High levels of acute phase proteins and soluble 70 kDa heat shock proteins are independent and additive risk factors for mortality in colorectal cancer

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Abstract Recently, we reported that high soluble Hsp70 (sHsp70) level was a significant predictor of mortality during an almost 3-year-long follow-up period in patients with colorectal cancer. This association was the strongest in the group of <70-year-old female patients as well as in those who were in a less advanced stage of the disease at baseline. According to these observations, measurement of the serum level of sHsp70 is a useful, stage-independent prognostic marker in colorectal cancer, especially in patients without distant metastasis. Since many literature data indicated that measurement of C-reactive protein (CRP) and other acute phase proteins (APPs) may also be suitable for predicting the mortality of patients with colorectal cancer, it seemed reasonable to study whether the effect of sHsp70 and other APPs are related or independent. In order to answer this question, we measured the concentrations of CRP as well as of other complement-related APPs (C1 inhibitor, C3, and

C9) along with that of the MASP-2 complement component in the sera of 175 patients with colorectal cancer and known levels of sHsp70, which have been used in our previous study. High (above median) levels of CRP, C1 esterase inhibitor (C1-INH), and sHsp70 were found to be independently associated with poor patient survival, whereas no such association was observed with the other proteins tested. According to the adjusted Cox proportional hazards analysis, the additive effect of high sHsp70, CRP, and C1-INH levels on the survival of patients exceeded that of high sHsp70 alone, with a hazard ratio (HR) of 2.83 (1.13–70.9). In some subgroups of patients, such as in females [HR 4.80 (1.07–21.60)] or in ≤70-year-old patients [HR 11.53 (2.78–47.70)], even greater differences were obtained. These findings indicate that the clinical mortality–prediction value of combined measurements of sHsp70, CRP, and C1-INH with inexpensive methods can be very high, especially in specific subgroups of patients with colorectal cancer.

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Introduction

Recently, we reported that high soluble Hsp70 (sHsp70) level [hazard ratio (HR): 1.88 (1.20–2.96, $p=0.005$)] was a significant predictor of mortality during a follow-up period of 33.0 (24.4–44.0) months in 179 patients with colorectal cancer (Kocsis et al. 2010). Further analysis revealed that the significant association between high Hsp70 levels and poor survival was the strongest in the group of <70-year-old female

patients. These novel findings indicate that the serum level of sHsp70 might prove a useful, stage-independent prognostic marker in colorectal cancer without distant metastasis.

Many findings have been published lately to indicate that a similar association exists between the mortality of patients with colorectal cancer and some APPs or other inflammatory markers. Allin et al. (2009) in their large-scale prospective study performed in the general Danish population found a significant association between high baseline CRP levels and the development of different types of malignancy including colorectal cancer. Moreover, in patients with advanced cancer (mostly in those without a distant metastasis), high baseline CRP levels were found associated with early death from colorectal cancer. Japanese authors also observed a strong correlation between high preoperative CRP levels and poor survival of patients with colorectal cancer (Nozoe et al. 2008). Similar findings were reported by Chinese authors for different types of cancer including colorectal cancer (Wang and Sun 2009). The predictive value of CRP was found to be independent of CEA levels (Koike et al. 2008). Moreover, not only CRP but also different scores derived from inflammatory markers were found strongly related to the survival of patients with colorectal cancer (Ishizuka et al. 2009).

The aim of our present work was to study whether the high predictive value on the poor survival of colorectal cancer patients with high sHsp70 levels is related to or is independent of the similar effect of the high levels of APPs. Therefore, concentrations of CRP, as well as of other complement-related APPs (C1 inhibitor, C3, and C9), were measured in the sera of colorectal cancer patients with known levels of sHsp70. Furthermore, the concentration of an additional non-acute phase complement protein, MASP-2, was determined in the same serum samples, as the concentration of this protein had been found associated with the mortality of patients with colorectal cancer (Ytting et al. 2005, 2008).

Materials and methods

Patients

This study was performed in the outpatient oncology clinic of the 3rd Department of Internal Medicine, Semmelweis University, between October 2000 and March 2005. A number of 175 consecutive patients, diagnosed with colorectal cancer, who were willing to give informed consent for the study, were enrolled regardless of tumor stage. This patient cohort was the same, which we already tested for the association between sHsp70 levels and survival (Kocsis et al. 2010), except four patients with no available serum samples for the present study. In the majority of cases, the

primary tumor was removed surgically, according to relevant international guidelines, and patients were enrolled 4–6 weeks after surgery. In 16 cases, the primary tumor could not be removed before inclusion; these patients had advanced, metastatic tumors and were referred for primary chemotherapy. All but 30 patients received adjuvant or first-line, fluorouracil (5-FU)-based chemotherapy according to current national guidelines. Those who did not receive chemotherapy had a very early stage tumor (Dukes A, B1, $n=22$) or had refused treatment ($n=8$). Patients were prospectively followed up for 33.0 (23.5–43.5) months (with an acceptable drop-out rate of $8/175=4.6\%$). Reasons for leaving the study included moving to another location or were unknown. Baseline clinical characteristics of all patients as well as of two subgroups of the study population (i.e., patients who had died before the median survival time for the whole group, 33 months elapsed, and those who survived this period) are summarized in Table 1.

Tumor staging was done according to the system described in the paper of Greene (2007). Briefly, we define TNM-T staging as: stage 0 no evidence of a primary tumor, stage 1 the tumor invades submucosa, stage 2 the tumor invades muscularis propria, stage 3 the tumor infiltrates the muscularis propria and propagates to the subserosa or to non-peritonealized pericolic or perirectal tissues, and stage 4 the tumor directly invades other organs or structures and/or breaches the visceral peritoneum. The TNM-N staging was defined as: stage 0 no regional lymph node metastasis, stage 1 metastasis in 1 to 3 regional lymph nodes, stage 2 metastasis in four or more regional lymph node. The TNM-M staging was defined as: stage 0 no distant metastasis, stage 1 distant metastasis.

Laboratory methods

The sHsp70 levels in the serum were measured using the ELISA kit (DYC163E, R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions as detailed earlier (Molvarec et al. 2006). The detection range of the assay was 0.05–10 ng/ml; and the intra-/inter-assay variability was $<10\%/<16\%$, respectively. Serum concentration of the highly sensitive CRP was determined with the particle-enhanced immunoturbidimetric assay (Roche Cobas Integra 4000). Serum levels of C1 inhibitor, C3, and C9 were measured by radial immunodiffusion, as described in detail previously (Biro et al. 2000; Derzsy et al. 2010; Szeplaki et al. 2007). Serum concentration of MASP-2 was determined using the method described in Varga et al. (2008).

Statistical analysis

The nonparametric Mann–Whitney test was used for group comparisons. Categorical data were compared

Table 1 Demographic and clinical characteristics of patients with colorectal cancer

	Patients who died in ≤33months (median)	Patients who survived 33months (median)	<i>p</i> Values ^a	All patients
Number of patients	90	85	–	175
Males/females	52/33	47/43	0.232	99/76
Age at baseline, years (mean ± S.D.)	67.8±10.0	64.4±11.5	0.056	66.3±10.7
TNM-T stage, 0/1/2/3/4/not available	0/0/15/49/14/12	1/4/18/53/5/4	0.047	1/4/33/102/19/16
TNM-N stage, 0/1/2/not available	23/38/12/17	45/29/7/4	0.010	68/67/19/21
TNM-M stage, 0/1/not available	58/25/7	73/8/4	0.001	131/33/11
Grade 1/2/3/not available	6/35/23/26	11/48/13/43	0.015	17/83/36/39
Localization of the primary tumor				
Colon	49 (54%)	62 (73%)	0.008	111
Sigmoid	11 (12%)	2 (2%)		13
Rectum	30 (33%)	21 (25%)		51
CRP, ng/ml,	8.54 (2.07–25.33)	3.05 (1.32–6.69)	0.003	4.83 (1.56–16.89)
C1-INH, mg/ml	0.29 (0.24–0.34)	0.27 (0.19–0.32)	0.044	0.28 (0.23–0.34)
C3, g/l	2.05 (1.37–2.63)	1.77 (1.14–2.21)	0.015	1.89 (1.20–2.40)
C9,% of NHS pool	142 (111–168)	118 (80–172)	0.153	1.33 (99–168)
MASP2, ng/ml	105 (67–152)	118 (84–171)	0.204	112 (79–170)
sHsp70, ng/ml	1.62 (1.22–2.58)	1.74 (1.21–2.37)	0.775	1.68 (1.21–2.39)

^a χ^2 test (discrete variables, *t* test, or Mann–Whitney test (continuous variables))

using Fisher's exact test or the χ^2 test for trend. Multiple logistic regression and Cox proportional hazards models were applied to evaluate potential confounders and to correct the *p* values of univariate analyses. All the tests were two-tailed. Statistical analysis was performed using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, www.graphpad.com) and SPSS 13.0 (SPSS, Chicago, IL) softwares. *p* < 0.05 was considered as significant.

Results

Relationship between the mortality of colorectal cancer patients and the serum concentrations of the proteins tested

This patient cohort was the same, which we already tested for the association between sHsp70 levels and survival (Kocsis et al. 2010) except four patients with no available serum samples for the present study. Demographic characteristics, as well as clinical and laboratory findings, in the two subgroups of patients (patients who died before the median survival time for the whole group, i.e., 33 months had elapsed, and those who survived that period) are shown in Table 1. Survivors had less advanced disease according to all staging and grading systems, as well as there were fewer patients with colon cancer in the survivor group. Serum concentrations of

CRP, C1-INH, and C3 were significantly lower in the second (survivor) than in the other group, whereas there were no significant differences in C9 and MASP-2 levels between the two groups (Table 1). Localization of the tumor did not influence the serum concentrations of the CRP (*p* = 0.25) and sHsp70 (*p* = 0.581), while serum level of C1-INH was slightly but significantly (*p* < 0.001) higher in patients with rectal cancer [0.30 (0.25–0.37) mg/ml, median (IQ range)] than in those with colon cancer [0.26 (0.20–0.31) mg/ml].

High CRP, C1-INH, and sHsp70 levels independently predict survival

Evaluating the aggregate effect on survival of the variables, which were significantly different between the two groups—using age- and sex-adjusted Cox-regression analysis—together with sHsp70, we found that not only high sHsp70 [HR 1.98 (1.13–3.49, *p* = 0.018)] but also high CRP [HR 2.06 (1.16–3.66), *p* = 0.014] and (with a marginal significance) high C1-INH levels [HR 1.67 (0.93–3.00), *p* = 0.084] were independent predictors of mortality. However, no such association with high C3 levels could be detected.

sHsp70 levels did not correlate to C1-INH concentration [Spearman correlation coefficient: 0.063 (*p* = 0.40)] and only weakly to CRP (*R* = 0.196, *p* = 0.022), whereas no significant correlation was found between C1-INH and CRP concentrations (*R* = 0.028, *p* = 0.744).

The combination of high CRP, C1-INH, and Hsp70 levels is a better predictor of mortality than high sHsp70 level alone

Next, we studied whether the addition of concomitant, high levels of the two mortality-associated APPs—CRP and C1-INH—increases the mortality-predictive value of sHsp70 measurement alone (Table 2). A risk due to two or more variable is additive: (a) when there is no strong correlation between them; and (b) if at the multiple regression models (such as in Cox proportional hazard model analysis used in the present study), addition of a variable (covariate) to a given model significantly increases the predictive power of the model. Therefore, in the following, successive models (blocks) were built using Cox proportional hazards analysis (Table 2). The addition of high/low CRP concentration significantly ($p=0.009$) increased the predictive power of the model containing high/low sHsp70, age, and sex. The addition of high/low C1-INH also resulted in a marginally significant ($p=0.077$) increase (Table 2).

The combined effect of CRP, C1-INH, and sHsp70 was significantly higher than that of sHsp70 alone (Fig. 1). Since we have observed previously that the sex and age of

Table 2 Baseline CRP and C1-INH levels independently and additively increase the predictive value of high sHsp70 levels on the mortality of 175 patients with colorectal cancer (as calculated by Cox regression analysis)

Variables	HR (95% CI)	<i>p</i> Values
Model 1: $\chi^2=6,595$ $p=0.010$		
sHsp70, above/below median*	1.82 (1.14–2.89)	0.012
Model 2: $\chi^2=12,177$ $p=0.007$		
sHsp70, above/below median*	1.73 (1.15–2.86)	0.031
Sex (males/females)	1.48 (0.91–2.39)	0.114
Age, years	1.04 (1.01–1.07)	0.009
LT test (change from previous block $\chi^2=10,804$, $p=0.005$)		
Model 3: $\chi^2=19,960$ $p=0.001$		
sHsp70, above/below median*	1.85 (1.06–3.23)	0.031
Sex (males/females)	1.31 (0.76–2.25)	0.330
Age, years	1.03 (1.00–1.06)	0.099
CRP, above/below median**	2.10 (1.19–3.74)	0.011
LT test (change from previous block $\chi^2=6,763$, $p=0.009$)		
Model 3: $\chi^2=23,123$ $p<0.001$		
sHsp70, above/below median*	1.98 (1.13–3.49)	0.017
Sex (males/females)	1.42 (0.82–2.46)	0.208
Age, years	1.02 (0.99–1.05)	0.137
CRP, above/below median**	2.06 (1.16–3.66)	0.014
C1-INH above/below median***	1.67 (0.93–3.00)	0.084
LT test (change from previous block $\chi^2=3,121$, $p=0.077$)		

* >1.67 vs. <1.67 ng/ml, ** >4.71 vs. <4.71 mg/l, ***** >0.27 vs. <0.27 mg/ml

Fig. 1 Survival of patients with combinations of high and low (see Table 1) levels of CRP, C1-INH, and sHsp70 serum concentrations. 0 Neither is high, 1 only sHsp70 is high, 3 all three are high. Hazard ratio: Cox regression analysis adjusted to sex and age for the comparison of combinations 3 vs. 1 is indicated

patients markedly influenced the effect of high sHsp70 levels on the survival of patients with colorectal cancer (Kocsis et al. 2010), we performed the same stratified analysis for the combined effect of CRP, C1-INH, and sHsp70 as compared to the separate effect of sHsp70 (Fig. 2). The combined effect of the three variables significantly exceeded that of sHsp70 alone only in women and in patients under 70 years of age at baseline. In the latter group, an extremely high HR [11.53 (2.78–4.70), $p=0.001$] was calculated (Fig. 2).

Finally, we studied whether the relationship between the mortality of colorectal patients and concomitant, high serum levels of CRP, C1-INH, and sHsp70—as compared to high sHsp70 level alone—remains significant after adjustment for different clinical parameters (Table 3). According to the Cox regression analysis, adjustment for neither TNM-T nor TNM-M stages decreased the significance of the association. Similarly, adjustment for tumor grade, localization, or therapeutic strategies (chemotherapy or surgical operation) did not affect the association, whereas after adjustment for TNM-N stage, the effect of the combined sHsp70/CRP/C1-INH effect remained only marginally significant (Table 3).

Discussion

Our present findings indicate that measuring some APPs, in addition, can markedly increase the predictive value of sHsp70 determination on the survival of patients with colorectal cancer. In our previous study (Kocsis et al. 2010), high baseline Hsp70 levels were found associated with an age- and sex-adjusted HR of 1.88 (1.20–2.96), $p=0.005$. By contrast, in the present study, the HR for early

Fig. 2 Survival of male vs. female patients (*upper row*), as well as of patients aged less than or equal to 70 years of age or older than 70 years (*lower row*), with high and low (see Table 1) levels of CRP, C1-INH, and sHsp70 serum concentrations. 0 Neither is high, 1 only sHsp70 is high, 3 all three are high. Results of Cox regression analysis adjusted to sex or age for the comparison of combinations 3 vs. 1 are indicated

mortality of patients who had high CRP and C1-INH levels, in addition to a high sHsp70 level, was significantly higher [HR 2.83 (1.13–6.87)] than the separate effect of high sHsp70 alone. Similar and even more substantial differences were observed between the isolated effect of high sHsp70 and the combination of high sHsp70, CRP, and C1-

INH levels, when the analysis was restricted to female patients or to those aged ≤ 70 years [HR 4.80 (1.07–21.60) and 11.53 (2.78–47.70)], respectively. In our previous paper (Kocsis et al. 2010), we already discussed the eventual role of the observed gender differences and speculated that it is due to the interference of the high sHsp70 levels with the

Table 3 Correlation of the high serum levels of CRP, C1-INH, and sHSP70 to the mortality of 175 patients with colorectal cancer during the 33.0 (24.0–44.0)-month-long follow-up period

Model	Variable	Odds ratio (OR)	95% CI	p Values
Adjusted for age, sex, and TNM–T stage	sHsp70 above median*, CRP above median**, and C1-INH above median*** (combination 3) vs. sHsp70 above median, but CRP and C1INH below median (combination 1)	4.10	1.40–11.03	0.010
Adjusted for age, sex, and TNM–N stage	Combination 3 vs. 1	3.43	0.88–13.45	0.077
Adjusted for age, sex, and TNM–M stage	Combination 3 vs. 1	2.74	1.07–7.03	0.036
Adjusted for age, sex, and grade	Combination 3 vs. 1	2.84	1.14–7.35	0.026
Adjusted for age, sex, and localization (colon/sigmoid/rectal)	Combination 3 vs. 1	3.82	1.42–10.29	0.008
Adjusted for age, sex, and the type of chemotherapy (FU-based or another)	Combination 3 vs. 1	2.89	1.14–7.35	0.026
Adjusted for the age, sex, and the surgical operation (yes/no)	Combination 3 vs. 1	2.62	1.26–5.42	0.010

The effect of different other demographic and clinical variables was calculated by Cox regression analysis

* >1.67 vs. ≤ 1.67 ng/ml, *** >4.71 vs. ≤ 4.71 mg/l, ***** >0.27 vs ≤ 0.27 mg/ml

protective effect of the estrogens (Newcomb and Storer 1995). By contrast, serum concentrations of three other complement proteins—C3, C9, and MASP-2—were not found to be independently related to the mortality of patients with colorectal cancer. These latter findings with MASP-2 are at variance with earlier results from a Danish group (Ytting et al. 2005, 2008), probably because of either ethnic differences or the much longer follow-up (7–9 years) in the latter studies.

Similar to the measurement of sHsp70 alone, the predictive value of combined sHsp70+ CRP+ C1-INH measurements was found independent of clinical stage, except the TNM-N stage (Table 2).

These data indicate that the influences of the levels of the soluble 70 kDa heat shock protein and the APPs are independent from each other, and the effects of these variables are additive.

As for the independent and additive association between high sHsp70 and acute phase protein levels as well as poor patient survival, it seems that different processes are related to the two types of serum variables. In our previous paper (Kocsis et al. 2010), we put forward different, tentative explanations for the increase in the levels of extracellular sHsp70 levels in colorectal cancer and assumed that this results from the injury and necrosis of tumor cells, just as it is seen in other diseases where tissue damage occurs. Examples include wound discharge; the peripheral blood of patients with soft tissue trauma (Flohe et al. 2007; Pittet et al. 2002); peripheral and renal vascular disease (Wright et al. 2000); or patients after myocardial infarction (Dybdahl et al. 2005; Satoh et al. 2006) and coronary bypass grafting (Dybdahl et al. 2002). Intracellular Hsp70 is known to have a major role in anti-tumor immunity. Additionally, it is an adjuvant to tumor-associated antigens, and as such, it may induce specific tumor cell killing by cytotoxic T cells (Calderwood et al. 2005). Thus, we speculate that sHsp70 molecules may interact with the specific T cells and inhibit their function. High sHsp70 level in the serum might in this way inhibit cellular anti-tumor immunity and may therefore be responsible for the rapid disease progression to death.

As regards the connection between the high serum concentration of CRP and other inflammatory markers found earlier by many groups (Allin et al. 2009; Ishizuka et al. 2009; Koike et al. 2008; Nozoe et al. 2008; Wang and Sun 2009), several possible explanations were suggested. Acute phase proteins (APPs) are produced and release into circulation predominantly by the hepatocytes due to inflammation, tissue destruction (e.g., trauma, myocardial infarction) infections, or cancer. Under these conditions, the levels of the so-called positive APPs (such as CR or C1-INH) increase while that of the negative APPs (such as albumin or transferrin) decrease. According to Nozoe et al. (2008), the elevation of serum CRP significantly correlated

with a decreased lymphocyte count in the peripheral blood of patients with colorectal cancer (Nozoe et al. 2000), suggesting a possible relationship between elevated serum CRP level and impaired immunity in tumor-bearing patients. CRP is an acute phase protein produced by the liver, and its level is elevated in response to inflammatory cytokines. Therefore, as it was pointed out by Ishizuka et al. (2009), the CRP concentration changes in response to injury, infection, and neoplasia. It is well known that cancer promotes the release of proinflammatory cytokines from tumor cells (Balkwill and Mantovani 2001) or from the immunovascular system, leading, in the extreme, to cachexia (Kotler 2000) and malnutrition.

Although the role of inflammation in cancer is poorly understood, some organs stand a greater risk of developing cancer, when subjected to chronic inflammation (Coussens and Werb 2002; Eaden et al. 2001). Moreover, there is some evidence that anti-inflammatory drugs can reduce the risk of colorectal cancer (Baron et al. 2003). These findings indicate a close relationship between inflammation and cancer.

Several mechanisms can be responsible for of the additive effect of high sHsp70 and high APPs levels on the mortality of patients with colorectal cancer. First, it is possible that extracellular Hsp70 stimulates the production of APPs. Since, however, there was only a weak correlation ($R=0.196$) between Hsp70 and CRP levels [similar to our previous findings in patients with preeclampsia (Molvarec et al. 2009)] and no significant correlation between sHsp70 and C1-INH was found, this explanation seems improbable. It is more likely that the presence of tumor cells and/or an immune reaction against these cells concomitantly induce a stress response and inflammation, and the resulting increased levels of sHSP70 and APPs can both lead to poor patient survival.

The independent effect of the two uncorrelated APPs, CRP, and C1-INH seems intriguing. However, in our earlier work, we had similar findings; for example, while C1-INH levels were predicted, CRP levels did not predict inflammation-associated restenosis of the carotid artery after eversion endarterectomy (Szeplaki et al. 2007).

Clearly, much more studies are necessary to find a definite explanation for the relationship between high levels of some serum proteins and the mortality of colorectal cancer and other cancer types. Our present findings—the independent and additive influences of the APP and sHsp70—however, suggest that different mechanisms are responsible for this association. Regardless of the mechanism, the predictive value of serum protein determination may have an important implication in clinical practice, since identifying patients with poor-risk carcinomas and a low chance for survival at initial diagnosis could make early and individualized therapy possible and would thereby improve clinical outcome. The clinical value of the determination can be even higher in some

subgroups of patients, e.g., in relatively young patients or in female patients with no distant metastases at baseline.

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