Comparative Analysis of Hsp10 and Hsp90 Expression in Healthy Mucosa and Adenocarcinoma of the Large Bowel

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Abstract. *Background: Heat shock proteins (Hsps) assist other proteins in their folding and drive the degradation of defective proteins. During evolution, these proteins have also acquired other roles. Hsp10 is involved in immunomodulation and tumor progression. Hsp90 stabilizes a range of "client" proteins involved in cell signaling. The present study evaluated the expression levels of Hsp10 and Hsp90 in normal mucosa and adenocarcinoma samples of human large bowel. Materials and Methods: Samples of normal mucosa and adenocarcinoma were collected and Reverse transcriptase-polymerase chain reaction RT-PCR, western blotting (WB) analyses, as well as immunohistochemistry were performed to evaluate the expression levels of Hsp10 and Hsp90. Results: RT-PCR showed a higher gene expression of Hsp10 and Hsp90 in adenocarcinoma samples compared to healthy mucosa. WB results confirmed these findings. Immunohistochemistry revealed higher levels of Hsp10 in adenocarcinoma in both the epithelium and the lamina propria, while Hsp90 expression was higher in the adeno-carcinoma samples only in the lamina propria. Conclusion: Hsp10 and Hsp90 may be involved in large bowel carcinogenesis.*

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Colorectal cancer is a malignancy with significant social and health care-related effects. The carcinogenetic model of the large bowel is a multi-step process in which the transformation of normal mucosa into invasive tumor entails the accumulation of different genetic alterations (1). Many studies have suggested the involvement of heat shock proteins (Hsps) in large bowel carcinogenesis (2, 3). Hsps are among the most evolutionarily conserved proteins. They are an important class of proteins that have different functions essential for cell life and survival. Their classic role is to assist other proteins in folding and re-folding and, in the case of defective or irreversibly misfolded proteins, to drive their degradation (4). For this reason, some Hsps are also known as molecular chaperones. During evolution, this class of proteins has also acquired "extra-chaperoning" roles, such as participating in immune system regulation (5), cell senescence (5), cell differentiation, programmed cell death and carcinogenesis. These molecules have also been implicated in the pathogenesis of a number of chronic inflammatory and autoimmune diseases, like inflammatory bowel disease (IBD), in which Hsps have been identified as potential biomarkers for diagnostics, prognostics and etiopathogenetic factors, or therapeutic targets (6, 7). Moreover, Hsps have other important functions such as their involvement in various metabolic mechanisms of neoplastic cells such as tissue invasion, induction of angiogenesis and metastasis. Hsp10 is a 10-kDa, highly conserved, mitochondrion-resident protein that co-chaperones with another mitochondrial heat shock protein, Hsp60, for protein folding and the assembly and disassembly of protein complexes (8). Moreover, Hsp10 plays other important roles in a variety of mechanisms such us

immunomodulation and cell proliferation and differentiation (9, 10). The latter are linked to its localization either in the cytosol (11) or the extracellular space. Hsp90 is a molecular chaperone that performs its functions inside cells by forming cytosolic chaperoning machines (4). This chaperone is involved in signal transduction and other key pathways critical for malignancy in several cancers (12, 13), including large bowel carcinoma (14). Hsp90, that also includes two isoforms, Hsp90α and Hsp90β, is an ubiquitous protein highly expressed (accounting for up to 1-2% of total cellular protein content) in the cytosol, both in normal and stress conditions (15). Hsp90 can accumulate in cancer cells and is implicated in the carcinogenesis process for many reasons. This molecule favors tumorigenicity and promotes cancer cell growth and survival (16, 17) by inhibiting programmed cell death and senescence (18). Hsp90 client proteins involved in such activities include, for example, mutated p53, BRAF and ErbB2 (19). Hsp90 also influences tumor neoangiogenesis because it stabilizes proteins important for the metabolism of endothelial cells such as vascular endothelial growth factor and nitric oxide synthase (20, 21).

In the present study, we evaluated by immuno-morphological experiments, western blotting (WB) and RT-PCR analyses the levels and expression of Hsp10 and Hsp90 in a series of large bowel mucosa samples obtained from healthy controls and patients with adenocarcinomas with moderate grade of differentiation.

Materials and Methods

Sample collection. A total of 40 large bowel mucosa biopsies from healthy controls (n=20) and adenocarcinomas with moderate grade of differentiation (n=20) were obtained from the DICHIRONS Department of the University of Palermo, Italy. The samples collected for WB and PCR analyses were kept frozen at –80˚C until use, while the samples for immunomorphological analysis were fixed in formalin and embedded in paraffin.

RT-PCR analysis. Total RNA extraction from all tissue samples was performed using TRI REAGENT® (Catalog Number T9424, Sigma-Aldrich, Saint Lous, MO, USA) according to the manufacturer's instructions. cDNA was synthesized using ImProm–II Reverse Transcriptase (Catalog Number A3800, Promega Corporation, Madison, WI, USA) and amplified using GoTaq® Flexi DNA Polymerase (Catalog Number M8291, Promega Corporation, Madison, WI, USA). PCR amplification was performed by adding specific primers for human Hsp10, Hsp90α and Hsp90β : for human Hsp10 cDNA, the forward primer sequence was 5'CTCCCAG AATATGGAGGCACC-3' and the reverse primer sequence was 5'-TGGAATGGGCAGCATCATGT-3'; for human Hsp90α cDNA, the forward primer sequence was 5'-GTCTAGTTGACCGTTCCGCA-3' and the reverse primer sequence was 5'-GAGGAGG CACCCTCAAGTTC-3'; for human Hsp90β cDNA, the forward primer sequence was 5'-CTCTCTCGAGTCA CTCCGGC-3' and the reverse primer sequence was 5'-GTACGTTCCTGAGG GTTGGG-3'). The PCR product was visualized on 1.5% agarose gel with the Syber stain (SYBER SafeTM DNA gel stain, Invitrogen, Carlsbad, CA, USA). Experiments were performed in triplicate. Quantitative measurements of bands were performed using the NIH Image J 1.40 analysis program (National Institutes of Health, Bethesda, MD, USA).

Western blotting analysis. 50 mg of tissue samples from normal mucosa and adenocarcinoma were incubated on ice in a Radio-Immunoprecipitation Assay (RIPA) lysis buffer (0.3M NaCl, 0.1% SDS, 25mm HEPES pH 7.5, 1.5 mm $MgCl_2$, 0.2 mm EDTA, 1% Triton X-100, 0.5mm DTT, 0.5% sodium deoxycholate) containing proteases and phosphatase inhibitors (0.1 mg/ml phenylmethylsulfonyl fluoride, 20 mg/ml aprotinin, 20 mg/ml leupeptin, 10 mg/ml NaF, 1 mM DTT, 1 mM sodium orthovanadate, 20 mM β-glycerol phosphate) for 1 h. Subsequently, the samples were centrifuged at 13,000 rpm for 10 min 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, and then electrophoretically transferred to a nitrocellulose membrane (Nitrocell Membrane, Bio-Rad Laboratories Inc. Karlsruhe, Germany). After blocking, the membranes were incubated overnight with primary antibodies (rabbit anti-Hsp10 polyclonal antibody, sc-20958 and mouse anti-Hsp90, F-8 clone,sc-13119, Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA) diluted 1:1000 in Tris Buffered Saline with Tween 20 (T-TBS) containing 1% dry milk. Blots were washed in T-TBS and incubated for 1 h with Horseradish Peroxidase (HRP) conjugated secondary antibodies (ECLTM anti-rabbit IgG HRP-conjugated whole antibody; ECLTM anti-mouse IgG HRP-conjugated whole antibody, Amersham Biosciences) diluted 1:5000 in T-TBS containing 1% dry milk. β-actin (mouse anti-β-actin monoclonal antibody, C4 clone, sc-47778, Santa Cruz Biotechnology, Inc.) was used as a loading control. The final detection procedure was carried out using the ECL Western Blotting Detection Reagent (RPMN2232, Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's instructions. Experiments were performed in triplicate.

Densitometric analysis of blots was performed using the ImageJ 1.41 software (National Institutes of Health, USA, http://rsb.info.nih.gov/ij).

Immunohistochemical analysis. Samples of normal mucosa and adenocarcinoma were fixed in formalin and embedded in paraffin. From the paraffin blocks, sections with a thickness of 4-5 mm were obtained using a cutting microtome. These sections were dewaxed in xylene for 10 min and rehydrated by sequential immersion in a descending scale of alcohols and transitioned in water for 5 min. Subsequently, the sections were immersed for 8 min in sodium citrate buffer (pH 6) at 95˚C for antigen retrieval and, subsequently, immersed for 8 min in acetone at –20˚C to prevent the detachment of the sections from the slide. All subsequent reactions were conducted at room temperature. The reactions were performed by a streptavidin–biotin complex method using Histostain®-Plus 3rd Gen IHC Detection Kit (Invitrogen Corporation, Cat no. 85-8943). The primary antibodies used were anti-human Hsp10 antigen (Gene Tex polyclonal, dilution 1:300) and anti-human Hsp90 antigen (Santa Cruz Biotechnology, clone F-8, dilution 1:200). Non-immune sera were substituted for negative controls. NCI-H292 cells were used as positive control. 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, Heidelberg, Germany.) connected to a digital camera (Leica DC 300F). Each tissue section was analysed on two separate occasions by two independent observers (FC and FR).

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

RT-PCR analysis. RT-PCR experiments carried-out on samples of normal mucosa and adenocarcinoma showed that mRNA expression of Hsp10, Hsp90α and Hsp90β were higher in adenocarcinoma samples than in normal mucosa (Figure 1). These data suggest that there is an increased gene expression of these molecules in colon adenocarcinoma.

Western blotting analysis. WB experiments showed that Hsp10 and Hsp90 levels increase significantly from normal mucosa to adenocarcinoma (Figure 2). The data obtained with the WB experiments are in agreement with the RT-PCR results, and suggest that there is an increased synthesis of these protein molecules in colon adenocarcinoma.

Immunohistochemical analysis. The immunohistochemical evaluation, carried-out on all samples, showed that the localization of cellular immunopositivity of Hsp10 and Hsp90 was cytoplasmic, and sometimes granular. The levels of Hsps were investigated in the epithelial and lamina propria cells. The percentage of positive cells was calculated in 10 random high power fields (HPF) at a magnification of 400× and expressed as means (Figure 3). Hsp10 levels, both in the epithelium and lamina propria, were higher in adenocarcinoma samples compared to normal mucosa. In contrast, Hsp90 levels resulted higher only in the lamina propria cells of the adenocarcinoma samples, compared to normal mucosa, while no differences in expression were observed between the epithelial cells of the normal mucosa and adenocarcinoma samples (Figure 4). Data obtained from immunohistochemistry were plotted using the Microsoft Excel software (Microsoft Italia, Milan, Italy). Standard statistical analyses were employed to calculate the means of positivity percentage and the standard deviations (SD).

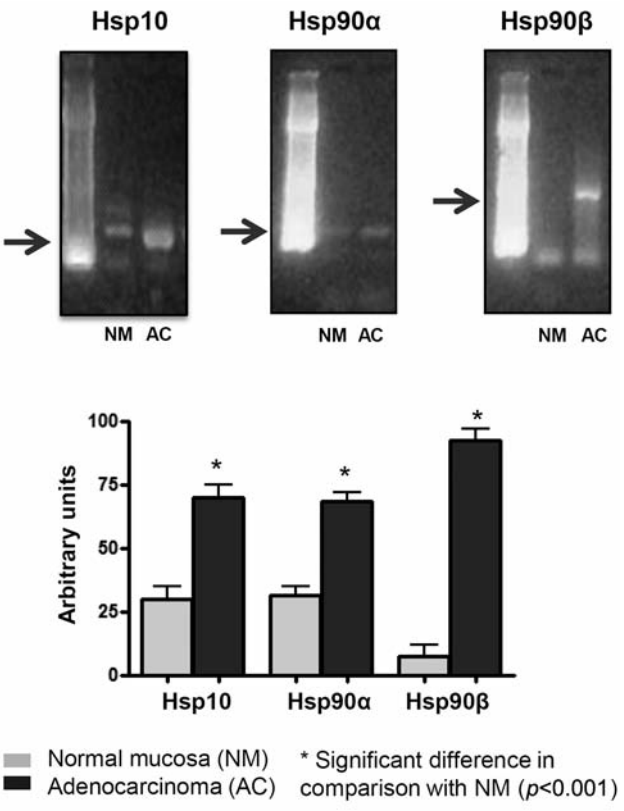

Figure 1. *RT-PCR analysis: 1.5% agarose gels of the gene HSP10, Hsp90 isoform α and Hsp90 isoform β from Homo sapiens amplified by RT-PCR. Histogram of quantitative measurements of bands of mRNA expression levels for Hsp10, Hsp90α and Hsp90β genes.*

Discussion

In the present work, we studied the gene expression of Hsp10 and Hsp90 (Hsp90α and Hsp90β isoforms) by RT-PCR analysis in samples of normal large bowel mucosa and adenocarcinoma of large bowel. The data obtained showed a higher gene expression of Hsp10 and Hsp90 in adenocarcinoma samples than in healthy mucosa. Moreover, the Western blotting analysis confirmed greater concentrations of Hsp10 and Hsp90 proteins in the adenocarcinoma samples compared to healthy mucosa. The results obtained from comparative evaluation of the protein levels and gene expression of Hsp10 and Hsp90 confirmed that these proteins seem to be implicated in the carcinogenesis of large bowel. However, the RT-PCR and WB techniques have the limitation of not differentiating the levels of proteins and RNA between the different tissue structures, *i.e.*, the epithelial and lamina propria components. Therefore, we performed further immunohistochemical experiments to evaluate the immunopositivity in the epithelial and in the lamina propria cells. In the currently available literature, several reports support the idea that Hsps are involved in the pathogenesis and the progression of different human neoplasms for many reasons. Possibly the most important reason is that the Hsps have acquired, probably during cellular evolution, various functions within the cell such as

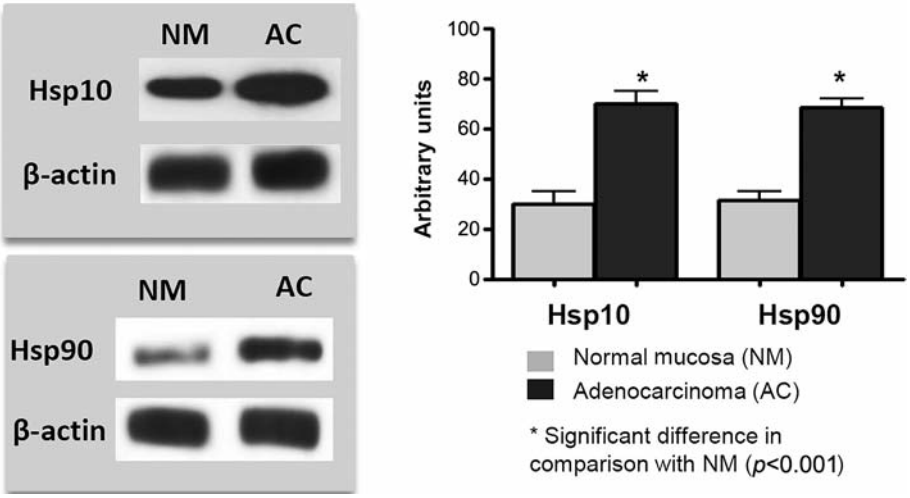

Figure 2. *Western blotting Analysis: Representative immunoblots for Hsp10 and Hsp90 (on the left) and densitometric quantification of Hsp10 and Hsp90 protein levels (on the right) in normal mucosa (NM) and adenocarcinoma (AC). β-actin was used as a loading control.*

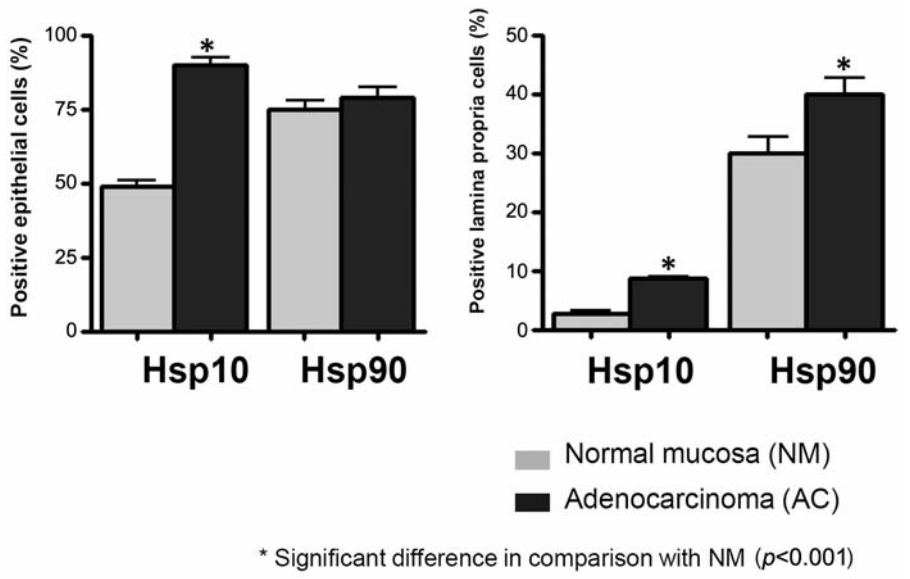

Figure 3. *Histogram showing immunohistochemical results: The percentage of positive cells was calculated in 10 random high power fields (HPF) of epithelial cells (EC) and lamina propria cells (LPC) of normal mucosa (NM) and adenocarcinoma (AC) samples of large bowel at a magnification of ×400.*

participating in immune system regulation (22-24), cell proliferation and differentiation (25, 26) and carcinogenesis (27, 28). Higher Hsp10 levels have been found in different malignancies (29) such as large bowel cancer (11, 30), exocervical cancer (30), prostate cancer (31), mantle cell lymphoma (32) and serous ovarian cancer (33), while lower expression levels of this protein have been detected in bronchial cancer (29, 34). In our experiments, Hsp10, which in normal cells is generally localized in the mitochondrial matrix, accumulated in the cytosol of cancerous cells and the lamina propria of adenocarcinoma, while Hsp90 showed a higher immunopositivity in the lamina propria of the tumor. This finding, in agreement with other data present in literature (11, 30, 35) is of importance because it suggests an active trafficking of Hsp10 between the inner and outer regions of the tumor cell. Moreover, the expression of Hsp10 and Hsp90

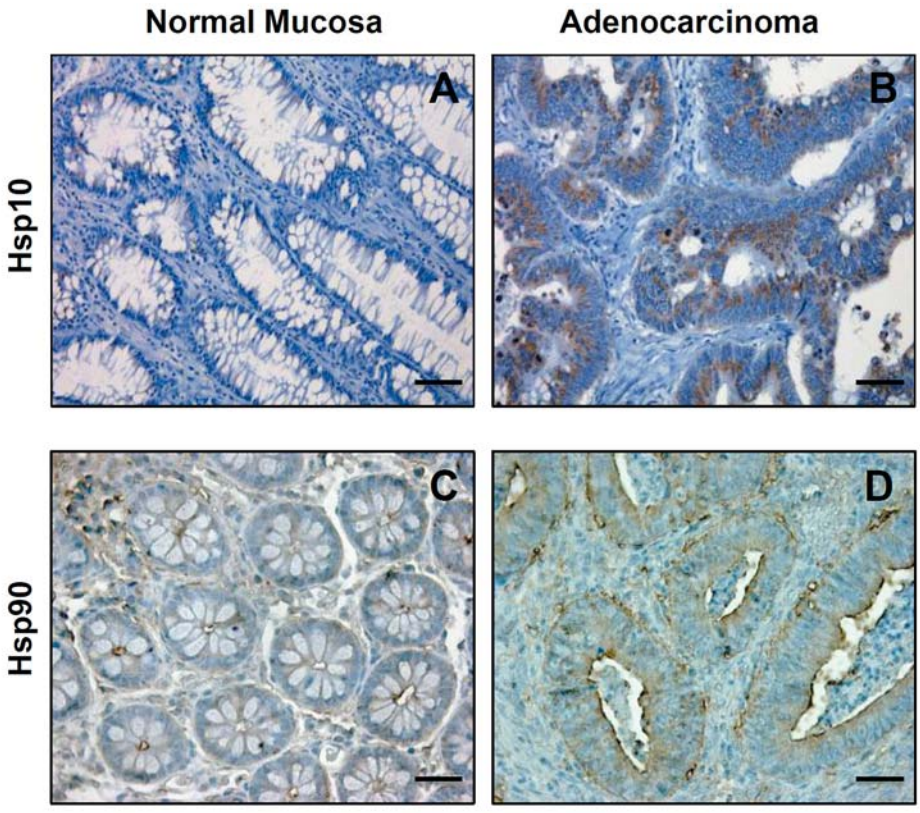

Figure 4. *Representative images of immunohistochemical results: Representative images of immunohistochemical experiments performed for Hsp10 and Hsp90 in large bowel mucosa samples of Hsp10 immunostaining: A) Normal mucosa. B) Adenocarcinoma with moderate grade of differentiation. Hsp90 immunostaining: C) Normal mucosa. D) Adenocarcinoma with moderate grade of differentiation. Magnification ×200; scale bar 100 μm. Histograms show statistical results for the evaluation of immunopositivity for Hsp10 and Hsp90 in epithelial cells (upper histogram) and in lamina propria cells (lower histogram) in normal mucosa (NM) and adenocarcinoma (AC) samples of human large bowel.*

in the lamina propria cells suggests a possible role of these proteins in tumor progression and immunomodulation. Many studies have suggested that Hsp10 could be involved in the immunomodulation mechanism that occurs in tumors (36, 37). For example, in a study performed in patients with ovarian cancer, the authors suggest that Hsp10 plays an immunosuppressive role being considered as a critical factor in the suppression of T-cell activation acting as a means to escape immune surveillance in cancer (38). Both Hsp10 and Hsp90, like other Hsps, protect cancer cells against apoptosis (17, 39). In particular, Hsp10 seems to be a molecule that inhibits the proapoptotic activity by interacting with the Raf signaling cascade and shifting the balance towards cell survival (40, 41). The anti-apoptotic action of Hsp90 involves the inhibition of cytochrome c-mediated activation of pro-caspase-9 (42) and its interaction with proteins able to generate signals in response to growth factor stimulation (43, 44). Other studies have shown that tumor cells overexpressing Hsps have an increased tendency to invade surrounding tissues and to spread to distant organs (45, 46). In particular, the higher expression of Hsp90 in the lamina propria cells in large bowel adenocarcinoma samples may suggest an involvement of this protein in the mechanisms of tumor progression and angiogenesis. In fact, some previous reports demonstrate that Hsp90 stabilizes vascular endothelial growth factor and nitric oxide synthase in endothelial cells (45) and may induce neovascularization (46). We have carried-out a study based on gene and protein expression analyses and immunomorphological experiments, obtaining results that are in accordance with various previously published studies. Further investigations are certainly needed to better understand the molecular mechanisms that underlie the involvement of Hsp10 and Hsp90 in large bowel carcinogenesis. Once the role of these molecules in large bowel carcinogenesis has been established, this type of cancer may be considered a "chaperonopathy by mistake" (47) and Hsps could become a key target in the search for new therapeutic strategies for cancer (48, 49).

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