Expression of heat shock protein 70 (HSP70) in patients with colorectal adenocarcinoma – immunohistochemistry and Western blot analysis

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The role of heat shock protein 70 (HSP70) expression has been investigated in various types of tumors. There are only little and controversial data about its clinical relevance in colorectal carcinoma, one of the most common carcinomas observed in humans. In this study we investigated expression of HSP70 in human colonic carcinoma and possible correlation with clinicopathology.

To assess patterns (cytosolic and membrane) of HSP70 expression, the 48 surgically removed colorectal adenocarcinomas and 12 normal colonic and rectal mucosal samples were examined by immunohistochemistry and Western-blot.

According to results of immunohistochemistry, expression of cytoplasmic HSP72 was significantly higher in colorectal carcinoma compared with normal and adjacent mucosa ($p<0.01$). In addition, there was significant increase in HSP72 expression in lymph node-positive compared to node-negative group ($p<0.001$). Dukes C2 stage of colonic cancer showed significantly higher immunohistochemical score than Duke's B2 and B1 stage groups ($p< 0.05$ i.e. $p< 0.02$). There was no relation between expression of HSP72 and degree of tumor differentiation.

Using Western blot analyses, we noticed elevated levels of cytosolic HSP70 in colorectal cancer cells compared to normal. Densitometric analysis of blots of plasma membrane HSP70 expression has shown decrease in colorectal cancer cells compared to normal mucosa.

According to our results, overexpression of HSP72 in malignant tissues of patients with colorectal carcinoma is related to tumor progression, suggesting that these proteins could play an important role not only in tumorigenesis but also in the development of drug resistance. Further research is necessary to clarify the mechanisms responsible for differential HSP70 expression as well as its definitive role in colorectal cancer.

Key words: HSP70 – Colorectal carcinoma – Western-blot – Immunohistochemistry

HSP70 are stress proteins that cooperate as chaperones in mammalian cells and have important cell biologic roles. They function as the intracellular detergents for the aggregated and denatured molecules caused by variety of stressors, and as the molecular chaperones in peptide and protein transport between cell organellas, and also contribute in the formation of three-dimensional structure of proteins. Those proteins are associated with p53 (especially mutant p53) and pRb110 proteins, suggesting that they may have roles in cell growth control and differentiation (1–4). Constitutive form of HSP70 (HSC70) may have anti–oncogenic potential (4). It has been revealed that HSP70 is an antiapoptotic chaperone protein. Namely, it appears as a promising target for the treatment of cancers resistant to classic caspase-mediated apoptosis (5). HSP70 contributes to the import and subsequent degradation of denatured proteins (6). This chaperoning function indicates a role in antigen processing and presentation (7).

Beside these intracellular chaperoning functions, several studies have suggested a positive correlation between HSP expression and tumor immunogenicity. It has been reported that HSP70 surface positive tumor exosomes stimulate mi-

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gratory and cytolytic activity of natural killer (NK) cells (8). Membrane-bound HSP70 served as a recognition site for the cytolytic attack mediated by NK cells (9). The 14 amino acid sequence (TKD) of HSP70 was identified as a tumor-selective recognition structure for NK cells. Incubation of peripheral blood lymphocyte cells with TKD plus low-dose interleukin 2 (IL-2) enhances the cytolytic activity of NK cells against HSP70 membrane-positive tumors, *in vitro* and *in vivo*. Treatment of colonic and lung cancer patients with *ex vivo* heat shock protein 70-peptide activated autologous NK cells (10).

Finally, many studies have shown that HSP purified from tumors can be used as a tumor-specific vaccine. These observations demonstrate that manipulation of HSP (i.e. HSP110) expression in tumors, specifically when combined with GM-CSF, represents a potentially powerful approach to cancer vaccine formulation (11). Also, increased expression of inducible HSP70 in appoptotic cells is correlated with their efficacy for antitumor vaccine therapy (12).

Colorectal cancer is one of the most common carcinomas observed in humans. Recently it was reported that increased oxidative stress is associated with human colorectal cancer. There are few and controversial studies on the clinical relevance of expression of the 70kDa member of heat shock protein family, HSP70, in colorectal cancer (13–16). The present study was designed to determine the both expression levels of HSP70 in colorectal cancer by immunohistochemistry and Western blot analysis, and correlations between this expression and clinicopathological parameters of colorectal cancer.

Materials and methods

Subjects. Forty-eight surgically removed colorectal adenocarcinoma and 12 normal colonic and rectal mucosal samples were examined, 32 patients were male, and 16 were female. The mean age of the patients was 62 years (range 33-80 years). The anatomical site of primary tumor was rectum in 32, sigmoid colon in 8, descending colon in 4, transverse colon in 2, ascending colon in 1, and cecum in 1 of the cases. The histological classification of the tumors was established according to previous criteria (17). Thus, 10 (20.83%) were well-differentiated type adenocarcinoma (grade 1, G1), 19 (39.58%), were moderately differentiated adenocarcinomas (grade 2, G2) and 19 (39.58%). were poorly differentiated adenocarcinomas (grade 3, G3). In addition, tumors were classified according to the Astler-Coller modification of Dukes' classification (18). The distribution of these tumors was stage B_1, 6 cases (12.5%), stage B_2, 26 cases (54.16%) and stage C_2, 16 cases (33.33%). Among the cases, 16 (33.3%) had regional lymph node metastasis, and no patients had remote metastasis. In addition to the colorectal adenocarcinoma, 12 normal colonic and rectal mucosal samples were selected from macroscopically normal areas; 6 samples were taken from the areas more than 10 cm away from tumors, and 6 from normal mucosa of non-tumor patients. All patients had given informed consent before surgery at the Clinical Center of Serbia and Military Medical Academy- Belgrade.

Tissues and cellular fractionation. The normal mucosa and colorectal cancer tissues were surgically dissected and frozen. The nuclear fraction of normal and tumor tissues were obtained by differential centrifugation, and the plasma membrane fraction was obtained by modified method of Bauer and Hurtenbach (19). Briefly, the samples of normal and neoplastic material were minced by fine dissection and homogenized in 0.25 M sucrose, 5 mM $MgCl_2$, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 50 mM Tris-HCl (pH 7.4) followed by centrifugation of the homogenate at 800 x g for 10 min. The supernatants were spun at 11000 x g at 4°C for 25 min and the resulting supernatant was again centrifuged at 20000 x g at 4°C for 45 min. The pellet, containing plasma membranes and associated proteins, was resuspended in 10 mM Tris-HCl (pH 7.4) and stored at -20°C. The cytosolic fraction was obtained by further centrifugation of supernatant at 100000 x g, at 4°C for 60 min. The pellet obtained after first centrifugation was homogenized in ice-cold buffer (2.2M sucrose, 5mM $MgCl_2$, 0.5% Triton X-100, 1mM PMSF, and 50mM Tris-HCl, pH 7.4) and further purified by centrifugation. After this step, the pellets representing the soluble nuclear proteins were resuspended in 0.25M sucrose, 5mM $MgCl_2$, 0.5% Triton X-100, 1mM PMSF, and 50mM Tris-HCl, pH 7.4. The protein concentration was determined by Lowry method (20) and samples were aliquoted and stored at -20°C. The presence and purity of plasma membranes isolated from human colorectal cancer tissues and normal mucosa was examined by membranous NaK-ATPase activity, according to the methods described earlier (21, 22). Only the samples which displayed NaK- ATPase activity were considered as pure and taken for further analyses.

Immunohistochemistry. Frozen tissues were cut at 6μm on cryostat. Sections were air dried and fixed in cool (4°C) acetone for 10 min. Immunohistochemical staining was carried out using the ABC technique (23). Pretreatment with wet autoclaving was performed. Slides were incubated with the primary monoclonal antibody N27 F3-4 or C92 F3A-5 (a gift from Dr.W.Welch, Lung Biology Center, San Francisco, USA) diluted 1:450 for 1h at room temperature followed by biotinylated rabbit anti-mouse immunoglobulin (DAKO) and the avidin-biotin-peroxidase (streptAB complex/HRP) complex for 30 and 45min, respectively. Peroxidase activity was developed by a solution of 5mg of 3,3'-diaminobenzidine tetrahydrochloride (Sigma) dissolved in 10 ml of Tris buffer (0.05M, pH 7.6) and 0.03% H_2O_2. Methyl green was used to counterstain the slides. Negative control slide were processed with each slide run and excluded the primary antibody but included all other steps of the procedure. Note that used antiserum C92 F3A-5 are antibodies against inducible form of HSP70 (HSP72) and the antiserum N27 F3-4 are antibodies against both constitutive and inducible forms of HSP70 (HSP72/73).

Both cytoplasmic and nuclear staining were noted in order to detect a possible different localization of HSP70. The score of immunmoreactivity was assessed as follows: complete lack of HSP70-positive cells was scored as 0, scattered (up to 10%) as 1, focal moderate to strong HSP70 positivity (up to 50%) as 2, and more than 50% HSP70-positive cells as 3.

Western-blot. Proteins isolated from normal mucosa and colorectal cancer cells were suspended in Laemmli sample loading buffer (0.1M Tris, pH 6.8, 20% glycerol, 4% SDS, 0.04% bromphenolblue, 10% β-mercaptoethanol), and separated electrophoretically on 12% acrylamide gels, according to procedure of Laemmli (24). Approximately 50 μg of nuclear, 20 μg of cytosolic and 50 μg of plasma membrane proteins was loaded per lane. Separated proteins were transferred onto nitrocellulose (HYBOND, Amersham) according to the procedure of Towbin et al (25) The membranes were incubated for 2.5h at room temperature with mouse monoclonal antibodies N27 F3-4 (a gift from Dr. William J. Welch, Lung Biology Center, San Francisco, USA), diluted 1:1000, or with BB70 (a gift from Dr. David O. Toft, Mayo Clinic, Rochester, USA) diluted 1:500. After incubation with a secondary alkaline phosphatase-conjugated antibody (1:400) for 1.5h at room temperature HSP70 immunoreactive bands were detected by BCIP/NBT substrate. The bands representing the HSP70 immunoreactivity were densitometrically scanned, and the values obtained for normal mucosa and colorectal cancer were compared. Western blots were scanned by a computer-based laser densitometer (Ultra Scan XL scanning laser densitometer Pharmacia LKB, Uppsala, Sweden). The quantitification was performed using an Origin 3,5 PC software package.

Statistical analysis. The significance of differences between different experimental values was assessed by means of ANOVA and Student's *t* tests. Results were considered to be significant if the p-value was less than 0.05. In all cases the same degree of significance was obtained with both tests.

Results

In order to examine the HSP70 expression in the cytosolic, membranous and nuclear fractions of colorectal cancer cells we performed immunohistochemical and Western blot analyses.

Immunohistochemical staining showed that HSP70 was expressed in 44 of 48 primary tumors (91.6%) which was significantly higher than in normal mucosa (six of 12 i.e. 50%). Using the mAb C92 F3A-5, inducible form of HSP70 (HSP72) was detected mainly in the cytoplasm of both colorectal cancer cells (Fig. 1) and control tissue specimens, by a diffuse pattern. The HSP70 immunoreactivity was seen throughout the neoplastic epithelium, whereas the HSP70 expression in normal control colon epithelium was in the apical half of the crypt. In contrary to weak-to-moderate immunoreactivity detected in the cytoplasm of control colorectal epithelial cells, the cytoplasmic immunoreactivity in colorectal carcinoma cells was mainly moderate-to-strong. The statistically significant difference in the score of cytoplasmic HSP72-immunoreactivity was found between the lymph node-negative and lymph node-positive tumors (Fig. 2a). In addition, the Duke's stage C2 tumors express HSP72 at significantly higher level than Duke's B1 and B2 stage groups (Fig. 2b). There was no relation between expression of HSP70 and histological grade of tumors (data not shown).

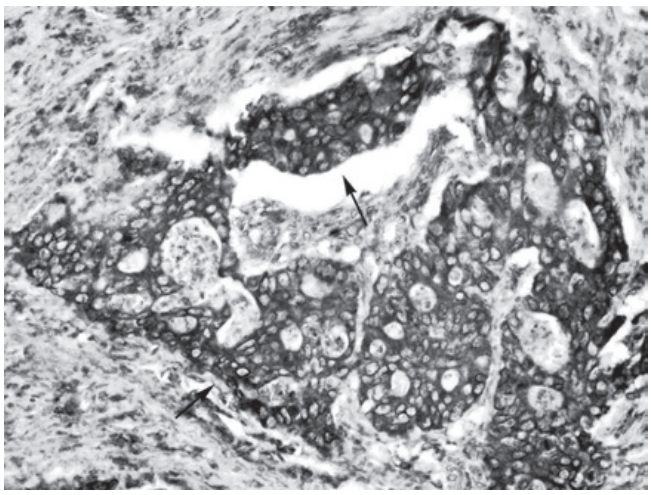


Figure 1. HSP72 immunostaining in malignant cells from a colorectal carcinoma Immunoreactivity specific to HSP72 detected in the cytoplasm of the cancer cells (arrows) of poorly differentiated colorectal adenocarcinoma, stage G3C2. Avidin-biotin-peroxidase complex (ABC) method, x20.

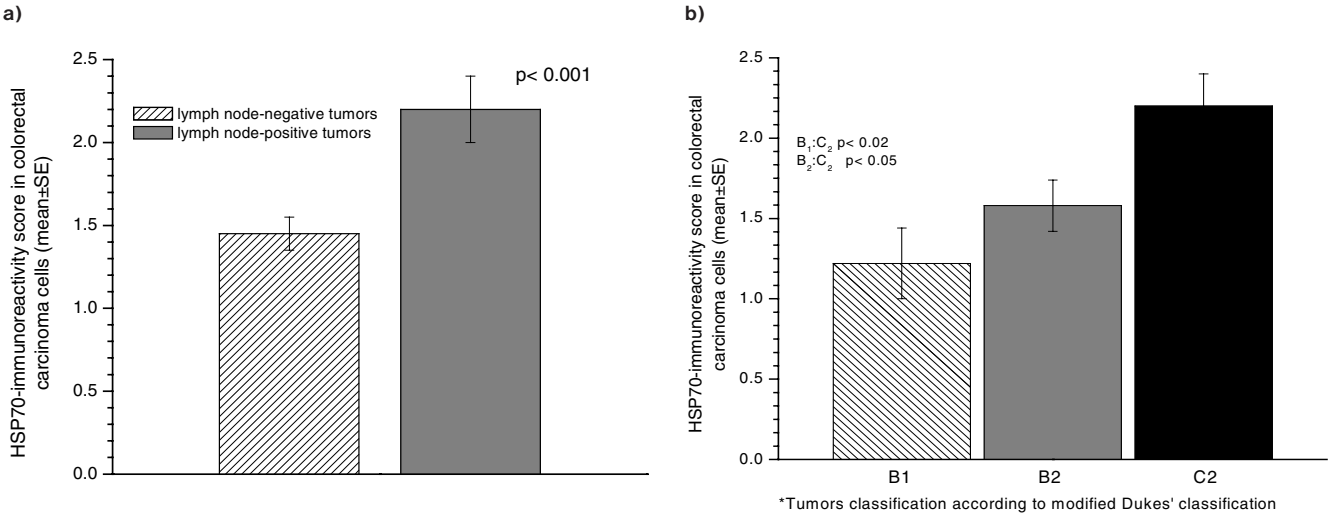
We detected weak HSP70 immunoreactivity in the plasma membrane area of tumor cells (Fig. 3) and in five of six (83.3%) cases weak-to-moderate expression in normal colonic mucosa.

Immunoreactivity specific to HSP70 was observed in certain nuclei of colorectal cancer cells, but there was no relation with histopathological characteristics of the tumors. In the nuclei of normal mucosa cells, were detected HSP70s by mAb N27 F3-4, which recognize both forms of 70 kDa heat shock protein.

Cytoplasmic and nuclear immunostaining of HSP72/73 in the tumor cells did not significantly differ among groups of tumors (data not shown).

Results of Western blot analysis of HSP70 in nuclear (Lane 1, 2, 3), cytosolic (Lane 4, 5, 6) and plasma membrane (Lane 7, 8, 9) fractions of colorectal normal and cancer cells (probed with mAb BB70 which recognize both the constitutive and inducible forms of HSP70) are shown in Figure 4. In the cytosolic fractions of most of the colorectal cancer cells elevated level of HSP70, compared with normal mucosa was detected.

HSP70 proteins, both forms, were detected in 60% of plasma membranous fractions obtained from colorectal cancer cells (Figs. 4 and 5). Their expression was lowered compared to fractions from normal mucosa tissues as obtained by densitometrical analysis of blots (Fig. 6). HSP70 proteins



Figures 2a, b. Expression of cytoplasmic HSP72 in colorectal tumors of different stages Tumors were classified according to the Dukes' classification (Astler-Coller modification) as B_1, B_2, and C_2. The expression of cytosolic HSP72 was elevated in lymph node-positive cancers compared to negative (Fig. 2a) and significantly increased in more advanced tumor stage (Fig 2b).

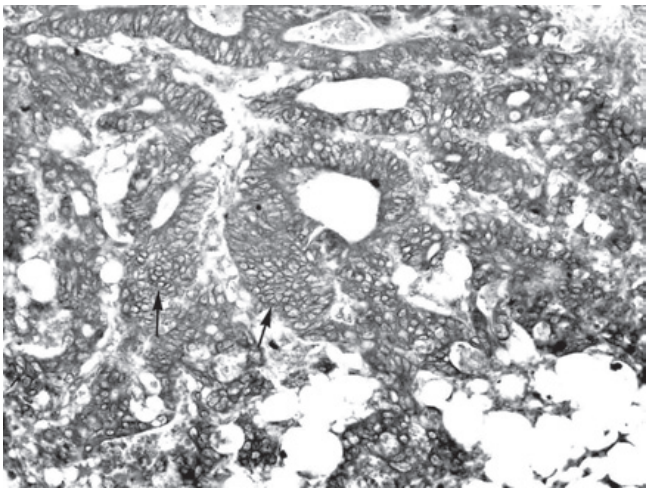


Figure 3. Expression of HSP72 in membranes of colorectal carcinoma cells Immunoreactivity specific to HSP70 was detected in the cell membrane of cancer cells (arrows) of poorly differentiated colorectal adenocarcinoma stage G2C3. Avidin-biotin-peroxidase complex (ABC) method, x10.

were detected in some of plasma membranous fractions obtained from adjacent normal mucosa (Figs. 4 and 5) and from non-tumor tissues (Fig. 5).

The cytosolic HSP70 expression is elevated in more advanced stages of colorectal carcinoma, and this result is comparable to that obtained by immunohistochemistry. Some additional bands were detected at lower molecular mass, which are probably the degradation products of HSP70.

Discussion

In this study, we assayed by immunohictochemistry and Western blot the expression of both constitutive and inducible forms of HSP70 (HSP72/73) as well as inducible form only (HSP72) in 48 samples of colorectal adenocarcinoma and 12 adjacent normal tissues or non-tumor colorectal tissue, in order to determine the expression pattern and level of expression. Histopathological results were also considered to establish the clinical relevance of HSP70 in colorectal cancer.

Colorectal cancer is a heterogeneous group of tumors made up of cancer cells with diverse growth rates and metastatic potential. Because clinical behavior of these tumors is often different among patients classified in the same pathological or clinical stage, there are continuous research efforts to provide additional information to aid in diagnosis and to predict the clinical behaviors of colorectal carcinoma.

Multiple proto-oncogenes, oncogenes, regulatory factors, and tumor suppressor genes have been suggested to play a role in the progression of colorectal tumors (26–28). Recently, Stoler et al found approximately 11,000 genomic mutations per colon carcinoma cell (29), although whether or not all of those mutations contributed to the malignant state of the cells, remains to be determined. Also, hypoxia increases ability of tumor cells to metastasize and strongly influences malignant progression of tumors by modulating different patterns of gene expression (30). Many malignant solid tumors, including colorectal tumors, contain significant fractions of hypoxic cells, which is apoptosis-resistant population of cells and which presence has been considered a problem in cancer treatment (31). Generally, molecular pathogenesis of colorectal cancer still remains to be elucidated.

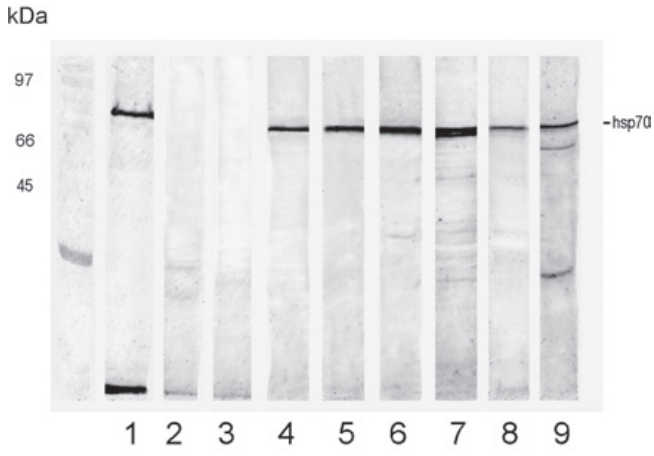


Figure 4. Western blot analysis of HSP72/73 in nuclear, cytosolic and membrane fractions of colorectal normal and cancer cells.
Lane 1 – nuclear fraction of normal mucosa, 50 μg/lane; Lane 2, 3 – nuclear fraction of colorectal cancer, *G2B2, G3B2, 50 μg/lane; Lane 4 – cytosolic fraction of normal mucosa, 20 mg/lane; Lane 5, 6 – cytosolic fraction of colorectal cancer cells, G2B2, G3C2, 20 μg/lane; Lane 7 – membrane fraction of normal mucosa, 50 μg/lane; Lane 8, 9 – membrane fraction of colorectal cancer cells, G2B2, G3B2, 50 μg/lane.
***Dukes' stage and degree of differentiation Left margin is molecular mass standard (kDa). Proteins with 72/73 kDa are indicated in the figure as HSP70.**

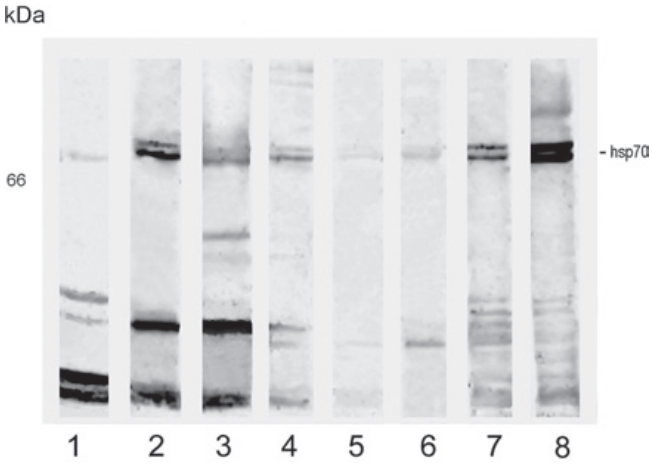


Figure 5. Western blot analysis of HSP70 in membrane fractions of colorectal cancer cells according to different stages of tumors.
Lane 1 – molecular mass in kDa of the standard proteins; Lane 2, 3 – membranes of normal mucosa; Lane 4, 5, 6, 7, 8 – membranes of colorectal cancer cells with different stage, *G1B1, G2B2, G1C2, G1C2 and G3C2, respectively.
***Dukes' stage and degree of differentiation**

There is evidence that HSP is a group of highly-conserved proteins synthesized after heat induction. During the growth and development of normal cells, including molecularly normal colon, HSP70 is constitutively expressed at low or moderately levels (13), but the expression is dramatically enhanced by stressful condition, such as heat, hypoxia, neoplasia and virus infection (33, 34). Constitutively expressed HSPs act as molecular chaperones which function, mainly, by assisting in folding newly synthesized proteins and their translocation into organelles. HSPs have also been considered to play general role in protection from cellular injury (4).

Many studies suggested that HSPs are continuously expressed at high levels in tumor cells without any stimulation, and that there is a possible correlation between the expression of HSP70 and the growth and progression of tumor cells. HSP70 has been reported to be overexpressed in various cancers such as breast (35), lung (36), stomach (37), melanoma (38), and hepatocelular carcinoma (39). However, limited and contradictory information is available in the HSP70 expression pattern in colonic cancer tissues, as well as in the correlation between clinicopathology and this expression (13–16, 32).

We presently demonstrate that the inducible form of HSP70 was detected mainly in the cytoplasm of epithelial colorectal cells of control tissue specimens. Namely, a weak-to-moderate immunoreactivity was detected in the cytoplasm with a diffused pattern, mostly in the apical half of the crypt. These results are in accordance with results of the study of Hwang

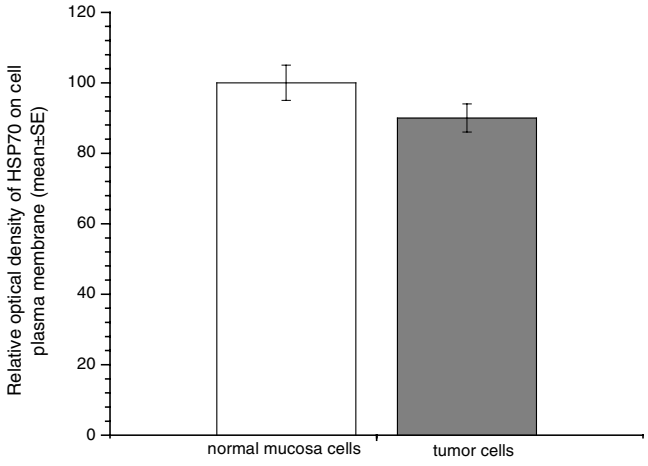


Figure 6. Densitometrical analysis of HSP70 plasma membrane expression of normal mucosal and colorectal cancer cells
Data represent the quantities of HSP70 on the plasma membrane of normal and colorectal cancer cells.

et al. (13). They were analyzed by immunohistochemistry 81 pairs of primary colorectal carcinoma and adjacent, paired normal tissues, and revealed a weak and diffused cytoplasmic expression of HSP70 in the majority of normal colorectal epithelial cells. Murphy et al (2001) identified protein expression patterns, including HSP70,what indicates that a cell is

normal, in aim to defining a molecularly normal colon. In contrary to our results, it was found that in grossly uninvolved colonic musoca HSP70 was expressed in very high level (32). It is very known that the constitutively expressed HSP70 acts as molecular chaperone which functions by assisting in folding newly synthesized proteins and their translocation into organelles, and may have anti–oncogenic potential (4).

In our study, immunohistochemical staining showed that HSP70 was expressed in 44 of 48 primary colorectal tumors (91.6%), which was significantly higher than in normal mucosa. Predominantly pattern of immunoexpression in colorectal tumor cells is cytoplasmic expression. Using Western blot analyses, we noticed elevated levels of cytosolic HSP70 in colorectal cancer cells compared to normal. These results are in accordance with some previous studies (14, 16). Namely, results of Kanazawa et al (2003) concerning frequency of HSP70 immunopositive colorectal carcinoma, are little lower than our results: among 50 colorectal cancer tissue studied, 80% of tumors showed specific immunoreactivity to HSP70 (14). Wang et al (2005) observed, very similarly to our results, that among 80 colorectal carcinomas 92.5% express HSP70 (16). In those both studies, HSP70 was overexpressed in cancer tissue samples compared with normal tissue or with adjacent mucosal epithelial cells. Apparently these results remain at variance with the observation by Ozdemirler Erata et al. (2005), who proposed, by using Western blotting procedure, that inducible HSP70 expression was suppressed under induced oxidative stress conditions in malignant tissue of 20 colorectal carcinomas, as compared with paired samples of adjacent normal tissues (15). Also, Murphy et al. (2001) revealed upregulation early, but downregulation later in colon adenocarcinoma (32).

There are a few and controversial studies on the clinical significance of HSP70 expression in human colorectal cancer (13–16, 40, 41), as well as in other tumors (35, 37, 42, 43). According to our results, the association between inducible HSP70 (HSP72) expression and the Dukes' stage of colorectal carcinoma exist. It was found that cytoplasmic HSP72 immunohistochemical score, as well as expression of HSP72 determined by Western blotting, were significantly elevated in colorectal cancer cells, particularly in more advanced stages with lymph node involvement. We did not find a significant relation between HSP72 expression and degree of tumor cell differentiation. These results are consistent with some researches (13, 16) but are not with others (14, 15). To explore to potential role of HSP family members in the malignant metastatic progression of human colorectal carcinoma, Hwang et al (2003) have initiated their study by analyzing the expression profiles of HSP70 and HSP110 proteins, by Western blot analysis, in weakly metastatic colorectal cancer cell lines (Clone A and MIP-110), and highly metastatic cell lines (CX-1 and HT-29). Those authors subsequently examined the expression profiles of these proteins in 81 surgical specimens of human colorectal carcinoma tissues, by immunohistochemistry. Results obtained from colorectal carcinoma cell lines have shown that HSP70 and HSP110 were preferently elevated in highly metastatic CX-1 and HT-29 cell lines. Interestingly, consistent with results obtained with those cell lines, immunohistochemical results obtained from surgical specimens of colorectal carcinoma revealed that expression of both HSP70 and HSP110 were strongly correlated to positive regional lymph node involvement and advanced clinical stages of the cancers, but there was no correlation with tumor differentiation (13). Similarly, results of the study of Wang et al (2005) also revealed that Dukes C and D stages of colonic cancers showed higher positive rates than Dukes A and B stage groups and that there were differences in HSP70 expression between metastatic and non-metastatic groups (16). During the progression of colonic cancer, inducible HSP synthesis increases gradually (40). Similarly to our results, it is suggested that the HSP70 could be an indicator of the disease recurrence in patients with breast carcinoma (35), and that HSP70 correlated with adverse prognostic indicators, i.e. relatively high tumor grade and presence of nodal metastases in mouse fibrosarcoma (42). Taken together with our results, observations mentioned above suggested that HSP70 might play some role in the malignant metastatic progression of colorectal cancer in a stage-dependent manner. Considering that colorectal carcinoma is a heterogeneous neoplasm whose clinical behavior is often different among patients classified in the same clinical stage, we also believe, according to Hwang et al, that analysis of HSP70 expression would provide, at least in a part, additional information about prediction the clinical behaviors of colorectal carcinoma (13).

However, in contrary to our results, it has been reported that HSP70 is not associated with the stage and/or differentiation of colorectal tumors (14, 15, 41) as well as with advanced characteristics of tumors in gastric cancers (37).

Why is the HSP70 overexpression in cytosol reciprocal in terms of the clinical stage of colorectal carcinoma and what might be the mechanisms of this expression patterns? It is well known that tumor cells progress gradually through mutant oncogene products. The existence of mutant or oncogene products may stimulate HSP synthesis because HSPs acts as molecular chaperones in regulating and stabilizing these products during tumor growth (1, 13, 39, 41). Namely, HSPs appear to be integrated with mutant p53 to stabilize its function (1). It is postulated that a complete regulatory loop involving p53 and HSP70 might be involved in machinery that controls cell cycle progression and tumor cell proliferation, including colorectal tumors (39, 41). In our study overexpression of HSP70 is also associated with abnormal expression of mutant tumor suppressor p53 protein in colorectal cancer cells (unpublished observation), which could confirm the malignant potential of these specimens. From these observations, we can speculate that HSP70 may be useful in predicting clinical behaviour of the colorectal carcinoma's clinical behavior, since HSP70 expression could characterize the biological phenotype of particularly aggressive cancers, which either manage to

immunoescape, or elicit an immune response not efficient enough to suppress tumor progression.

In our study, densitometric analysis of blots of plasma membrane HSP70 expression has shown decrease in colorectal cancer cells compared to normal mucosa. We and others have determined a tumor-specific plasma membrane localization of HSP70, including colon carcinoma cell lines (8, 44, 45). The role of the plasma membrane expression of HSPs, the transport mechanism and membrane anchorage is still an open question. Recently, many studies demonstrated that release of HSP70 from viable tumor cells could be enhanced by exogenous stress, and alternative vesicular pathway of HSP70 export from colon carcinoma cells was hypothesized, not involving the endoplasmic reticulum–Golgi compartment (45, 46). Also, many studies provided evidence that HSP70 surface-positive tumor exosomes stimulate migratory and cytolitic activity of NK cells and that NK cells preincubated with HSP70 surface-positive exosomes initiated apoptosis in tumors through granzyme B release. In addition, exosome-mediated lytic activity of NK cells was blockable by HSP70 specific antibody (8, 47). Exosomes are internal vesicles produced by inward budding of endosomal membranes in a process sequestering particular proteins and lipids. With respect to their biogenesis, exosomal surfaces resembled that of the plasma membranes from the tumor sublines from which they originated (48).

By using autologous tumor sublines with different HSP membrane expression pattern, earlier studies of Multhoff's group have demonstrated that surface appearance of HSP70 immunogenic determinants (i.e. TKD) correlates with susceptibility of tumor cells to lysis by NK cells in non-MHC dependent fashion, and that HSP70 high-expressing tumor cells are killed significantly better by NK cells as compared with their low-expressing counterparts (49–52).

Also, it is shown that the cytolytic activity of NK cells against HSP70 membrane-positive colon carcinoma cells was enhanced after TKD/IL-2 stimulation in 9 of 11 patients with metastatic colorectal cancer who had failed standard therapies and it is postulated that reinfusion of HSP70-activated autologous NK cells is safe in these cases (10). Finally, functionally, HSP70/Bag-4 and HSP70/HSP40 membrane-positive tumor cells appeared to be better protected against radiation-induced effects, including G2/M arrest and growth inhibition. On the other hand, membrane-bound HSP-70, but neither Bag-4 nor HSP40, served as a recognition site for the cytolytic attack mediated by NK cells (9).

Taken together from the above observation concerning both functions of membrane-expressed HSP70 and biological properties of membrane-positive colon carcinoma, we have speculated that decrease of plasma membrane HSP70-expression of colorectal cancer cells compared to normal mucosa detected in our study, may result in significantly lower tumor cell killing by NK cells, and in poor protection of tumor cells against radiation-induced effects, including G2/M arrest and growth inhibition.

Currently, the examination of HSP70 is of special scientific interest, because of their possible clinical applications in cancer prevention (vaccine) and therapy. Namely, recent studies provided evidence that increased expression of inducible HSP70 in apoptotic cells is correlated with their efficacy for antitumor vaccine therapy (12). Also, treatment of colon and lung cancer patients with ex vivo heat shock protein 70-peptide-activated, autologous natural killer cells is now in a clinical phase trial (10). In addition, it is evidence of eradication of glioblastoma, and breast and colon carcinoma xenografts by HSP70 depletion (5).

In conclusion, our study demonstrated a different cytosol and plasma membrane HSP70-expression in tumor cells of human colorectal carcinoma, i.e. stage depending overexpression of cytosolic HSP70, and decreased membrane expression of HSP70. The molecular mechanism underlying the HSP expression may offer novel models of strategy to prevent and manipulate the malignant progression of colorectal carcinoma.

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