infiltrating with predominantly intramural growth, diffusely infiltrative/linitis plastica with subtle endophytic growth, and annular with circumferential involvement of the colorectal wall and constriction of the lumen. Generally, polipoid cancers have a better prognosis than ulcerative cancers. Overlap among these types is common (16)

The World Health Organization classifies colorectal cancers in adenocarcinoma, mucinous adenocarcinoma, signet ring cell carcinoma, small-cell carcinoma, adenosquamous carcinoma, squamous cell carcinoma, medullary carcinoma, undifferentiated carcinoma (16). Adenocarcinoma is the most frequent histological type and, is gland-forming, with variability in the size and configuration of the glandular structures. This tumor can be gradated predominantly, on the basis of the extent of glandular appearances, and should be divided into well, moderately and poorly differentiated (Grading G). Poorly differentiated adenocarcinomas should show at least some gland formation or mucus production. The percentage of the tumour showing formation of gland-like structures can be used to define the grade. Well differentiated (G1) lesions exhibit glandular structures in > 95% of the tumour; moderately differentiated (G2) lesion has 50-95% glands; poorly differentiated (G3) adenocarcinoma has 5-50%; and undifferentiated (G4) carcinoma has < 5% (15) The tumor cells represent a combination of columnar and goblet cells. The carcinoma consistently elicits an inflammatory and desmoplastic reaction, which is particularly prominent at the border of tumor. Mucinous adenocarcinoma shows large lakes of extracellular mucin (> of 50% of the tumor mass). Signet-ring cells carcinoma is rare in large bowel and has a diffuse infiltration of cells with intracellular accumulation of mucin. Squamous carcinoma show squamous differentiation. The adenosquamous

carcinoma shows features of both squamous carcinoma and adenocarcinoma. Medullary carcinoma is characterized by sheets of malignant cells with abundant pink cytoplasm exhibiting prominent infiltration by intraepithelial lymphocytes. The undifferentiated carcinoma lack morphological evidence of differentiation and have variable histological features. In its growth, colorectal carcinoma extends through the muscularis propria into pericolic or perirectal soft tissue, involving contiguous structures. The two staging systems most commonly used by pathologists are the one proposed by the Dukes, modified by Asler and Coller, and TNM system. In both is considered the degree of invasion of intestinal wall, the degree of lymphatic node involvement and, the degree of metastasis.

## 3. HEAT SHOCK PROTEIN

Molecular chaperones, many of which are Heat Shock Proteins (HSP), are an important class of molecules essential for cell life and survival. Hsps were first discovered as the products of genes induced by heat shock (17), but the term Hsp is used nowadays with great flexibility to indicate the products of genes that are induced by a number of chemical, physical and biological stressor (18). Both terms Hsp and chaperones are used often as synonyms but not all HSPs are chaperones and not all chaperones are Hsps. Hsps are part of the chaperoning system, a physiological system composed of Hsp-chaperones, co-chaperones and co-factors of an organism (19). This physiological system is essential for the control of protein homeostasis and maintenance of correct and functional conformation of proteins in all fluids, cells, and tissues (19), and also, is important in some other processes not of necessity, related to protein homeostasis (20). Many Hsps have a chaperoning activity. As per the classic concept, the canonical function of Hsps is to assist nascent polypeptides in their correct folding and, when the protein is not properly folded or is defective, to assist protein refolding or degradation (21) (Fig5).

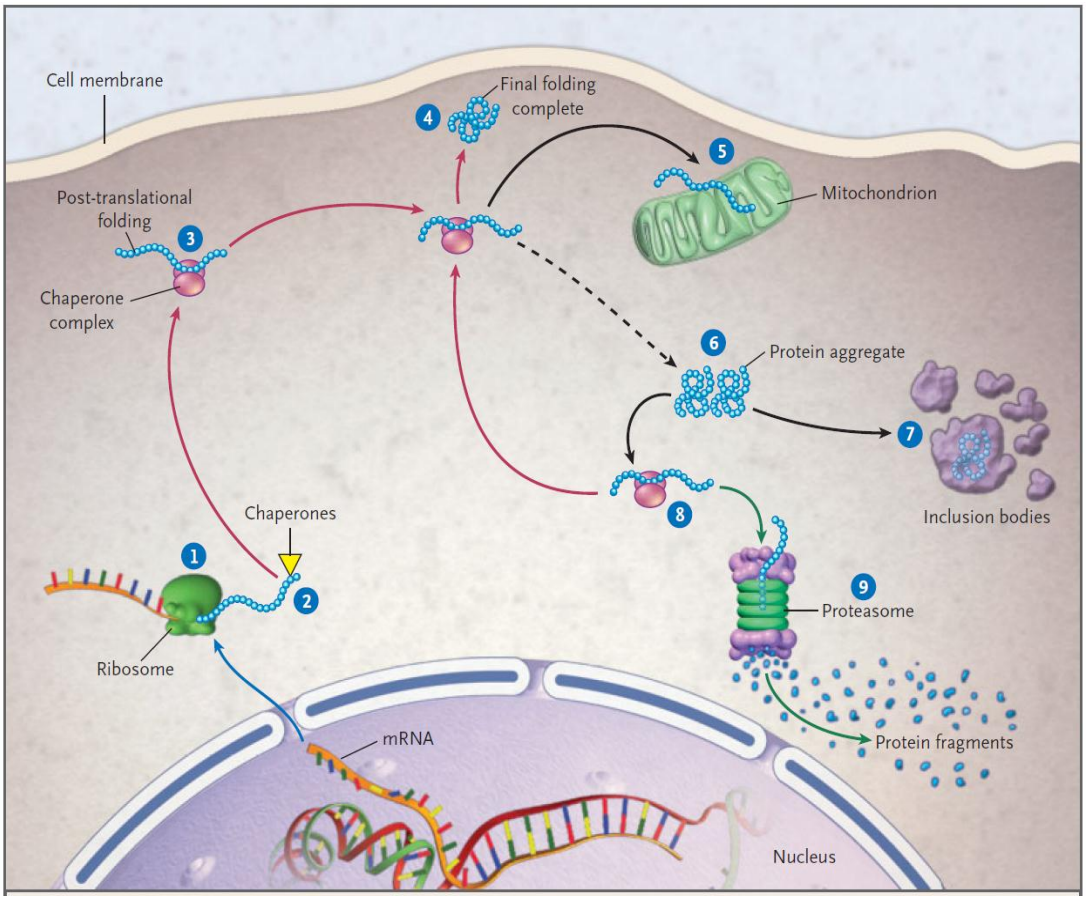


**Fig.5** Chaperoning functions of Hsps. From Macario and Conway de Macario, New Eng. J. Med.2005

Hsps have also other important "extra-chaperoning" functions such as participation in immune system regulation (20,23) cell differentiation (24), gene expression and DNA replication (25) programmed cell death (26,27), cellular senescence (20,28) and carcinogenesis ( 29,30). Chaperones usually do not work alone, but they form a team of molecules that works together. An example is the chaperonin complex of Hsp60 that is formed by identical subunits and co-works with Hsp10 and others small Hsps. Also Hsp70 form a team of co-worker molecules by interacting with Hsp40 and with a co-factor NEF (31) Hsps (including Hsp60, Hsp10, Hsp70 and Hsp90) are constitutively expressed in cells, and for many years were considered typical intracellular. In fact they have an intracellular and pericellular localization being present in the mitochondria, in the cytosol, in cell membrane, in the nucleus, in the cell surface, and, also, have an extracellular localization being present in fluids such as blood, lymph and cerebrospinal fluids (19). Hsps may be released outside cells through microvesicles (such as exosomes) that are important for the intercellular cross-talk (32,33). Hsps are classified in various ways. These proteins were initially classified according to molecular weight expressed in KiloDaltond (kDa) (Table 1), but the expanding number of Hsps has made it necessary to revise the nomenclature (34). However the nomenclature based on molecular weight is the most used.

| **Chaperone family** | **Molecular weight (kDa)** | **Main function(s)** |
|---|---|---|
| Heavy | 100 or higher | Prevents protein aggregation, helps protein folding. |
| Hsp90 | 81-99 | Protein folding, cytoprotection; intracellular signaling (e.g., steroid receptor); cell-cycle control. |
| Hsp70 | 65-80 | Prevents protein aggregation, protein folding, cytoprotection and anti-apoaptotic function. |
| Hsp60 | 55-65 | Prevention of protein aggregation, protein folding, cytoprotection, macrophage activator possibly through Toll-like receptor. |
| CCT | 55-63 | Protein folding. |
| Hsp40 | 35-54 | Protein folding and refolding together with Hsp70/Hsc70. |
| Small Hsp | 34 or less | Anti-apoptotic function; cytoprotection. |
| Hsp10 | 10 | Protein folding together with Hsp60; modulation of immune system. |

**Table 1** Classification of Hsps according to their molecular mass. From Rappa F et al. ANTICANCER RESEARCH 32: 5139-5150 (2012).

Hsps are implicated in the pathogenesis of a range of tumors. In fact, these proteins are involved in various metabolic mechanisms of neoplastic cells, such as proliferation,

invasiveness, induction of neoangiogenesis and metastasization. Hsps are involved in vital mechanisms of malignant cells and their elevated levels can protect cancerous cells against apoptosis. Regard to the mechanisms of apoptosis, many Hsps participates in both intrinsic and extrinsic pathways interfering with molecules essential in the apoptotic process. Hsp27 and Hsp70 interfere with apoptosis through inhibition of aptosome formation in intrinsic pathway (35,36), while Hsp90 has a role in modulating the function of TNF receptor-1, a molecular receptor involved in the extrinsic pathway of apoptosis (37).Moreover, Hsp60 favors the cell proliferation through its ability to survive to apoptotic stimuli (38); Hsp90 is associated with many of the key signaling molecules that mediate autonomous growth in cancer (39); Hsp27 and Hsp70 mediate endothelial cell mobility and favors tumor-invasiveness (40); Hsp90 influences tumor neoangiogenesis stabilizing vascular endothelial growth factor in endothelial cells (41), Hsp27 overexpression plays an important role in metastasis in colorectal carcinoma (42) and Hsp60 has a role in metastasis in head and neck squamous cell carcinoma (43). Moreover Hsps are expressed at higher levels in a wide range of cancers, compared to the normal tissutal counterpart, and they are closely associated with a poor prognosis and resistance to therapy. Hsps levels change during the carcinogenetic steps in various organs. For example, higher expression of Hsp60 was found in the adenoma-carcinoma sequence of the large bowel (44,45), in the dysplasia-carcinoma sequence of the uterine cervix (46) and during the carcinogenesis of prostate (47).

# 4. CHAPERONOPATHIES

Chaperonopathies are pathological conditions associated with the impairment of one or more molecular chaperones. In this case the molecular chaperone malfunction is an etiological-pathogenic factor (21). The Chaperonopathies are classified etiologically as genetic or acquired and pathogenically as by defect, excess, or mistake. In genetic forms, the defective function of a chaperone is due to a gene mutation (21,48) Examples of genetic chaperonopathies are neurovegetative disease, congenital malformation, dilated cardiomyopathies, and endoplasmic reticulum pathologies (21). In acquired forms, the malfunction of a chaperone may be due to pathological post-translational modifications, such as oxidation, glycosylation and acetylation, causing alteration of function (21). Among the acquired chaperonopathies there are diseases associated with aging and with dysfunction of immune system (20). Usually, genetic chaperonopathies have an early onset, while acquired forms have an elderly onset. In the chaperonopathies classified into by defect, excess or mistake, the alteration is in the quantity of the chaperone and in its level and type of its function (48). In particular, the chaperonopathies by mistake or collaborationism are an important group of diseases in which a molecular chaperones may be normal, but participates in a pathological mechanisms that favors disease development, rather than defend the organism from the disease. Among these chaperonopathies, there are autoimmune disease and some types of tumors. For examples, in some autoimmune diseases (such as inflammatory bowel disease), the molecular chaperone may act as immunogenic and antibodies may generate against it. These antibodies would recognize the chaperone, now acting as antigen, and will react with it. In some cases this antigen-antibody interaction can stimulate an exaggerated immune response. A chaperone can also bind a receptor localized on the surface of immune cells (such as neutrophil and macrophage) and thereby triggers production of cytokines and starts inflammation (49). Finally, in some types of tumors, molecular chaperones can favors the survival of malignant cells in various ways, thus collaborating with the neoplastic process. The roles of chaperones in cancer are different depending on the type of tumor and vary going from supporting tumor growth and metastasis to participating in antitumor mechanism by promoting tumor-cell apoptosis (50).

# 5. EXOSOMES: A WAY FOR INTERCELLULAR COMUNICATION

The intercellular communication is due to several mechanisms: cells can communicate with each other through soluble molecules that are in the extracellular environment (such as hormones and cytokines), through adhesion molecules that are localized on cellular surface (51), and through membrane vesicles released by one cell and captured by another. This mechanism of cellular communication is alternative and important because the extracellular vesicles can contain a large number of molecules that may transfer from one cell to another cell. The extracellular vesicles are constituted by a lipid bilayer that contains within different free proteins, as well as RNA and microRNA. Through different molecules present in their lumen, the vesicles could simultaneously activate different cellular pathways and can travel long distance before delivering their cargo between different cells within the organism (52). Based on their composition, size and mechanisms of release, extracellular vesicles have been named exosomes, apoptotic bodies, microvesicles, etc. Exosomes are a category of extracellular vesicles that have received most attention from researchers in this field. They have been implicated in different cellular functions and pathological conditions. Over the years they have been observed to be secreted into the extracellular medium from a large number of cell types, including T cells, B cells, dendritic cells, platelets, epithelial cells and cancer cells (53, 54). Exosomes are 30-200 nm wide membranous vesicles first discovered in 1984 by Pan and Johnstone in maturing mammalian reticolocytes as a mechanism for removal of plasma membrane proteins (55). The secretion of exosomes is carried out in two steps divided as endocytosis and exocytosis. Initially, the exosome is formed inside the cell, in which a tract of plasma membrane bends inward. During this process, the proteins of membrane are incorporating into the internalized membrane, while cytosolic molecules are enclosed into the vesicles. In a second moment the vesicles formed by internalization of membrane, are enclosed into multivesicular endosomes (MVEs) that are vesicle larger and, after, fuse again with the plasma membrane and released into extracellular space as exosomes (56) (Fig.6).

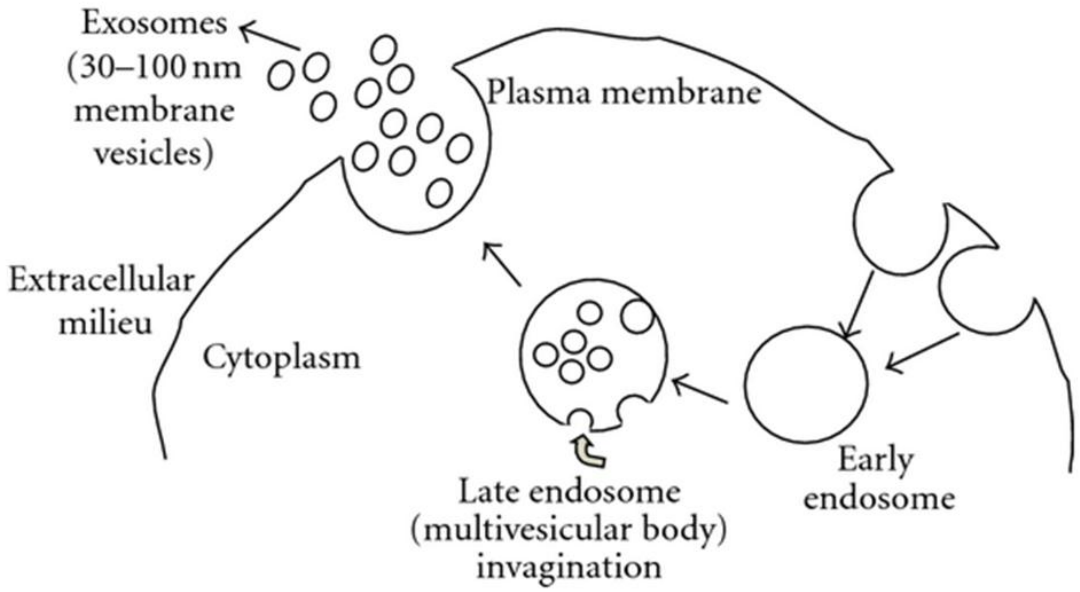


**Fig.6** Exosome biogenesis: a schematic view of the secretion of exosomes. The secretion of exosomes includes one step that consists in internalization of a portion of plasma membrane and formation of the multivesicular bodies (MVBs) The second step consists in the release of exosomes upon the fusion of MVBs with the plasma membrane. http://www.hindawi.com/journals/ijr/2012/573528.fig.001a.jpg

Lipid rafts or cholesterol and sphingolipid microdomains, enriched in plasma membranes, are shown to play an important role in the formation of exosomes *(*57,58). These components give greater stability to the exosomes, which are collected from physiological fluids such as normal urine, plasma, synovial fluid (59, 60) Exosomes also contain common components such as chaperone (Hsc70), cytoskeletal proteins (such as actin and tubulin), endosomal sorting complexes required for transport (ESCRT) proteins (such as Alix). Depending on the cell types from which they are derived, exosomes play a role in diverse physiologic and pathological processes serving as a novel form of cell-cell communication. For example, different types of immune cells (such as antigen presenting cells, APCs, B cells and dendritic cells) secret exoxomes in which are present MCH I and II and stimulatory factors that induce the antigen-specific immune response. Tumor cells also produce exosomes, evidently abundant in culture and malignant effusions (61). Exosomes derived by tumor cells show anti-tumorigenic proprieties such as inducing tumor cell apoptosis (62) and promoting anti-tumor immunity (63) Many studies have, also, demonstrated that exosomes could play an important role in cancer progression (64) Cancer cell derided exosomes are related to the secretion of growth factors into tumor microenvironment.

The exosomes can carry factors that stimulate the proliferation of tumor cells, can favor the onset of metastasis (65) and can improve tumor angiogenesis (66) (Fig.7).

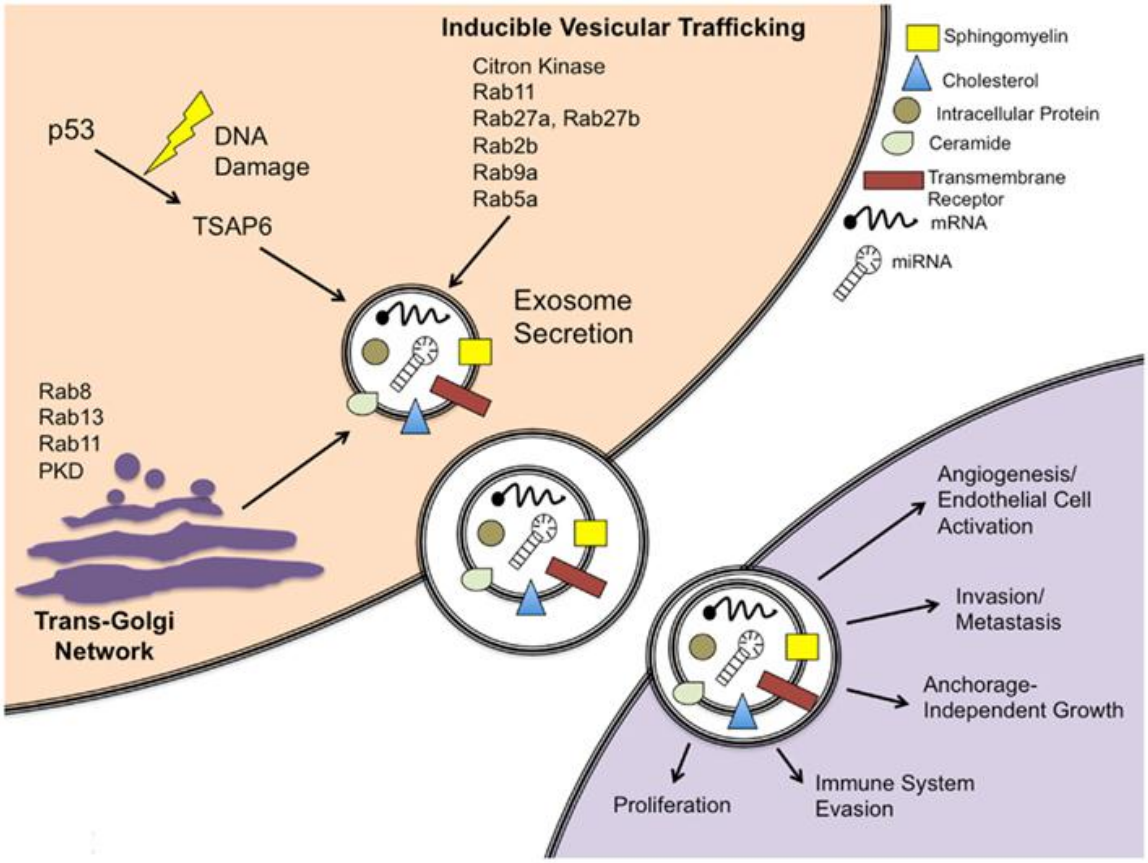


**Fig.7** A schematic picture that show the various functions in the intercellular cross-talk of exosomes. http://c431376.r76.cf2.rackcdn.com/17287/fonc-02-00038-HTML/image_m/fonc-02-00038-g001.jpg

Exosomes derived by cancer cells can contain biomarker that could be important for the diagnosis or for the detection of pathological status. A study carried out on exosomes isolated from plasma derived by patients with ovarian cancer has demonstrate that these vesicles contain a protein that can be used, with other screening methods, for the detection of this cancer (67). Among the proteins that can be carried by exosomes there are Hsps. Hsp60 is present in the exosomes released from human tumor cell (54) and from cardiac myocytes in both basal state and following mild stress (68). Moreover, another study performed on exosomes derived from tumoral cells has shown that Hsp60 is integrated in the exoxome membrane (32). These data suggest that a new role for extracellular Hsp60 in the cross talk between tumor cells and the immune system; in fact Hsp60 in exosome membrane would serve as a ligand for receptors of immune cells. The presence of Hsp60 in exosomes could also suggest a role of this protein as a

biomarker in assessing tumor progression. The molecular composition of exosomes reflects the specialized functions of the original cells (69).

## AIMS

The aims of this PhD thesis have been to evaluate:

1) the levels and the expression of a number of HSPs, including Hsp60, in samples of human large bowel mucosa obtained from healthy controls (normal mucosa) and from patients with tubular adenomas with moderate grade dysplasia (preneoplastic lesion) and with adenocarcinoma with moderate grade differentiation (neoplastic lesion) by immunomorphology, WB and PCR;

2) the subcellular localization of Hsp60 in normal and tumoral samples by immunogold experiments;

3) the presence and the levels of circulating Hsp60 in the serum of patients with tumors before and after surgery, compared to controls, by ELISA;

4) the presence and the levels of exosomal Hsp60 in the plasma of patients with tumors before and after surgery, compared to controls, by western blotting and other techniques.

The first set of experiments (1) showed that Hsp60 is the most significant among the researched HSPs in terms of expression and levels, both in the epithelium and the lamina propria of pre-neoplastic and neoplastic lesions and this was why we continued our experiments (2,3,4) focusing only on this protein.

Our intention was to (dis)prove the utility of Hsps, in particular Hsp60, in diagnostics, assessing the prognosis and follow-up in patients affected by colorectal neoplastic lesions. Moreover, our results should shed light on the usefulness of Hsp60 as target for a novel anti-cancer approach (chaperonotherapy) given its putative involvement in colorectal carcinogenesis.

# EXPERIMENTAL PROCEDURES

## 1. Specimen collection

Formalin-fixed paraffin embedded biopsies of tubular adenomas with moderate grade of dysplasia (n°20) were retrospectively collected from files of the Surgical Anatomy Unit of the Civico Hospital, Palermo, Italy, for histological and immunomorphological analyses.

Tissue biopsies from 40 large bowel mucosa of healthy controls (n°20) and adenocarcinomas with a moderate grade of differentiation (n°20) were collected prospectively for histological and immunomorphological analyses, western blotting and PCR analysis. The samples for histological and immunomorphological analyses were fixed in formalin and included in paraffin, while the samples for Western Blotting and PCR analyses were kept frozen at -80°C until use.

Tissue samples from 10 colon adenocarcinomas (G2) were collected for the evaluation of sub-cellular/sub-tissutal localization of HSP60 (by immunogold) with transmission electron microscopy (TEM).

Two blood samples from 20 patients with colon adenocarcinomas (G2) were also taken, the first one day prior to, and the second one week after, the surgical removal of the tumor; at the same time we collected blood samples from 20 healthy age-matched controls. Plasma was obtained from these samples to perform, respectively, ELISA test and exosome isolation.

All these biopsies were obtained from the DICHIRONS Department of the University of Palermo, Italy. All the procedures were in accordance with the ethical standards of the responsible committees (institutional and national) on human experimentation and with the Helsinki Declaration of 1975 (as revised in 2008). Written informed consents were obtained from all patients at the time of biopsy and blood sampling, and the study was approved by Ethics Committee of the Polyclinic Hospital of the University of Palermo, Italy.

## 2. Histological analysis

For histological analysis, the samples were fixed in formalin and embedded in paraffin. From all paraffin blocks sections with a thickness of 4–5 mm were obtained with a cutting microtome. These sections were dewaxed in xylene for 10 minutes and rehydrated by sequential immersion in a descending scale of alcohols and transition in water for five minutes. The sections were then stained with hematoxylin and eosin, mounted with coverslips and finally, observed with an optical microscope (Leica DM 5000 B) connected to a digital camera (Leica DC 300F).

## 3. Immunohistochemistry

Immunohistochemistry was performed on 5-micron thick sections of paraffin-embedded tissue, arranged with the microtome. The sections were dewaxed in xylene for 30' at 60°C and rehydrated, at room temperature, by sequential immersion in a graded series of alcohols and transferred into water for five minutes. Subsequently, the sections were immersed for 8' in Sodium Citrate Buffer (pH 6) at 95°C for antigen retrieval and, subsequently, immersed for 8' in aceton at -20°C to prevent the detachment of the sections from the slide. All subsequent reactions were conducted at room temperature. After a wash with PBS (Phosphate Buffered Saline pH7.4) for 5', the sections were treated for 5' with Peroxidase Quencing Solution (reagent A of Histostain®-Plus 3rd Gen IHC Detection Kit, Invitrogen) to inhibit any endogenous peroxidase activity. Another washing with PBS for 5' was carried out and the sections were treated with a blocking protein (reagent B of Histostain®-Plus 3rd Gen IHC Detection Kit, Invitrogen) for 10' to block non-specific antigenic sites. Subsequently, the sections were incubated overnight, with a primary antibody against human Hsp10 (Gene Tex polyclonal, dilution 1:300), human Hsp60 (mouse anti-Hsp60 monoclonal antibody, Sigma, St. Louis, MO, catalogue no. H4149, dilution 1:400), human Hsp70 (SANTA CRUZ BIOTECHNOLOGY, clone W27, dilution 1:200) and human Hsp90 (SANTA CRUZ BIOTECHNOLOGY, clone F-8, dilution 1:200). Appropriate positive and negative (isotype) controls, were run concurrently. After a wash with PBS (Phosphate Buffered Saline pH7.4) for 5', the sections were incubated with a universal biotinylated secondary antibody (Biotinylated Secondary Antibody reagent C Histostain®-Plus 3rd Gen IHC Detection Kit, Invitrogen) for 10'. After a subsequent washing with PBS for 5', the sections were incubated with streptavidin-peroxidase complex (Streptavidin-Peroxidase Conjugate reagent D Histostain®-Plus 3rd Gen IHC Detection Kit, Invitrogen) for 10', and following a further washing in PBS for 5', the slides were incubated in the dark for 5' with the DAB chromogen (diaminobenzidine) (DAB chromogen reagents E1 and E2 Histostain®-Plus 3rd Gen IHC Detection Kit, Invitrogen). Nuclear counterstaining was carried out using hematoxylin (Hematoxylin aqueosus formula, N. Cat. S2020, DAKO). Finally, the slides were prepared for observation with coverslips with an aqueous mounting solution. The observation of the sections was performed with an optical microscope (Leica DM 5000 B) connected to a digital camera (Leica DC 300F).

## 4. Western Blotting

50mg of tissue samples from normal mucosa and colon adenocarcinoma were incubated on ice in a RIPA lysis buffer (0.3M NaCl, 0.1% SDS, 25mm HEPES pH 7.5, 1.5mm MgCl2 , 0.2 mm EDTA, 1% Triton X-100, 0.5mm DTT, 0.5% sodium deoxycholate) containing proteases and phosphatase inhibitors (0.1 mg/ml phenylmethyl sulfonyl fluoride, 20 mg/ml aprotinin, 20 mg/ml leupeptin, 10 mg/ml NaF, 1 mM DTT, 1 mM sodium orthovanadate, 20 mM ß-glycerol phosphate) for 1 h. Subsequently, the samples were centrifuged at 13,000 rpm for 10' at 4°C and supernatants were isolated. Protein concentration was measured by the Bradford method. Proteins (40 mg) were separated on a 12% SDS-PAGE gel. The separated proteins were electrophoretically transferred to a nitrocellulose membrane (Hybond-C Extra, Amersham Biosciences, GE Healthcare, Little Chalfont, England (Nitrocell Paper, Bio-Rad). Equal protein loading was confirmed by Ponceau-S staining of the blotted membranes. After 1hr incubation at room temperature with a blocking solution, 5% milk (Target Retrieval Solution, DAKO) in TBS with 0.05% Tween 20 (Polyoxyethylene sorbitan monolaurate, Sigma-Aldrich) (T-TBS), the membranes were incubated with primary antibodies (rabbit anti-Hsp10 polyclonal antibody, Gene Tex; mouse anti-HSP60, LK1 clone, Sigma-Aldrich; mouse anti-Hsp70, W27 clone, SANTA CRUZ BIOTECHNOLOGY; mouse anti-Hsp90, F-8 clone, SANTA CRUZ BIOTECHNOLOGY) diluted 1:1000 in T-TBS containing 1% dry milk. Blots were washed in T-TBS and incubated for 1 h with HRP-conjugated secondary antibodies (ECLTM anti-mouse IgG HRP-conjugated whole antibody; ECLTM anti-rabbit IgG HRP-conjugated whole antibody, Amersham Biosciences) diluted 1:8000 in T-TBS containing 1% dry milk. The final detection procedure was carried out using the ECL Western Blotting Detection Reagent (Amersham Biosciences) according to the manufacturer's instructions. Band analysis was performed with the ImageJ 1.41 software (National Institutes of Health, USA, http://rsb.info.nih.gov/ij).

## 5. Total RNA extraction

Total RNA extraction from all tissue samples was performed using TRIzol® REAGENT (Sigma-Aldrich, USA), a mixture of guanidine thiocyanate and phenol in a mono-phase solution that dissolves RNA on homogenization of tissue sample. Tissue samples were then homogenized in TRI REAGENT (1ml for 50-100 mg of tissue) and subsequently centrifuged at 12000 x g for 10 minutes at 4°C to remove the insoluble material. 0,2 ml of chloroform was then added to the samples. After 15 minutes, the mixtures were centrifuged at 12000 x g for 15 minutes at 4°C, to separate the RNA (aqueous phase) from proteins and DNA. Subsequently, the aqueous phase was transferred to a fresh tube to which 0,5 ml of isopropanol was added. After 10 minutes, the samples were centrifuged at 12000 x g at 4°C. The supernatant was removed and the RNA pellet was added to 1 ml of 75% ethanol and then shaken. The mixture was centrifuged at 7500 x g for 5 minutes at 4°C. The RNA was eluted from the filter in 50μl of RNase-free H2O. The RNA extract obtained was stored at -20 ° C until use. The concentration of the RNA extracts was determined by spectrophotometer with a wavelength of 260nm. Only samples with an A260/A280 ratio of over 1.8-2 were considered usable for the following analyses.

## 6. RT-PCR (Reverse Transcription Polymerase Chain Reaction)

Qualitative RT-PCR was performed using ImProm –II Reverse Transcriptase (Promega) for the RNA retrotranscription, and GoTaq Flexi DNA Polymerase for the amplification of cDNA. For RNA retrotrascription, the reaction was performed according to the manufacturer's instructions. The RT reactions were cycled at 25°c for 5 minutes for annealing, at 50°C for 60 minutes for extension, and at 70° C for 15 minutes for inactivation of reverse trascriptase. PCR amplification was performed by adding specific primers for human Hsp60, Hsp10, Hsp70, Hsp90α and Hsp90β (for Hsp60 cDNA, the forward primer sequence was 5'-CGACGACCTGTCTCGCC- and the reverse primer sequence was 5'CTTTGGCATAAGCCCGAGTG-3'; for Hsp10 cDNA, the forward primes sequence was 5'CTCCCAGAATATGGAGGCACC-3' and the reverse primes sequence was 5'-TGGAATGGGCAGCATCATGT-3'; for Hsp70 cDNA, the forward primer sequence was 5'-AAAGATGGACCAACCACCCC-3' and the reverse primer sequence was 5'-CCTCCACTGCGTTCTTAGCA-3'; for Hsp90α cDNA, the forward primer sequence was 5'-GTCTAGTTGACCGTTCCGCA-3' and the reverse primer sequence was 5'-GAGGAGGCACCCTCAAGTTC-3'; for Hsp90β cDNA, the forward primer sequence was 5'-CTCTCTCGAGTCACTCCGGC-3' and the reverse primer sequence was 5'-GTACGTTCCTGAGGGTTGGG-3'). The reactions were cycled at 95°C for 2 minutes (1 cycle) for initial denaturation; at 95° C for 1 minute for denaturation, at 63-59,4 °C for 1 minute for annealing, at 72°C for 1 minute for extension (25 cycles), followed by a final extension at 72°C for 5 min (1 cycle). Finally, the PCR product was visualized on 1.5% agarose gel with the Syber stain (SYBER SafeTM DNA gel stain, Invitrogen).

## 7. ELISA Assay for Human Hsp60

ELISA was performed as previously described (70) using a commercial Hsp60 (human) enzyme immunoassay (EIA) kit (ADI EKS 600 ELISA kit from Enzo Life Sciences, Inc., Farmingdale, NY, USA). Blood samples from 20 patients with colon adenocarcinoma (G2) and 20 healthy age-matched controls were collected in EDTA-treated tubes. After a centrifugation at 2,000×g for 10 min, plasma was collected, aliquoted, and stored at −20 °C until use. A monoclonal mouse antibody for Hsp60 was pre-coated on the wells of anti-HSP60 Immunoassay plates. All reagents were used at 24°C and the standard Hsp60 was diluted in a sample diluent to perform a standard curve with six points, ranging from 3,125 to 100 ng/mL. Sample diluent alone was used as 0 (zero) standard. Primarily, 100µL of prepared standards and serum was added in duplicate to wells in incubation at 24°C for 1h. After washing six times with 1 x Wash buffer, 100µL of diluent anti-Hsp60 goat polyclonal antibody was added to each well and incubated at 24°C for 1h. After, 100µL of diluent horseradish peroxidase-conjugate anti-goat IgG was added to the plate and incubated at 24°C for 30 minutes. After washing, 100µL of 3,3′,5,5′-tetramethylbenzidine substrate was added and incubated for 15 min in the dark. Finally, 100 µL of Stop Solution was added and absorbance was measured at 450 nm in a microplate photometric reader (DV990BV4, GDV, Milan, Italy). Sample concentration was calculated by interpolating the sample concentrations in the standard curve. The sensitivity of the Hsp60 (human) EIA was determined to be 3.125 ng/mL. The intra-assay coefficient of variation of Hsp60 (human) EIA was determined to be <10% and the inter-assay coefficient of variation was also determined to be <10%.

## 8. Transmission electron microscopy (TEM).

### 8.1 LR-White inclusion

Samples of large bowel adenocarcinoma were fixed for 1 hour in 4% paraformaldehyde. Subsequently, the samples were washed in Millonig's buffer and stored in the buffer until they were processed. The samples were dehydrated with ascending methanol series (30, 50, 70, and 100%). Each step was repeated two times for 20 minutes. The samples were then treated with a solution of LR-white methanol two times for 30 minutes. Following this step, the samples were left in pure resin at 4°C overnight. Next day, the LR-WHITE resin was put into the accelerator and the specimens were subsequently put in embedding containers in LR-WHITE resin for 24 hours at 60°C.

### 8.2 Immunogold

Ultrathin sections, obtained by cutting the LR-WHITE resin blocks with an ultramicrotome, were mounted on gold grids to prepare them for the immunogold assay. The grids were washed for three times with distilled water, and incubated with hydrogen peroxide (0,3%) for 10 minutes. After washing twice in PBS, the grids were incubated in a serum blocking solution for 30 minutes and, without washing, the incubation was continued with a primary antibody diluted in a dilution buffer for 1 hour at room temperature. The primary antibody used was against Hsp60 and diluted at 1:15. Grids were rinsed three times with PBS for 5 min and incubated with a secondary antibody conjugated with 10nm colloidal gold particles for 30 minutes at room temperature. Subsequently, the grids were washed again for three times with PBS for 5 minutes and then fixed in a 2% glutaraldehyde solution in PBS for 10 minutes followed by three washings in distilled water for 5 minutes. The grids were then prepared for contrast staining by treating them with uranyl acetate (1%) for 5 minutes, followed by eight washings with methanol for 2 minutes, and treated with Reynolds' solution for 5 minutes and finally rinsed eight times in distilled water for 2 minutes. After this procedure, the grids were ready for electron microscopy (JEOL JEM 1220 TEM at 120kV).

## 9. Exosome isolation from plasma

Exosomes were isolated from plasma as previously described (71) with minor modifications. Blood samples of 20 patients (drawn one day prior to, and one week after surgical removal of the tumor) and 20 healthy controls were treated with EDTA and centrifuged at 4000 x g for 5 minutes and 11000 x g for 10 minutes and 20 minutes to separate the plasma. Samples were diluted with PBS and another centrifugation at 29,500 X g for 20 min was performed to pellet any remaining cells and debris. The supernatant was then filtered through a 0.2 µm filter (Millex GP, Millipore), followed by ultracentrifugation at 110,000 X g for 2 hrs to pellet the exosomes. The exosomes were then washed in cold PBS and resuspended in 50 µl of PBS.

## 9.1 Exosomes assessment procedure

To further ascertain the identity of the exosomes obtained from the plasma of the blood samples, exosomal preparations were examined by TEM and tested for markers considered characteristic of exosomes (Acetylcoline esterase activity, presence of Alix and Hsc70 proteins).

An assessment of exosome quality by transmission electron microscopy (TEM) was performed to ascertain the diameter of exosomal vesicles, which should be 100 nm or less (58,72). Pellets obtained by ultracentrifugation were resuspended in PBS with the addition of 100µl of freshly made fixative (2,5% glutaraldehyde in PBS) for 30 minutes. After fixation, the preparations were mounted on formvar nickel grids by layering grids over 10 ml drops of exosome preparations for 10 minutes at 24°C. Grid-mounted preparations were prepared for contrast staining by treating them with uranyl acetate (1%) for 5 minutes and with Reynolds' solution for 5 minutes and, finally, rinsing them eight times in distilled water for 2 minutes. After this procedure, the grids were ready for electron microscopy (JEOL JEM 1220 TEM at 120kV).

The assessment of exosome quality by acetylcoline esterase assay was performed to determine whether the vesicles purified showed Achetylcoline Esterase that is typically detected in exosomes (73). 15µl of the exosomal preparations were suspended in 100µl of PBS and incubated with acetylcoline (1,25mM) and 5,5'-dithio-bis-(2-nitrobenzoic) acid (0,1 mM) in a final volume of 1 ml. The incubation was performed in cuvettes at

37°C. The change in absorbance at 412 nm was monitored every 5 minutes for 30 minutes by a plate reader.

The assessment of exosome quality by identification of Alix and Hsc70 proteins was performed by Western Blotting analysis carried out on the exosomes purified by ultracentrifugation. For the Western Blotting analyses, 10µl (O 80µg) of exosome preparations were used, and the primary antibody used for Alix (mouse anti-Alix, 1A12 clone, SANTA CRUZ BIOTECHNOLOGY, INC, Santa Cruz, CA, USA, n° catalogue sc-53540) was diluted at 1:500 while the one for Hsc70 (mouse anti-Hsc70, B-6 clone, SANTA CRUZ BIOTECHNOLOGY, INC, Santa Cruz, CA, USA, n° catalogue sc-7298) was diluted at 1:500. The analysis was performed following the procedure described above (see paragraph n.4).

## 9.2 Western Blotting analysis of Hsp60 in Exosomes

10µl (O 80µg) of exosome preparations were used for western blotting analysis and a primary antibody for Hsp60 (mouse anti-HSP60, LK1 clone, Sigma-Aldrich) diluted at 1:1000 was used to detect the presence of Hsp60 in plasma exosomes. The analysis was performed following the procedure described above (see paragraph n.4).

## 10. Double immunofluorescence on adenocarcinoma tissue

Formalin fixed, paraffin-embedded sections of colorectal adenocarcinoma were run for the double immunofluorescence experiments. These experiments were two: the first was performed using primary antibodies anti-Hsp60 and anti-CD68, a marker for macrophages; the second was performed using primary antibody anti-Hsp60 and anti-CD34, a marker for endothelial cells. After dewaxing in xylene, rehydration in ethanol, and washing in phosphate-buffered solution (PBS), sections were incubated with unmasking solution (tri-sodium citrate 10 mM, 0.05%Tween 20) for 10 min at 58 °C and treated with blocking solution (3 % albumin bovine serum in PBS) for 30 min at 23 °C. Then, the sections were incubated with the first primary antibody (rabbit polyclonal antihuman HSP60, Clone H300, SANTA CRUZ BIOTECHNOLOGY, INC, Santa Cruz, CA, USA, catalogue n° sc-13966) diluted 1:100, overnight at 4 °C. The day after, the sections were incubated with the second primary antibody (For first experiment Mouse Monoclonal anti-CD68, clone KP1, SANTA CRUZ BIOTECHNOLOGY, INC, Santa Cruz, CA, USA, catalogue n° sc-20060 and for second experiment Mouse Monoclonal anti-CD34, clone B-6, SANTA CRUZ BIOTECHNOLOGY, INC, Santa Cruz, CA, USA, catalogue n° sc-74499) diluted 1:100, overnight at 4 °C. After washing twice in PBS all sections were incubated with fluorescent secondary antibodies such us rabbit IgG antibody conjugated with Texas Red (diluted 1:200; Gene Tex Inc, Irvine, USA) and mouse IgG antibody conjugated with fluorescein isothiocyanate (FITC; diluted 1:200; Sigma-Aldrich, Milan, Italy) respectively. The nuclei were counterstained with Hoechst (Sigma-Aldrich, Inc, Milan, Italy) for 15 min at 23 °C. Finally, all slides were mounted with cover slips using a drop of PBS, and readings and imaging were immediately performed with a Leica DM5000 upright fluorescence microscope (Leica Microsystems, Heidelberg, Germany). The cell positivity of both markers (“Merge”) was assessed through the program Adobe Photoshop CS5 version 12.0.4x64.

## 11. Statistical analyses

Statistical analyses were carried out using the GraphPad Prism 4.0 package (GraphPad Inc., San Diego, CA, USA). Standard statistical analyses were employed to calculate the means and the standard deviations (SD). One-way ANOVA (and nonparametric) analysis of variance was used to assess significant differences within the data. Differences between the means were considered significant when $P<0.001$ as indicated in the figures.

# RESULTS

## 1. Histological analysis

Histological analysis was carried to group the samples before further experiments. Three groups of samples were set up (1: normal mucosa; 2: tubular adenoma with moderate dysplasia; 3: adenocarcinoma with a moderate grade of differentiation) (Fig.8).

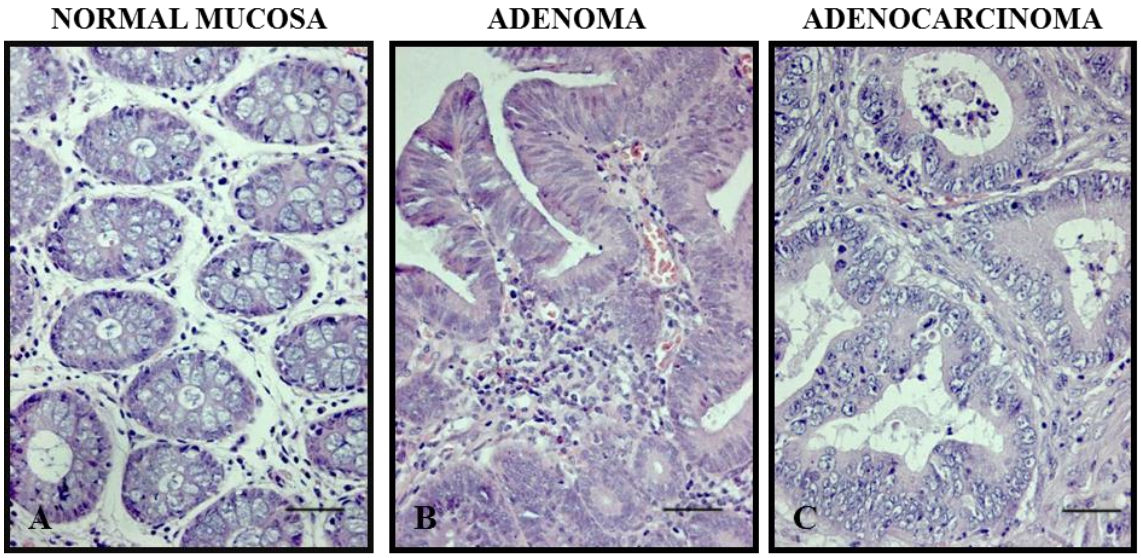


**Fig. 8** Representative pictures of hematoxylin and eosin staining A)Normal mucosa of colon; B) Adenoma with moderate dysplasia of colon; C) Adenocarcinoma with moderate grade of differentiation of colon. Magnification 200x. Scale bar: 100µm.

## 2. Immunohistochemical analysis

The immunohistochemical evaluation was carried out on all samples of normal mucosa, tubular adenoma and colon adenocarcinoma. Cellular expression of Hsp10 and Hsp90 was cytoplasmic and sometimes granular. Hsp60 immunopositivity was visible at cytoplasmic and membrane levels, while Hsp70 immunopositivity was visible at cytoplasmic and nuclear levels. The levels of expression of Hsps were investigated in the epithelium and lamina propria cells. The percentage of positive cells was calculated in 10 random high power fields (HPF) at a magnification of 400x, and was expressed as means (See Table 2). The immunohistochemical reactions show that Hsp60 and Hsp10 expression levels, both in the epithelium and lamina propria, increase gradually throughout the carcinogenic steps of large bowel cancer, from normal mucosa through tubular adenoma with moderate dysplasia to invasive adenocarcinoma (G2). Hsp70 immunopositivity levels increase significantly only in the epithelium of invasive adenocarcinoma, compared to normal and dysplastic mucosa. No change is detectable in the lamina propria. Finally, Hsp90 levels increase significantly only in the lamina propria cells of invasive adenocarcinoma, compared to normal and dysplastic mucosa, while the epithelium shows no changes (Fig.9). Data obtained from immunohistochemistry evaluations were plotted using Microsoft Excel software (Microsoft Italia, Milan, Italy). Statistical analyses were carried out using the GraphPad Prism 4.0 package (GraphPad Inc., San Diego, CA, USA). Standard statistical analyses were employed to calculate the means of percentage positivity and the standard deviations (SD) (Fig.10).

| **Immunostaining** | **NM** | | **AT** | | **AC** | |
|---|---|---|---|---|---|---|
| | **EC %** | **LPC %** | **EC %** | **LPC %** | **EC %** | **LPC %** |
| **Hsp10** | 48,5 | 2,1 | 67,5 | 6,2 | 90 | 10 |
| **Hsp60** | 6 | 1,9 | 65 | 5 | 95 | 10 |
| **Hsp70** | 30 | 3,5 | 30 | 3,5 | 65 | 5 |
| **Hsp90** | 70 | 30 | 75 | 25 | 78 | 40 |

**Table 2:** Results of immunohistochemical evaluations expressed as average percentage in Epithelial Cells (EC) and Lamina Propria Cells (LPC) in the groups of Normal Mucosa (NM), Tubular Adenoma (TA) and Adenocarcinoma (AC) of colon. The percentage of positive cells was calculated in 10 random high power fields (HPF) at a magnification of 400x, and was expressed as means.

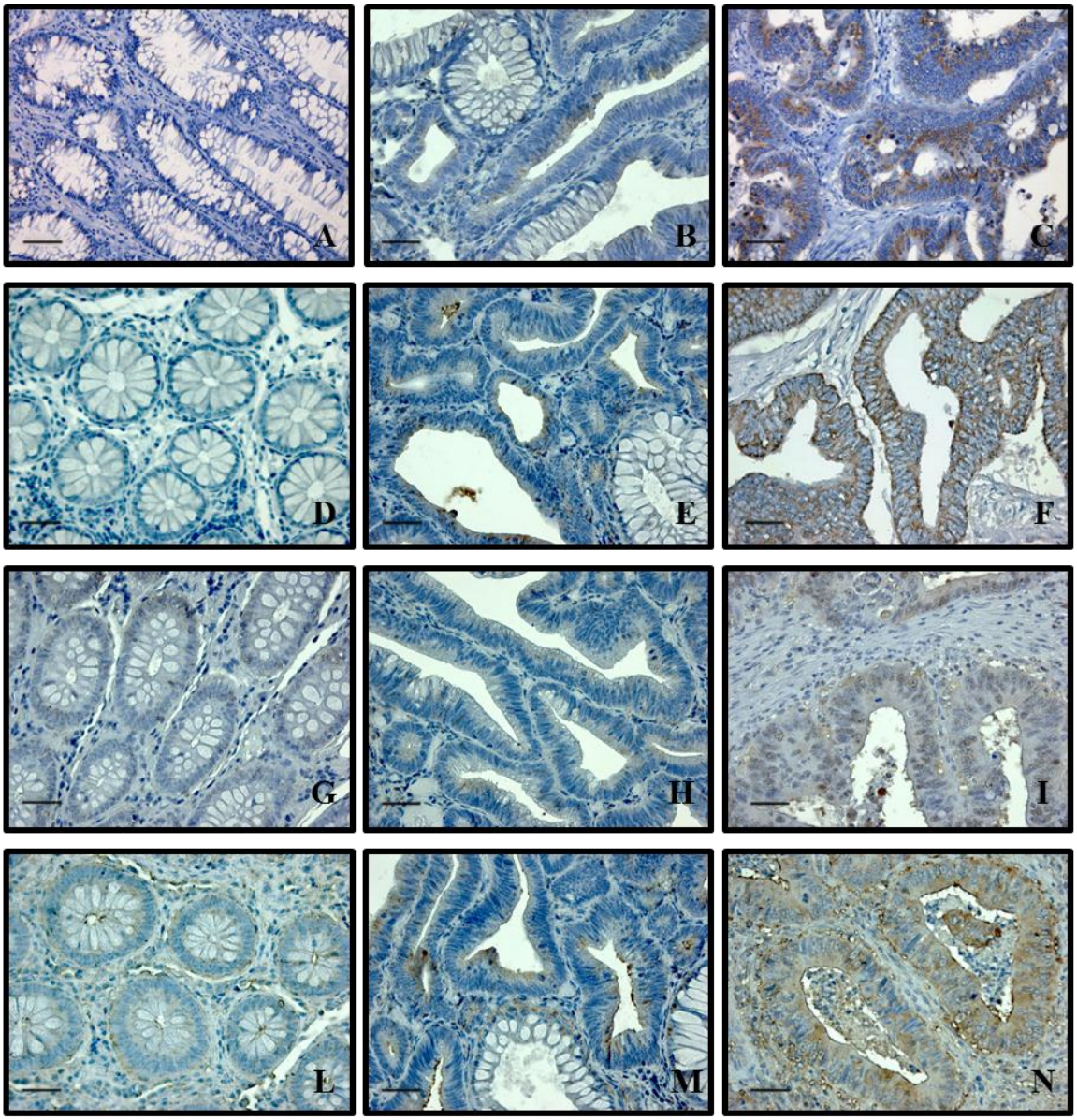


**Fig. 9** Representative pictures of immunohistochemical experiments performed for Hsps in samples of large bowel human mucosa. Hsp10 immunostaining: A) Normal Mucosa; B) Tubular Adenoma with moderate grade of dysplasia; C) Adenocarcinoma with moderate grade of differentiation. Hsp60 immunostaining: D) Normal Mucosa; E) Tubular Adenoma with moderate grade of dysplasia; F) Adenocarcinoma with moderate grade of differentiation. Hsp70 immunostaining : G) Normal Mucosa; H) Tubular Adenoma with moderate grade of dysplasia; I) Adenocarcinoma with moderate grade of differentiation. Hsp90 immunostaining: L) Normal Mucosa; M) Tubular Adenoma with moderate grade of dysplasia; N) Adenocarcinoma with moderate grade of differentiation. Magnification 200x. Scale bar 100µm.

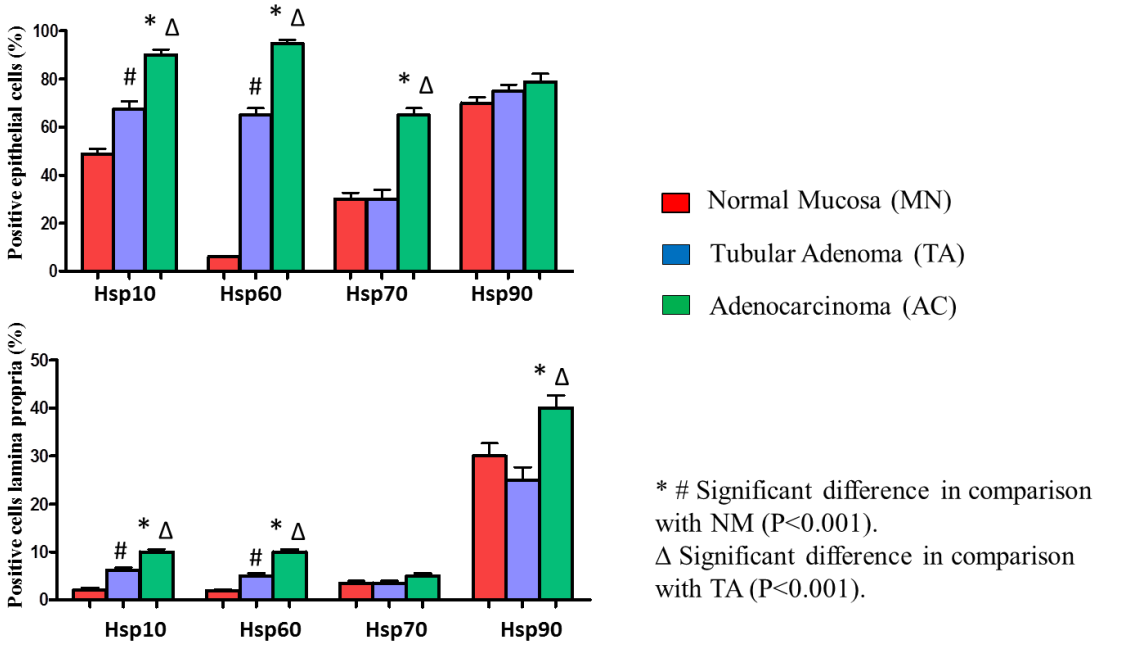


**Fig. 10** Histograms show statistical results for the evaluation of immunopositivity for Hsp10, Hsp60, Hsp70 and Hsp90 in epithelial cells (upper histogram) and in cells of lamina propria (lower histogram) in samples of normal mucosa (NM), tubular adenoma(TA) and Adenocarcinoma (AC) of human large bowel.

## 3. Western Blotting analysis

Western Blotting experiments were carried out on samples of normal mucosa and adenocarcinoma of colon. The results obtained by these analyses have showed that Hsp10, Hsp60, Hsp70 and Hsp90 levels increase significantly from normal mucosa to adenocarcinoma (Fig.11). Densitometric analysis of blots was performed using the NIH Image J 1.40 program (National Institutes of Health, Bethesda, MD,USA) (Fig.12). The data resulting by western blotting experiments are in agreement with the immunohistochemical results.

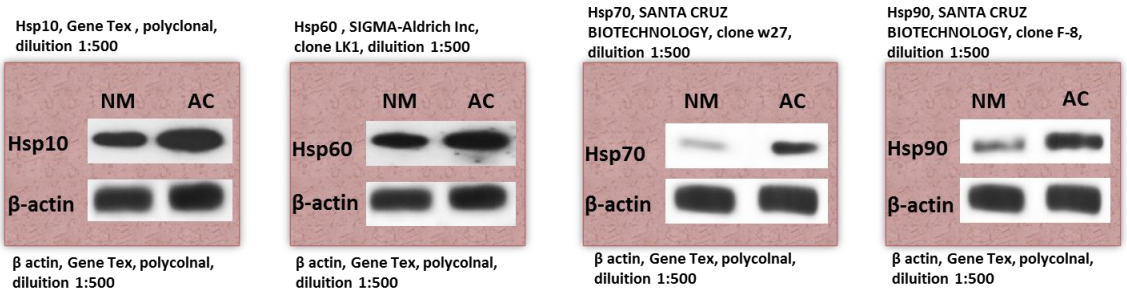


**Fig. 11** Representative immunoblots of western blotting analysis for Hsp10, Hsp60, Hsp70, Hsp90 in Normal Mucosa (NM) and Adenocarcinoma (AC). β-actin was used as a loading control.

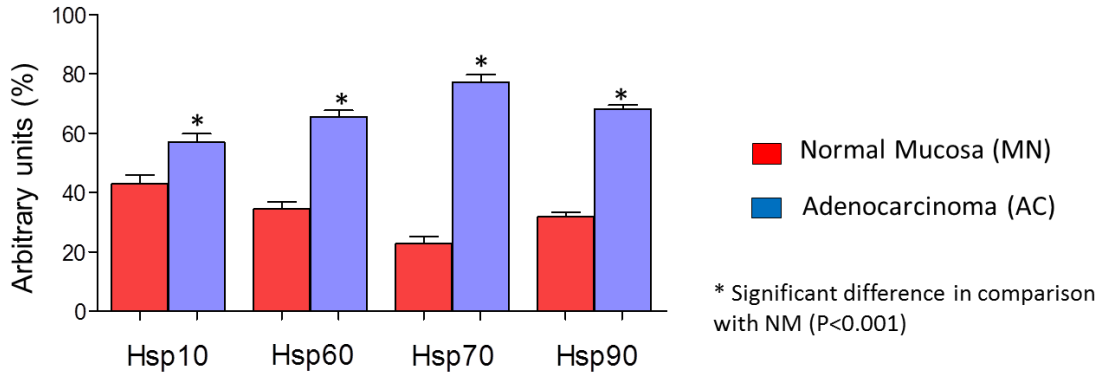


**Fig. 12** Densitometric quantification of Hsp10, Hsp60, Hsp70 and Hsp90 protein levels in Normal Mucosa (NM) and Adenocarcinoma (AC).

## 4. RT-PCR (Reverse Transcription Polymerase Chain Reaction)

RT-PCR experiments were carried out on samples of normal mucosa and adenocarcinoma of colon. The results obtained by RT-PCR analyses have showed that mRNA expression levels of Hsp10, Hsp60, Hsp70, Hsp90α and Hsp90β are higher in samples of adenocarcinoma than normal mucosa (Fig.13). Quantitative measurements of bands were performed using the NIH Image J 1.40 analysis program (National Institutes of Health, Bethesda, MD, USA) (Fig.14). These data suggest that increased levels (as shown by IHC and WB) are due to an increased gene expression of these molecules.

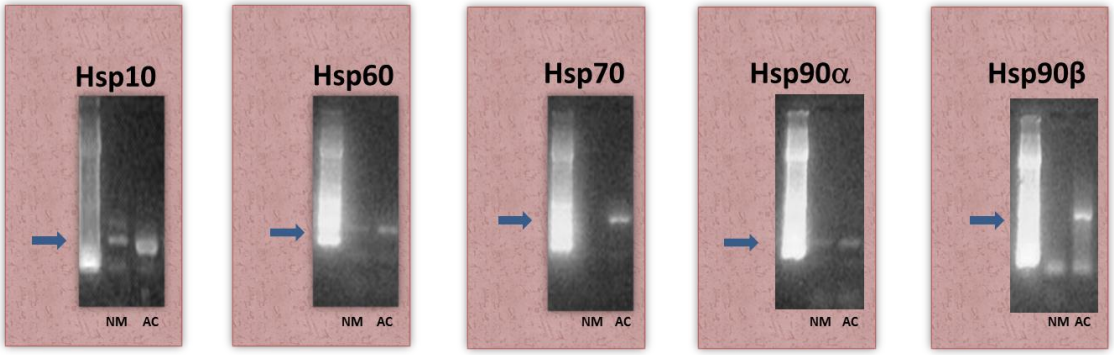


**Fig. 13** 1.5% agarose gels of the gene HSP10, Hsp60, Hsp70, Hsp90 isoform α and Hsp90 isoform β from Homo sapiens amplified by RT-PCR.

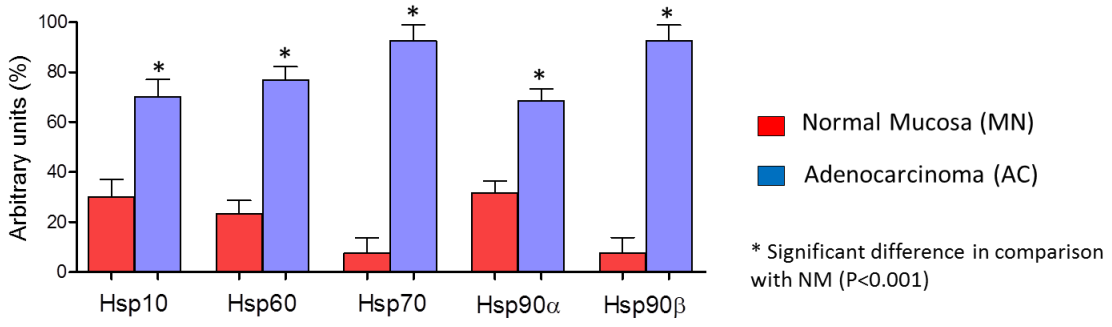


**Fig. 14** Histogram of quantitative measurements of bands of mRNA expression levels for Hsp10, Hsp60, Hsp70, Hsp90α,Hsp90β genes.

## 5. ELISA Assay for Human Hsp60

ELISA assay carried out on serum of 20 patients with adenocarcinoma and 20 healthy subjects has showed significantly ($p<0,001$) higher levels of Hsp60 in patients with colon cancer compared to healthy controls. However, one week after surgical removal of the tumor, Hsp60 levels significantly decreased reaching those of healthy subjects. The mean of Hsp60 plasma levels in healthy controls was 4,94 ng, in patients with colon adenocarcinoma was 31,45 ng in blood samples taken 1 day before surgical remove of tumor and, 3,2 ng in blood samples taken 1 week after surgical remove of tumor (Fig.15).

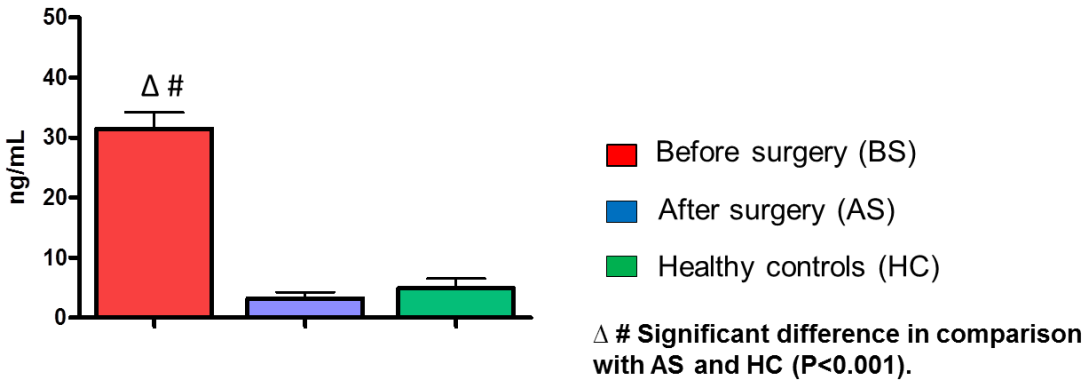


**Fig. 15** Histogram of Hsp60 plasma levels by Elisa Assay (ADI EKS 600 ELISA kit, Enzo Life Sciences, Inc., Farmingdale, NY, USA)

## 6. Immunogold experiments by Transmission electron microscopy (TEM)

Immunogold experiments were carried out to obtain a better ultrastructural localization of Hsp60 in adenocarcinoma tissue. Figure 16 shows that, in colon cancer cells, Hsp60 (black dots) is localized not only in the mitochondria (Fig.16 D) but also in the cytosol, in cytoplasmic regions very close to the membrane (Fig.16 B) and, to a large extent, also in the plasma membrane (Fig.16 A, C). These data suggest an active trafficking of Hsp60 in the cellular regions between the membrane and the neighboring areas of the cytoplasm.

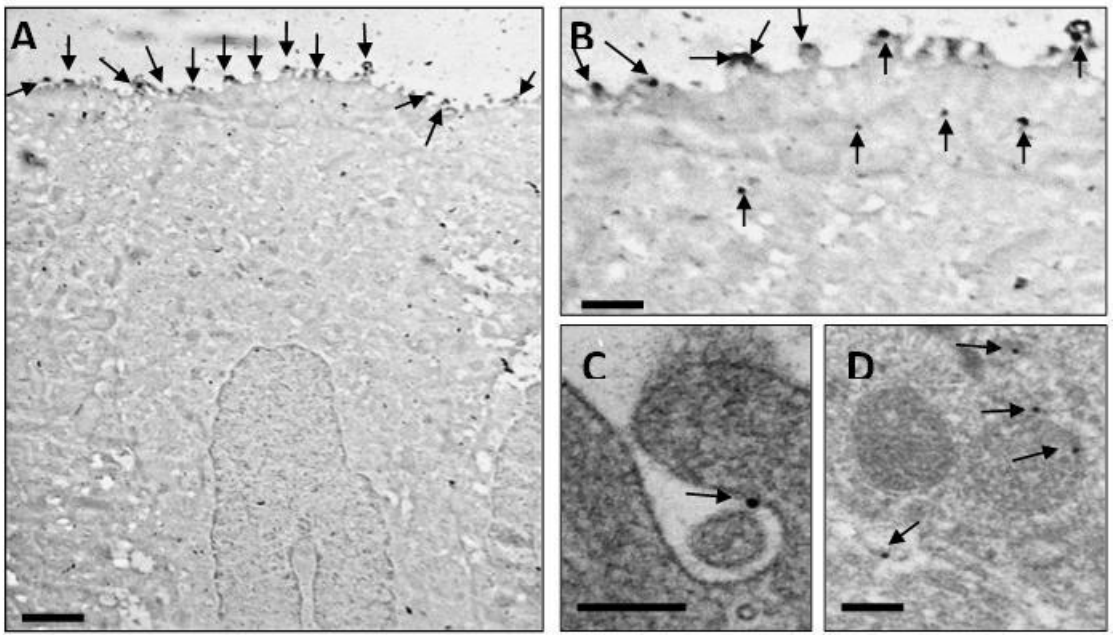


**Fig. 16** Immunogold stains for Hsp60 (black dots) in large bowel adenocarcinoma's tissue. A) Hsp60 localization in the membrane of adenocarcinoma cells. Bar: 1 µm B) Hsp60 localization in the cytosol very close to, and on the plasma membrane. Bar: 100nmC) Hsp60 localization on the cell membrane. Bar: 100nm. D) Hsp60 localization in the cytoplasm of cancer cells. Bar: 100nm.

## 7. Exosomes assessment procedure

To further ascertain the identity of the exosomes isolated from plasma samples, exoxomal preparations were examined by transmission electron microscopy (TEM). TEM analysis showed that the vesicles contained in the pellet isolated from plasma, were compatible with typical exosomes because presented the ultrastructural hallmarks of exosomes. Vesicles present in the pellet were of 30-90 nm in diameter (Fig.17).

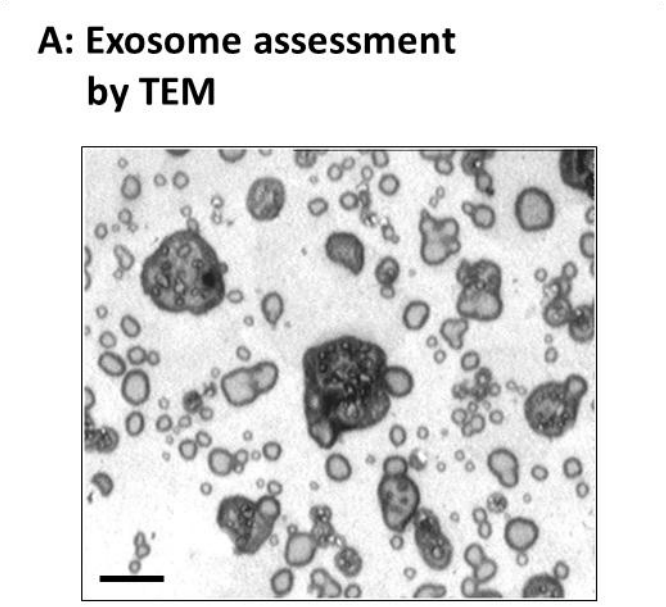


**Fig.17** Demonstration by TEM of exosomal preparation. An illustrative image of the exosomes isolated from plasma. Bar: 100nm.

Moreover the exosomal preparations were tested for Acetyl cholinesterase (AChE) activity that is typically detected in exosomes. Results obtained by AChE assay, confirmed the presence of exosomes in the pellet of ultra-centrifuged samples. In fact, the exosomes purified by ultra-centrifugation of plasma, showed an AChE activity that increased over the observation period of 30 minutes. In contrast the supernatant of ultracentrifugation products, showed very low AChE activity. Data are represented as mean ± SD of AChE activity measured at 412 nm every 5 minutes for 30 minutes in three independent experiments in duplicate (Fig.18).

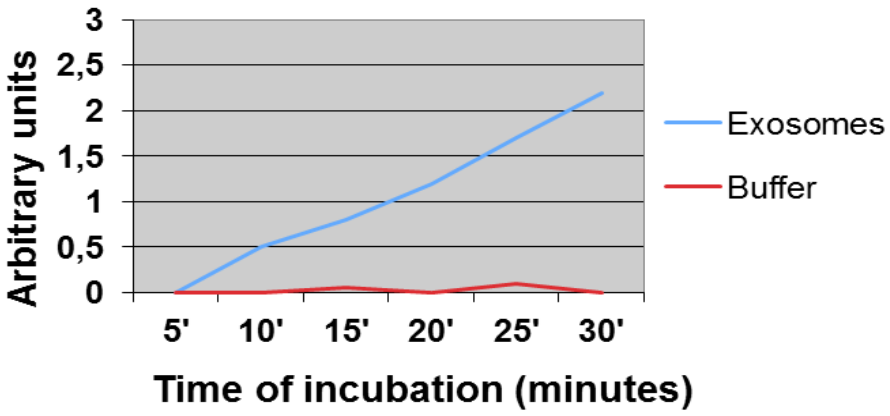


**Fig.18** AChE activity assay in exosomal preparations from the pellet and supernatants obtained after ultra-centrifugation of plasma.

Finally the Western Blotting analyses performed for Alix protein and Hsc70 protein, showed the presence of these markers in the pellet obtained from ultra-centrifugation (Fig.19).

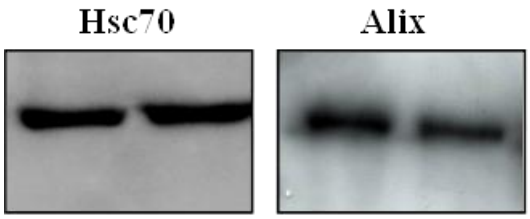


**Fig.19** Demonstration of Hsc70 and Alix poteins by Western Blotting in exosomal preparation.

## 8. Western Blotting analysis for Hsp60 in Exosomes

Western Blotting experiments for Hsp60 in exosomes showed that this protein was present in the exosomes isolated from the plasma of 20/20 patients with adenocarcinoma of colon (G2) before surgery. Its levels decrease significantly, in the same patients, one week after surgical removal of the tumor (Fig.20). While, Hsp60 was present in exosomal fraction of 1/20 healthy control (data not shown). These data are in agreement with the immunohistochemical results. Densitometric analysis of blots was performed using the NIH Image J 1.40 program (National Institutes of Health, Bethesda, MD,USA) (Fig.21).

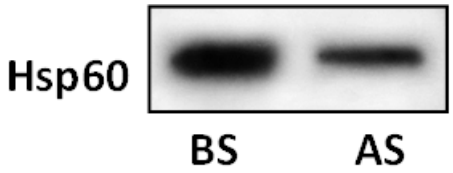


**Fig.20** Hsp60 was present in the exosomes isolated from the plasma before surgery (BS). Its levels decrease significantly one week after surgical removal of the tumor (AS).

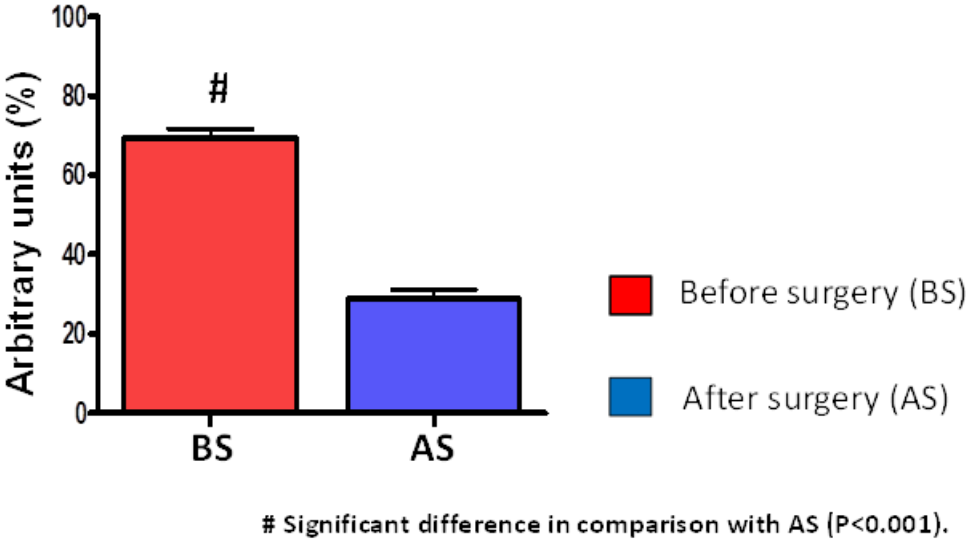


**Fig.21** Histograms show statistical results for the evaluation of Hsp60 levels in the exosomes isolated from plasma.

## 9. Double immunofluorescence analysis

Double immunofluorescence experiments for Hsp60 and CD68 performed on adenocarcinoma tissue sections, revealed that Hsp60 (red color) was present in almost all cancer cells and, also, in most of macrophages of the lamina propria that showed a double positivity for Hsp60 and CD68 (orange color) (Fig.22).

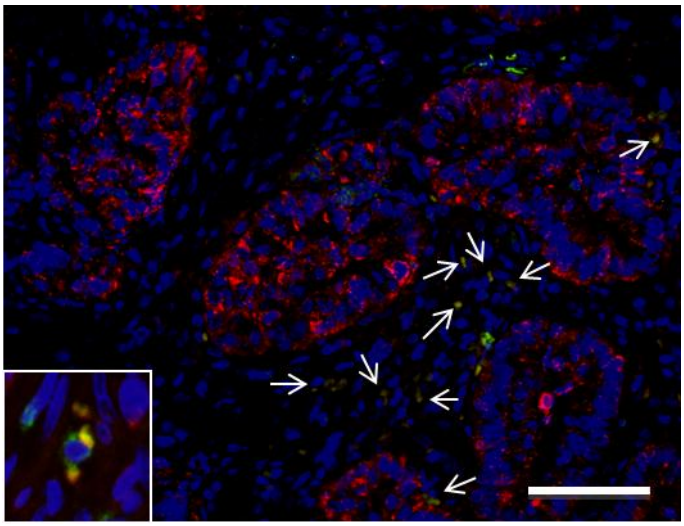

**Fig.22** Double immunofluorescence for Hsp60 and CD68: Hsp60 (red) was expressed in almost cancer cells, and also in most of macrophages (arrows on green-orange cells). Magnification 200x. Bar 100µm. Below and to the left of the figure shows a macrophage that presents a double positivity for Hsp60 (red color) and for CD68 (green color). The result of color merge is orange. Magnification 630x.

Double immunofluorescence for Hsp60 and CD34 showed that Hsp60 was present in almost cancer cells (red color). CD34 was expressed in the endothelial cells of the vessels of the lamina propria (green color). Hsp60 was expressed also in most of endothelial cells (that expressed a double positivity for Hsp60 and CD34) (Fig.23).

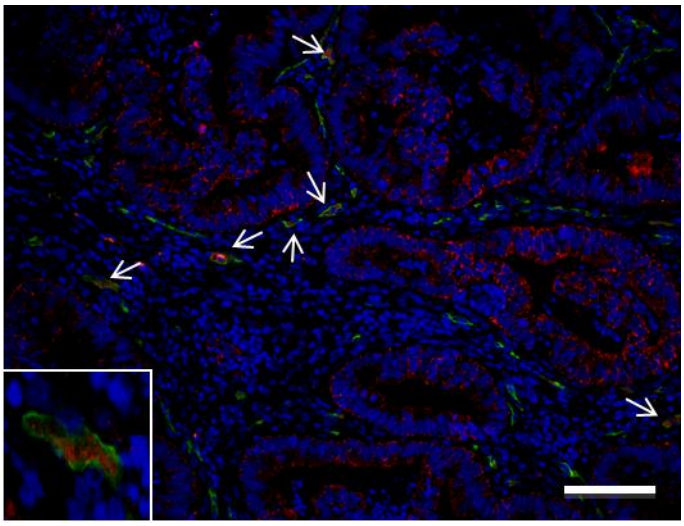

**Fig.23** Double immunofluorescence for Hsp60 and CD34: Hsp60 (red color) was expressed in almost cancer cells. CD34 was expressed in the endothelial cells of the vessels of the lamina propria (green color). Arrows are on lamina propria vessels. Magnification 200x. Bar 100μm. Below and to the left of the figure shows a vessel with endothelial cells positive for both markers Hsp60 (red color) and CD34 (green color). Magnification 630x.

# 6. DISCUSSION

Many reports support the idea that Hsps are implicates in the pathogenesis and in the progression of different human neoplasms, by uncertain metabolic mechanisms. Although Hsps perform their canonical “chaperoning” functions in both prokaryotic and eukaryotic cells, they have also acquired, probably during evolution, “extra-chaperoning” roles. Among these roles, there are some involved in the mechanisms of cancerogenesis of many types of cancers, as discussed in the Introduction section.

In this PhD thesis, we have been studies the protein expression of some Hsps (in particular Hsp10, Hsp60, Hsp70 and Hsp90) through experiments of immunohistochemistry in samples of human mucosa of large bowel taken from normal controls, pre-neoplastic lesions and invasive neoplasms. The results obtained have shown that Hsp10 and Hsp60 expressions are higher in pre-neoplastic lesion as well as in cancer lesion compared to normal mucosa. By contrast, Hsp70 and Hsp90 levels were increased only in the tumoral specimens, while there was not any difference between normal and preneoplastic lesions for these proteins. High levels of Hsp10 in the cytoplasm of cancer cells have been reported in the past (46) Also, Hsp60 has been shown to be overexpressed and localized in the cytoplasm of cancer cells (44). Hsp70 levels have shown an increase in expression only in epithelial component of cancer samples, and their immunohistochemical expression was localized in the cytoplasm and in the nucleus of cancer cells compared with normal cells. Hsp90 expression has shown only few significant differences between the samples studies, i.e. at the lamina propria levels of the tumoral component.

Hsp60 and Hsp10 are classically considered a mitochondrial molecule, while Hsp70 and Hsp90 are commonly present in the cytosol. The observation of an immunopositivity of Hsp60 at the cytoplasmic and membrane levels, as well as a nuclear immunopositivity of Hsp70 in cancer cells, confirms the concept that Hsps may change their intracellular distribution in comparison with normal cells. The presence of a diffuse immunohistochemical positivity for Hsp60 in the cytoplasm and on the plasma membrane and for Hsp70 in the nucleus have already been described in other forms of tumors (50,74,75) and have been correlated with tumor progression. These data indicate that the change of localization of Hsps in the cell may be an important marker of tumor progression. The immunoistochemical data obtained in this work were confirmed by

western blotting experiments that have shown increasing protein levels of all Hsps studied in the samples of invasive cancer compared with the samples of normal mucosa. Moreover the data obtained from the RT-PCR analyses conduct for Hsp10, Hsp60, Hsp70, Hsp90α and Hsp90β suggest that increased levels (as shown by IHC and WB) are due to an increased gene expression of these molecules. For the WB and RT-PCR experimental procedures, tubular adenoma biopsy samples were not used for two reasons. The main reason is that tubular adenoma is a dysplastic lesion that must be histologically evaluated as a whole. The second reason is that the characteristic presence of alternating normal and dysplastic glands within the same lesion may produce biased results.

The results obtained from the comparative evaluation of protein levels and genic expressions of Hsps have confirmed higher level of expressions of these proteins in the adenocarcinoma of colon. However, the limit of these techniques is that it does not permit to differentiate among levels of molecules and RNA in the different component of the tissues, i.e., epithelial and lamina propria components. However, these data are in agreement with many reports that have found changes of Hsps levels during the carcinogenetic process in various organs, such as oral cavity (76), uterine cervix (77), prostate (47) and large bowel (45). These evaluations imply that the all these Hsps are involved, directly or indirectly, in the carcinogenesis of these types of tumors. However, the increased levels of Hsp60 and Hsp10 also in preneoplastic lesions led us to hypothesize that these molecules are involved in the very early step of the carcinogenesis of this anatomical district.

Since other studies already showed *in vitro* that Hsp60 may be secreted from tumor cells and not from the normal counterpart cells via exosomes (that, as said in the Introduction section, are nanovesicles involved in tumor progression), and that Hsp60 is present in the membrane of exosomes (in turn becoming a suspicious player in tumor progression), we decided to focus our attention for the rest of the study on the localization of Hsp60 both in intracellular and extracellular sites in our specimens obtained by colon cancer *in vivo*. The ELISA assays performed for Hsp60 have shown significantly higher levels of this protein in the plasma samples of patients with adenocarcinoma of colon compared with healthy controls. This result suggests that the Hsp60, in some way, transits from the intracellular to the extracellular environment. However, one week after surgical removal of the tumor, Hsp60 levels significantly

decreased reaching those of healthy subjects. As a consequence, plasmatic levels of Hsp60 seemed to be more useful for monitoring disease progression and for the follow-up of patients with large bowel carcinoma. Hamelin C et al. has even proposed to consider the Hsp60 as a potential serum biomarker for colorectal carcinoma for determining prognosis or monitoring therapy (78). An increase in Hsp60 expression as compared with normal tissue has been shown for different types of tumor, including Hodgkin's lymphoma, and prostate, ovarian and breast adenocarcinomas (79). Zhao M et al. have furthermore investigated the levels of Hsp27 circulating in patients with epithelial ovarian cancer and have showed that the circulating Hsp27 levels were increased in epithelial ovarian cancer and correlated with peritoneal metastases. The authors have suggested that the evaluation of serum Hsp27 levels may be used as a potential additional indicator for peritoneal metastases in epithelial ovarian cancer and response to treatment (80). Moreover, the presence of Hsp60 in the circulation stimulates the question of why and how this protein passes from the cells into the bloodstream of tumor patients. We performed then a set of experiments to study the localizzation of Hsp60 in tumor samples, compared to normal tissue, by immunogold at TEM. In cancer cells Hsp60 was localized not only in and around the mitochondria as in normal cells, but also in the cytosol very close to the plasma membrane and in the plasma membrane. These evidences have suggested an active trafficking of this chaperon in the regions between the cell cytosol and plasma membrane. This trafficking of Hsp60 may be involved in the mechanisms of secretion of the same protein in the extracellular space. The presence of Hsp60 on the cellular membrane prompted us to research the presence of this protein in exosomes isolated from plasma obtained from patients with adenocarcinoma of large bowel, to verify whether these vesicles, which originate from the plasma membrane, may be involved in the mechanisms of Hsp60 secretion in the extracellular space. Hsp60 was found at higher levels in the exosomes isolated from plasma of all patients studied before tumor removal and, most interestingly, these protein levels decreased after surgery. These results have confirmed the data obtained from the ELISA assays for Hsp60 and have suggested that the secretion of Hsp60 outside the cells may involve the exosomal vesicles. These extracellular vesicles are important for the cell-to-cell communication and, Hsp60 would be considered a key player in the intercellular cross-talk. Exosomal Hsp60 could be a potential colon cancer biomarker, for instance, in assessing disease progression and response to treatment (follow-up). In a study of human tumor cells, the authors have

shown that the secretion for Hsp60 from tumor cells is not due to cell death or destruction, but to an active secretion mechanism (54). The authors have showed also that Hsp60 is present in the exosomes released from tumor cell in culture. In another study of tumor cells, Hsp60 was found integrated in the exosomes membrane (32) and, the authors have suggest that Hsp60 in the exosome membrane may play a role as a ligand for a receptor on the cells of the immune system or on other tumor cells. In this manner Hsp60 could gain an important role in the cross-talk between the cancer cells or between cancer cells and immune cells. Moreover, if Hsp60-bearing exosomes will be demonstrated to be able to enter in other tumor cells, we can hypothesise to use these nanovesicles as vector for a novel targeted anticancer therapy.

Finally, to better understand the latest data obtained, we performed double immunofluorescence experiments for CD68 and Hsp60 on sections of adenocarcinoma tissue and in normal counterpart. We focused on CD68 since this is a common marker used to detect macrophages, that it is known have receptors for Hsp60, such as TLR2, TLR4 and CD14 (81-84). The results obtained have shown that Hsp60 was present in almost all cancer cells and, also, in most of macrophages of the lamina propria that expressed the positivity for both CD68 and Hsp60 markers. These data reinforce the concept that Hsp60 transported by exosomes may, in some way, is transferred within immune cells and, therefore, play a role in the mechanisms of cross-talk between cancer cells and the cells of the immune system. Further studies will confirm these data and assess the presence of Hsp60 also in other types of immune cells (such as lymphocytes or NK cells). Subsequently, the immunofluorescence experiments conducted for the antigens CD34 and Hsp60 showed positivity in most of endothelial cells for both antigens. CD34 is a common marker used to detect vascular endothelial cells. This observation suggests that the Hsp60 is able to cross endothelial cells and to pass into the bloodstream. Other experiments will permit to better understand the molecular mechanisms of this process as well as to shed light about the putative involvement of Hsp60 in tumor neoangiogenesis, as already postulated (45).

Overall our results suggest that Hsp60 may be considered a novel biomarker for assessing colon cancer early diagnosis and, possibly, also in follow-up. Moreover, it could become also a target for a novel anti-cancer approach (chaperonotherapy) since its participation in the mechanisms of colorectal carcinogenesis. The chaperonotherapy is a new strategy of anticancer therapy that is gaining increasing importance for some forms

of cancer that may be considered as “chaperonopathies by mistake” in which Hsps/chaperones are involved in carcinogenesis (85). Chaperonotherapy consists of the utilization of Hsps/chaperones for treating chaperonopathies such as cancer. In the “negative chaperonotherapy” a drug is used to block the action of chaperones that favor the development of cancer, while, in the “positive chaperonotherapy”, the chaperone is used to fight against cancer by enhancing normal cellular defenses or stimulating the immune system (50). Current anticancer drug-development strategies involve identifying, and blocking, novel molecular targets crucial for tumor progression. Hsp60 seem to be involved in colorectal carcinogenesis, and for this reason it could become the target to be hit in the anticancer therapy. A drug that blocks the action of Hsp60 would inhibit the mechanisms by which this chaperone enhances tumor growth or tumor progression (84). At the same time, cancer specific Hsp60-bearing exosomes might be used as vector for anticancer drugs. However, further studies should be conducted to better understand the molecular mechanisms that underlie the involvement of Hsp60 in colorectal carcinogenesis and the ways in which this protein may be involved in therapeutic strategies.

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