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ORIGINAL PAPER

Serum level of soluble 70-kD heat shock protein is associated with high mortality in patients with colorectal cancer without distant metastasis

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Abstract Many findings indicate that measuring the serum concentration of soluble 70-kD heat shock protein (soluble HSP70) may provide important information in cardiovascular, inflammatory, and pregnancy-related diseases; however, only scarce data are available in cancer. Therefore, using a commercial ELISA kit, we measured soluble HSP70 concentration in the sera of 179 patients with colorectal cancer. We investigated the relationship between soluble HSP70 concentration and mortality, during 33.0 (24.4–44.0)months long follow-up. High (>1.65 pg/ml, median concentration) soluble HSP70 level was a significant (hazard ratio: 1.88 (1.20–2.96, p=0.005) predictor of mortality during the follow-up period. When we compared the soluble HSP70 levels in patients with non-resected primary tumors as compared to those who were recruited into the study 4–6 weeks after the tumor resection they were found to be significantly (p=0.020) higher in the former group. Since the patients with non-resected primary tumors had also distant metastasis and died early, we limited the further analysis to 142 patients with no distant metastasis at the beginning of the follow-up. This association remained significant even after multiple Cox-regression analysis had been performed to adjust the data for age and sex (p=0.028); age, sex, and TNM-T stage (p=0.041); age, sex, and TNM-N stage (p=0.021); age, sex, and histological grade (p=0.023); or age, sex, and tumor localization (p=0.029). Further analysis showed that the significant association between high HSP70 levels and poor survival is in the strongest in the group of <70-year-old female patients (HR: 5.52 (2.02-15.15), p=0.001), as well as in those who were in a less advanced stage of the disease at baseline. These novel findings indicate that the serum level of soluble HSP70 might prove a useful, stage-independent prognostic marker in colorectal cancer without distant metastasis.

Keywords Colorectal cancer · Mortality · Heat shock proteins · HSP70 · Predictive marker

Introduction

Heat shock proteins (HSPs) are intracellular, evolutionary conserved proteins with a most important role in maintaining homeostasis of the cells by holding and folding other proteins as well as by protecting the genetic information. Heat shock proteins are usually divided into families according to their molecular weight; currently, 10 kD (HSP10) 27 kD (HSP27), 40 kD (HSP40), 60 kD (HSP60), 70 kD (HSP70), 90 kD (HSP90), and 110 kD (HJSP110) heat shock protein families are known. All these HSPs—HSP27, HSP70, and HSP60—primarily play essential, but diverse roles in tumorigenesis and metastasis formation, by promoting autonomous cell proliferation and inhibiting programmed cell death (Calderwood et al. 2006). Due to the loss of p53 function and the greater expression of the proto-oncogens HER2 and c-Myc, the transcription of heat shock proteins is increased in certain tumor cells (Calderwood et al. 2006).

Although HSPs are intracellular, they can be released from the cells and become detectable in the blood of healthy individuals as soluble HSP (Pockley et al. 1998, 1999). Since the serum levels of soluble HSPs can markedly decrease or increase in different diseases, measurement of HSP concentration may provide clinically important infor-

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mation. Most studies focused on soluble HSP70, the concentration of which can be reliably measured using commercial kits. Measurement of soluble HSP70 was found useful for studying the pathomechanism of cardiovascular disease, and since its level was found to be decreased in patients with coronary artery disease, this HSP is considered cardio-protective (Pockley et al. 2003; Zhu et al. 2003). Accordingly, low HSP70 levels are associated with greater longevity (Terry et al. 2006). On the other hand, infection, inflammation, and necrosis are usually associated with elevated levels of soluble HSP70 (Njemini et al. 2003; Dybdahl et al. 2005; Satoh et al. 2006). Recently, our group reported on the importance of measuring sHSP70 level in pregnancy and pregnancy-related diseases (Molvarec et al. 2006, 2007; Madach et al. 2008).

Abundance of data indicate that the HSP70 content of different cancers is a useful marker of the course of the disease and may predict progression (reviewed in Ciocca and Calderwood (2005)). On the other hand, there are only scarce data on the measurement of soluble HSP70 in cancer. In a prospective study performed in Japan, baseline serum concentration of soluble HSP70 was associated with an increased risk of lung cancer (Suzuki et al. 2006). Plasma level of soluble HSP70 was found to be a potential biomarker for prostate cancer, although its clinical utility is uncertain (Abe et al. 2004). In the present work, we measured serum levels of soluble HSP70 in the blood of patients with colorectal cancer. There are many data indicating that HSP70 expression is high in colorectal tumors and it is related to progression of the disease (Hwang et al. 2003; Dundas et al. 2005; Rau et al. 1999). Moreover, the expression of HSP70 on the membrane of colorectal tumor cells is associated with different routes of metastasis and it is a predictor of patient survival (Pfister et al. 2007). To our best knowledge, however, no data are available on the levels of soluble HSP70 in colorectal cancer and therefore, we decided to investigate this in our study. Clinical follow-up of 179 patients for a median period of 33 months was undertaken along with the recording of clinical events. The relationship between soluble HSP70 levels and patient survival was analyzed. High-baseline concentration of soluble HSP70 in the serum was found to be a useful clinical marker predicting low chance of survival independently of sex and age, as well as most importantly, of clinical predictive markers, such as TNM stages.

Materials and methods

Patients

This study was performed in the outpatient oncology clinic of the Third Department of Internal Medicine, Semmelweis University, between October 2000 and March 2005. One-hundred and seventy nine consecutive patients, diagnosed with colorectal cancer, willing to give informed consent for the study, were enrolled independently of the stage of their tumor. In the majority of cases, the primary tumor was removed surgically according to relevant international guidelines; patients were enrolled 4–6 weeks after surgery. In 16 cases, the primary tumor could not be removed before enrollment, these patients had advanced, metastatic tumor and were referred for primary chemotherapy. All but 31 patients received adjuvant or first-line, 5-FU-based chemotherapy according to current national guidelines. Those who did not receive chemotherapy had very early stage tumor (Dukes A, B1 $n=23$) or had refused treatment ($n=8$). The patients were prospectively followed for 33.0 (24.4–44.0) months (yielding an acceptable drop-out rate of 8/187= 4.3%). Reasons for leaving the study included moving to another location or were unknown. Baseline clinical characteristics of the patients are summarized in Table 1.

Staging of the patients was done according to the system described in the paper of Greene (Greene 2007). Briefly, *TNM-T* stage 0: no evidence of primary tumor, stage 1: tumor invades submucosa, stage 2: tumor invades muscularis propria, stage 3: tumor invades through the muscularis propria into the subserosa, or into non-peritonealized pericolic or perirectal tissues, stage 4: tumor directly invades other organs or structures, and/or perforates visceral peritoneum. *TNM-N* stage 0: no regional lymph node metastasis, stage 1: metastasis in one to three regional lymph nodes, stage 2: metastasis in four or more regional lymph node. *TNM-M* stage 0: no distant metastasis, stage 1: distant metastasis

Table 1 Demographic and clinical characteristic of patients with colorectal cancer

Number of patients	179
Males/females	101/78
Age at baseline, years (mean ± S.D.)	65.6±10.1
TNM-T stage, 0 /1/2/3/4/not available	1/4/35/104/19/16
TNM-N stage, 0/1/2/not available	71/68/19/21
TNM-M stage, 0/1/not available	135/33/11
Grade 1/2/3/not available	17/86/36/40
Tumor resection yes/no	16/163
Localization of the primary tumor	
Colon	107
Sigmoid	11
Rectum	49
Colon+rectum	3
Sigmoid+rectum	1
Colorectal+other tumor	8

Laboratory methods

Soluble HSP70 levels in the serum were measured using the ELISA kit of R&D Systems (DYC163E, Minneapolis, MN, USA), according to the manufacturer's instructions as described in detail earlier (Molvarec et al. 2006). The detection range of the assay was 0.05 to 10 ng/ml; intra-/inter-assay variability was <10%/<16%, respectively. Serum concentration of the IgG type antibodies against HSP70 were determined as described in Molvarec et al. (2009).

Statistical analysis

The non-parametric Mann–Whitney test was used for group comparisons. Categorical data were compared using Fisher's exact test or the χ^2 test for trend. Multiple logistic regression and Cox hazard models were used to evaluate potential confounders and to correct the p values of univariate analyses. The power of our study to observe a 0.7 ng/ml difference in mean HSP70 levels (controls versus patients, Fig. 1) with a type I error of alpha = 0.05 was >0.99; whereas it was 0.78 for the difference between survivors and non-survivors (a difference of 0.52 ng/ml).

All the tests were two-tailed. Statistical analysis was performed using the GraphPad Prism 3.0 (GraphPad Software Inc, San Diego, CA, www.graphpad.com) and SPSS 13.0 (SPSS Inc., Chicago, IL) software.

Results

Serum soluble HSP70 concentration in patients with colorectal cancer according to stage of the disease and grade of cancer

We studied whether baseline serum concentration of soluble HSP70 is dependent on the stage of the disease or on the grade of cancer (Table 2). No significant differences were found between patients in different stages of the disease. Patients with high histological tumor grade had higher soluble HSP70 levels, than those with low-grade tumors, but the difference was only marginally significant. There was no significant correlation between the serum levels of soluble HSP70 and IgG anti-HSP70 antibodies (Fig. 1).

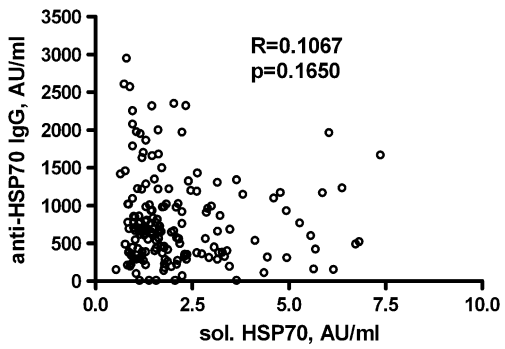

Fig. 1 Relationship between the levels of soluble HSP70 and the IgG type anti-HSP70 antibodies in the sera of 179 patients with colorectal cancer

As it was mentioned above, the primary tumor could not removed before enrollment in 16 cases. We compared soluble HSP70 levels in these patients to those with resected tumors. The median soluble HSP70 level (IQ range) of the patients was significantly (p=0.02) higher in the first (2.34 (1.91–4.51) AU/ml) than in the second group (1.64 (1.18–2.25) AU/ml.

Survival of patients with low and high soluble HSP70 levels

Next, we compared soluble HSP70 levels of the 95 patients who survived and of the 84 patients who did not survive the follow-up period. Median (25th–75th percentile) soluble HSP70 levels were: 1.51 (1.16–2.17) ng/ml and 1.84 (1.22–3.16) ng/ml, respectively (p=0.014, Mann–Whitney test). Next, the 179 patients were divided into two groups, according to median serum HSP70 level (1.65 ng/ml). The *low HSP70* group had serum soluble HSP70 concentration below, whereas the *high HSP70* group had levels equal to or higher than the median. Then, we compared the survival of the patients in the two groups during the 33.0 (24.0–44.0) months long observation period (Fig. 2a).

Patients in the *high HSP70* group had a significantly—almost twice—higher chance to die during the follow-up period, as compared to patients in the *low HSP70* group (Fig. 2a).

As it was described above, the original group of the 178 patients was heterogeneous: 16 patients had tumors at the onset of the study while tumors were resected in the rest of the patients 4–6 weeks earlier. The reason of non-resection of the tumor was that these 16 patients had very advanced metastatic cancer. Considering this fact, we decided to change the form of evaluation and restricted the analysis to patients who had not distant metastasis at the time of the recruitment to the study. This approach restricted the number of patients from 178 to 142. Since all patients of this group belonged to the TNM-M = 0 stage, this stage system was not considered at further analysis.

When the relationship between the serum level of soluble HSP70 and survival was studied in these 142 patients, a comparable hazard ratio (2.17 (1.13–3.99) vs. 1.88 (1.20–2.96)) of non-survival was found for the patients with high soluble HSP70 levels (Fig. 2b)

Next, we studied in these 142 patients whether the association between soluble HSP70 level and survival is dependent on the age and sex of the patients. Age groups

Table 2 Soluble HSP70 levels in the sera of 142 patients with colorectal cancer without distant metastasis, according to stages of the disease or grade of cancer

Stage or grade	Number of patients	Soluble HSP70, ng/ml median (25th–75th percentile)	*p* values Kruskal–Wallis test
TNM-T	37		
0, 1 or 2	92	1.68 (1.32-2.17)	
3	9	1.60 (1.14-2.36)	0.540
4	4	1.57 (0.99-2.74)	
Not available			
TNM-N			
0	68	1.60 (1.18-2.15)	
1	54	1.73 (1.24-2.71)	0.581
2	14	1.55 (1.21-2.14)	
Not available	6		
Grade of cancer			
Low (1 or 2)	90	1.58 (1.1.7)	
High (3)	22	1.94 (1.44)	0.078[a]
Not available	30		

[a] Mann–Whitney test

were defined as less than 70 years or ≥70 years (Fig. 3). Marked differences were observed: this association was restricted to the younger age group. Within the younger age group, female patients had a high (5.52 (2.02–15.15), and highly significant (p=0.001) hazard ratio for non-survival while in the male patients of this group, non-significant (p= 0.980) hazard ratio was obtained

Multivariate analysis of the association between soluble HSP70 levels and survival

In addition to HSP level, patient survival during the almost 4-years-long follow-up period was correlated with TNM-T (p=0.014), and TNM-N (p<0.001) stages. Tumor grade, by contrast, was not related to survival (p=0. 148).

Multivariate analysis was performed to ascertain whether the association observed between high soluble HSP70 concentration and excessive mortality (calculated by univariate analysis) remains significant after adjustment for various demographic and clinical confounders (Table 3).

Different regression models were developed. As evident from Table 3, high soluble HSP70 levels were found to be significantly associated with poor survival, even after adjustment for sex, age, and TNM-T and TNM-N stages; sex, age, and tumor grade; age, sex, and tumor localization, or age, sex, and the type of chemotherapy (FU-based or other).

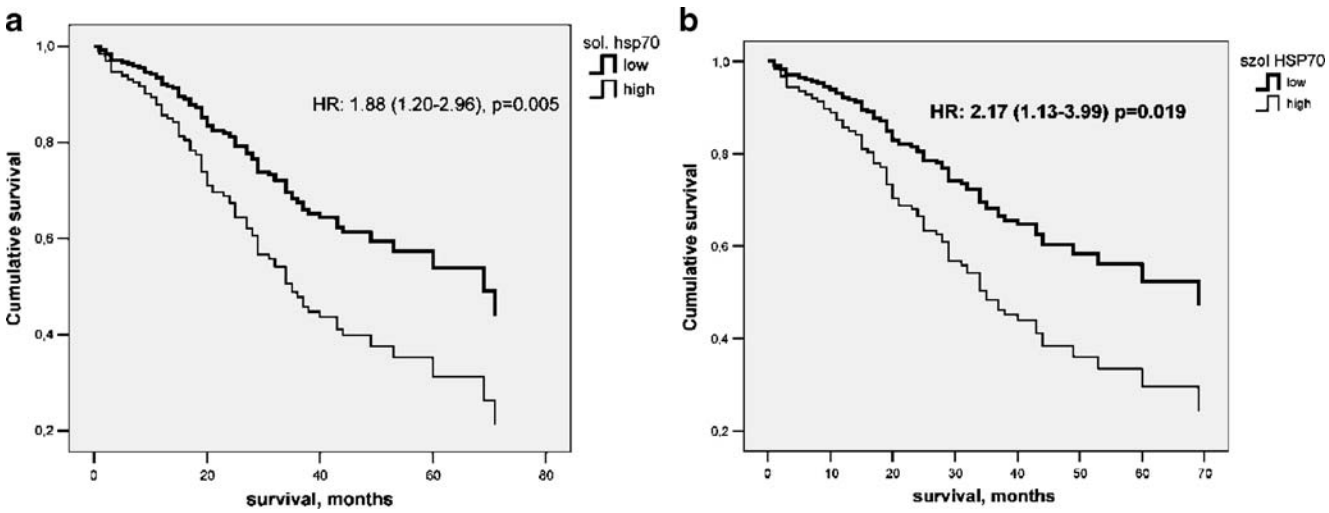

Fig. 2 Survival of colorectal cancer patients with low (<median 1.65 ng/l) or high (≥1.65 ng/ml) soluble HSP70 levels over a 33.0 (24.0–44.0)months long follow-up period. Hazard ratio (HR) with 95% CI for Cox-regression analysis is indicated. **a** All 178 patients, **b** 142 patients without distant metastasis

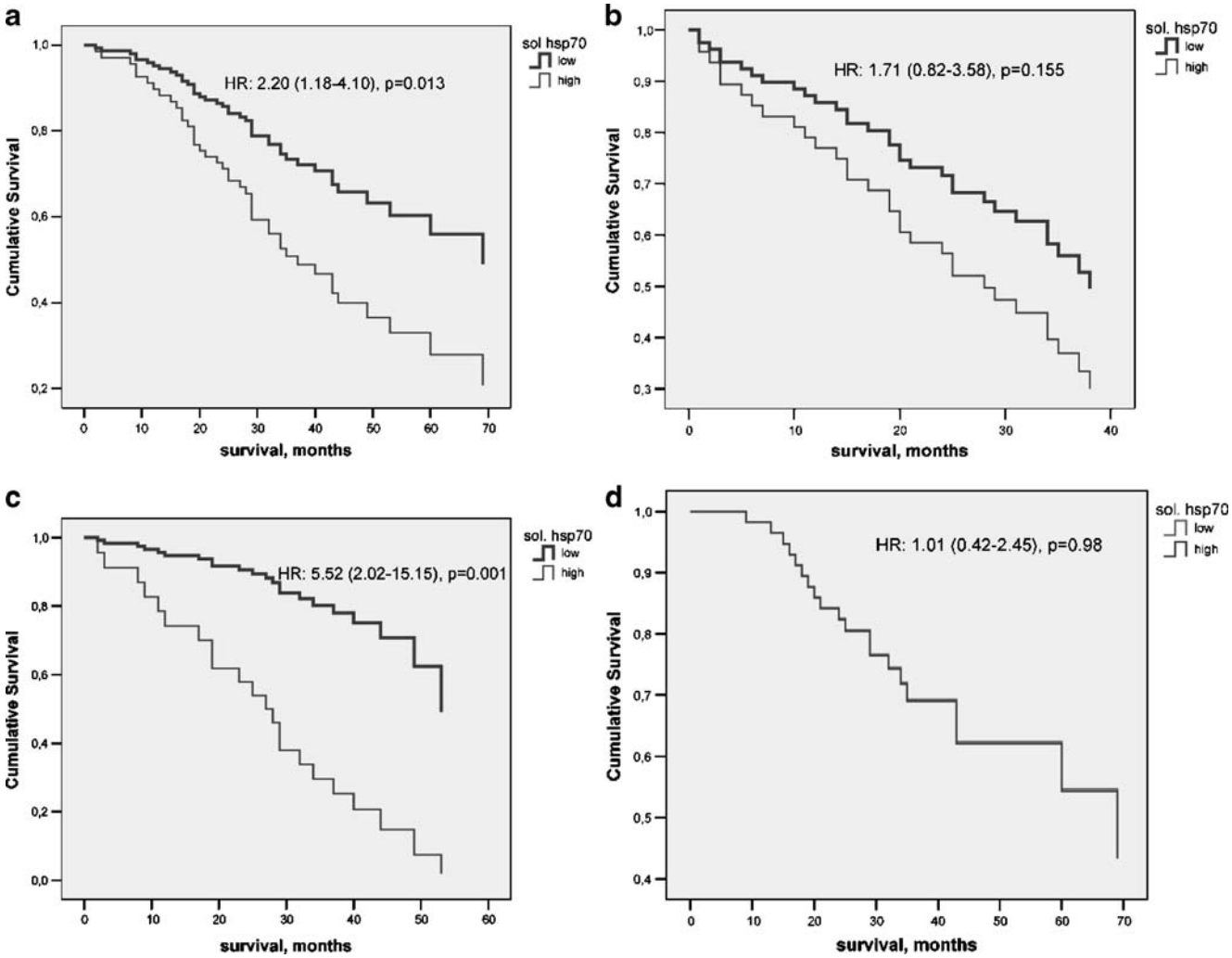

Fig. 3 Survival of patients with no distant metastasis with low (<median 1.65 ng/l) or high (≥1.65 ng/ml) soluble HSP70 levels over a 33.0 (24.0–44.0)-month-long follow-up period. **a** Less than 70-year-old patients; **b**≥70-year-old patients; **c** <70-year-old female patients; **d** <70-year-old male patients. Hazard ratios (HR) with 95% CI for Cox-regression analysis are indicated

It seems therefore that high serum HSP70 concentration is associated with a low survival rate independently of disease stage, histological tumor grade, or cancer localization.

Interaction between high HSP70 levels and the demographic and clinical characteristics of patients in predicting mortality

As high mortality was predicted not only by high soluble HSP70 levels, but by advanced stage of the disease (according to different staging systems) and no difference was found between the HSP70 levels of patients in different stages (Table 2), it seemed worthwhile to test whether soluble HSP70 levels interact with demographic variables and tumor stage in predicting mortality. The interaction tool of the multiple logistic regression was used for this calculation. In addition to age and sex (Fig. 3), highly significant ($p<0.001$) interactions were found with the TNM-T and TNM-N stages. These observations indicate that the strength of the association between high soluble HSP70 levels and patient survival can be quite different in different stages of the disease, whereas neither the grade, nor the localization of the tumor influences this association. Therefore, sex- and age-adjusted association between high soluble HSP70 levels and survival of patients in different TNM stages was calculated. High HSP70 levels were found to be associated with low survival rate in patients in lower (0 to 3) TNM-T stages (hazard ratio—HR, 2.60 [1.22–5.59], $p=0.014$), but not in those in the highest (4) TNM-T stage ($p=0.470$). Similarly, a significant association was observed in patients in the lower (0 or 1), but not in the higher (2) TNM-N stages (HR, 3.58 [1.52–8.47], $p=0.004$ vs. $p=0.971$). This indicates that the association between higher mortality and high soluble HSP70 levels exists in all patients, except those who were in an advanced stage of the disease already at baseline.

Table 3 Relationship between the serum level of soluble HSP70 and the mortality of 142 patients with colorectal cancer without distant metastasis during the 33.0 (24.0-44.0) months long follow-up period. Cox-regression analysis adjusted to different variables

Model	Variable	Odds ratio (OR)	95% CI	*p* value
Unadjusted	Soluble HSP70 high[a]/low[b]	2.17	1.13–3.99	0.019
Adjusted for age and sex	Soluble HSP70 high[a]/low[b]	2.14	1.09–4.22	0.028
Adjusted for age, sex and TNM-T stage	Soluble HSP70 high[a]/low[b]	2.07	1.03–4.17	0.041
	TNM-T stage	1.60	0.82–3.13	0.168
Adjusted for age, sex and TNM-N stage	Soluble HSP70 high[a]/low[b]	2.50	1.15–5.43	0.021
	TNM-N stage	2.26	1.36–3.75	0.002
Adjusted for age, sex and grade	Soluble HSP70 high[a]/low[b]	2.12	1.17–4.22	0.023
	grade	1.01	0.91–1.33	0.851
Adjusted for age, sex and localization (colon/sigmoid/rectal)	Soluble HSP70 high[a]/low[b]	2.18	1.08–4.18	0.029
	localization	1.33	0.95–1.85	0.097
Adjusted for age, sex, and the type of chemotherapy (FU-based or other)	Soluble HSP70 high[a]/low[b]	2.10	1.06–4.15	0.033
	Type of chemotherapy	0.75	0.38–1.49	0.413

[a] >1.65 ng/l

[b] ≤1.65 ng/ml

Discussion

This report discusses the predictive value of measuring baseline levels of soluble HSP70 in the sera of patients with colorectal cancer. High concentration of this protein was associated with a poor survival—patients with a serum concentration above the median (1.65 ng/ml) had more than twice higher age-, gender-, and localization-adjusted chance not to survive the almost 3-years-long follow-up period. Since the original group was found to be heterogeneous as for the presence of absence of tumor at baseline, we repeated the analysis by limiting it to the patients (142/179) who had no distant metastasis (TNM-M=0) at recruitment. Since comparable findings were obtained (Fig. 2), further analysis was restricted to that group. Importantly, this association between high soluble HSP70 level and poor survival was found to be independent of disease stage defined by TNM staging systems, even after adjustment for individual stages. The adjusted chance for the survival of patients with high HSP70 serum concentration was approximately half of those with lower HSP70 levels. Moreover, we found an interaction between high HSP70 serum level and stage of the disease—that is, the *association between high HSP70 level and survival* could be detected only in the group of patients with less advanced disease at baseline (i.e., with tumors not invading other organs or structures and/or propagating beyond the visceral peritoneum, as well as with four or more tumor-free regional lymph nodes).

Interestingly, we found that the association between serum HSP70 and mortality is dominant in women under the age of 70. It was observed in many large studies including a meta-analysis that the risk of colorectal cancer in current users of postmenopausal hormones is significantly 30% to 40% lower compared with non-users (Newcomb and Storer 1995; Grodstein et al. 1999; Rossouw et al. 2002; Newcomb et al. 2007). It seems that the estrogen is the active agent (Newcomb and Storer 1995). It is tempting to speculate that high level of serum HSP70 may in some way be modulating the protective function of estrogen in pre-menopausal women. In animal experiments high gender-related differences were found in the HSP70 expression, indeed which were mostly due to the estrogen (Voss et al. 2003; Nickerson et al. 2006)

Our findings are in line with the results of the prospective study by Suzuki et al. (2006). These authors measured the serum levels of soluble HSP70 and CRP in a case-control study conducted in Japan and studied the relationship between these serum markers and the clinical course of lung cancer. Odds to develop lung cancer by patients in the highest quartile of soluble HSP70 levels was significantly higher than that found in the rest of the patients; in males, the odds ratio was close to 2.5. No similar study has been published yet for other types of cancer.

Abundant data have been published on the increased expression of HSP70 in several tumors, such as breast cancer (Takahashi et al. 1994; Lazaris et al. 1997; Vargas-Roig et al. 1997), ovarian cancer (Elpek et al. 2003), carcinoma of the uterine cervix (Kaur and Ralhan 1995; Park et al. 1999), lung cancer (Zhong et al. 2003), prostate cancer (Abe et al. 2004; Cornford et al. 2000), and other types of cancer (reviewed in Ciocca and Calderwood (2005)) . Colorectal tumors also exhibit increased HSP70 expression (Hwang et al. 2003; Dundas et al. 2005; Pfister et al. 2007).

Unusual membrane expression of HSP70 occurs only on the surface of tumor cells, but not in normal tissues (Multhoff et al. 1995; Gehrmann et al. 2003). In principle,

increased membrane expression of HSP70 can be beneficial for the patients, because it can promote lysis of these tumor cells by NK cells (Multhoff et al. 2000). On the other hand, it can be associated with a greater metastatic potential and hence, unfavorable prognosis (Gehrmann et al. 2003; Farkas et al. 2003). In colorectal cancer, Hwang et al. (2003) compared the heat shock protein content of weakly vs. highly metastatic colorectal cell lines and found that HSP70 expression was elevated in the highly metastatic cell line. Over-expression of mortalin, a mitochondrial HSP70, was shown in colorectal adenocarcinomas and found to correlate with poor survival (Dundas et al. 2005). According to recent studies by Pfister et al. (2007), the relationship between HSP70 expression by the tumor cells and patient survival depends on the localization of the tumor. Specifically, HSP70 membrane expression correlated significantly with an improved overall survival in patients with colon cancers, whereas a marginally significant negative association was found in those with lower rectal cancer.

Theoretically, the elevation of soluble HSP70 levels in colorectal cancer patients can be explained by different mechanisms. In principle, it can be associated with the humoral immune response against the protein. In accordance with the earlier work of Pockley et al. performed in patients with hypertension (Pockley et al. 2002), we did not find significant correlation between the serum concentrations of the soluble HSP70 and IgG type anti-HSP70 antibodies in patients with colorectal cancer either. It is also possible that high soluble HSP70 levels results from increased expression of the protein by tumor cells. Under certain circumstances, HSP70 proteins escape from the cytoplasm, appear on the cell membrane and then, detach from the tumor cells (Pockley et al. 1998; Wright et al. 2000) through non-classical (endoplasmic reticulum–Golgi-independent) protein mechanisms, via intact lipid rafts (Broquet et al. 2003). To our best knowledge, however, no comparison of tumor-associated and soluble HSP70 was ever reported. In the present study, we found the serum levels of the soluble HSP70 significantly higher in those patients with non-resected primary tumors (primary chemotherapy) than in those with resected tumors before enrollment, indicating that in these patients, probably a part of the protein is derived from the tumors cells. On the other hand since the majority of the patients was recruited 4–6 weeks after the removal of the tumors, soluble HSP70 protein probable was originated from other sources. Furthermore, it cannot be excluded that a fraction of the tumor-derived protein was still detectable in the circulation after 4–6 weeks.

Accordingly, there is no direct evidence to support the neoplastic origin of high soluble HSP70 concentration in the blood of patients with colorectal cancer. An alternative explanation could be that the increase in HSP70 levels results from injury and necrosis of tumor cells, such as it is seen in other diseases where tissue damage occurs, e.g., in the wound discharge and peripheral blood of patients with soft tissue trauma (Pittet et al. 2002; Flohe et al. 2007), in peripheral and renal vascular disease (Wright et al. 2000)] or after myocardial infarction (Dybdahl et al. 2005; Satoh et al. 2006) and coronary bypass grafting (Dybdahl et al. 2002).

The association between high serum HSP70 levels and poor survival of patients with colorectal cancer is a novel observation for which no definite explanation can be offered at the time being. It is well known that extracellular HSP70 has a major role in anti-tumor immunity, as well as 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, it is tempting to speculate that high soluble HSP70 level in the serum may inhibit cellular anti-tumor immunity. Further studies are needed to investigate this assumption directly.

The identification of early, molecular markers, which can predict cancer-related mortality, is an urgent and important goal in the development of cancer diagnostics and therapy. Ideally, identifying patients with high-risk carcinomas and a low chance of survival at initial diagnosis could afford early and individualized therapy that would improve clinical outcome. Our findings indicate that the prognostic value (adjusted odds ratios of about 2; $p<0.01$; see Table 3) for the overall survival of the soluble HSP70 measurement is comparable to that of the known tumor markers, such as CEA and CA 19-9 for disease-free survival in early stage colorectal cancer. (Wang et al. 2002, 2007;Ogata et al. 2009) For example, in a recent study by Takagawa et al. (2008) performed in 638 patients with colorectal cancer, high (>10 ng/ml) CEA values were found an independent risk factor for disease-free survival with an adjusted odds ratio of 3.21 ($p<0.001$)

Due to the limited size of the study population, our present work should be considered a preliminary investigation only. If, however, our findings will be confirmed by subsequent studies conducted on larger cohorts of patients, measurement of the serum level of soluble HSP70 might prove a useful, non-invasive, and inexpensive predictive marker in patients with colorectal cancer who have no distant metastasis at the time of recruitment

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