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ORIGINAL ARTICLE

Heat shock protein 70 is a useful marker for predicting colorectal cancer

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Summary

Purpose: *In colorectal cancer (CRC), as in most of other malignancies, heat shock proteins (HSPs) are overexpressed and are associated with apoptosis, cancer cell proliferation, differentiation, invasion, and metastasis. HSP70 is one of the HSPs and has a promising future in cancer studies for both diagnostic and therapeutic applications. In this study, we tried to evaluate the serum levels of HSP70 in CRC patients, and to evaluate its predictive value of detecting CRC.*

Methods: *This prospective study was consisted of 33 patients diagnosed with CRC and 31 healthy subjects who were matched for age. Enzyme-linked immunosorbent assays (ELISA) were used to evaluate the serum levels of HSP70 in patients with CRC and in the healthy control group. A cut-off value for HSP70 was also determined using receiver operating characteristic (ROC) curve analysis.*

Results: *Patients with CRC had significantly higher HSP70 concentrations compared with the control group (4.52 ± 1.83 vs 1.22 ± 0.48 ng/ml, p=0.001), the cut-off value was ≥2.25 ng/ml (95% CI 0.993-1.003, p<0.001). The sensitivity and specificity of elevated serum HSP70 in the CRC group were 96.77 and 96.96%, respectively. Also, HSP70 levels were significantly higher with rectal disease localization (p=0.01).*

Conclusion: *This study shows that the serum level of HSP70 is elevated in patients with CRC. HSP70 may be utilized as an adjunct to other diagnostic or screening tests.*

Key words: *colon cancer, colorectal cancer, cut-off value, diagnosis, heat shock protein 70, rectal cancer*

Introduction

CRC ranks third in incidence and second in cancer-related deaths both in men and women. The majority of patients are diagnosed at an advanced stage [1-5]. CRC is one of the cancer types exerting an association between stressful life events and disease development [6].

As a consequence of diverse growth rate and metastatic potential of CRC, its clinical behavior may be different among patients classified in the same pathological or clinical stage [7-9].

CRC contains significant fractions of hypoxic cells, making thus itself somewhat apoptosis-resistant [10]. Multiple protooncogenes, oncogenes, regulatory factors, and tumor suppressor genes have been accused of playing a role in the progression of CRC [7,8,11,12].

Recent studies have shown that some proteins, such as HSPs, play an important role in the antiapoptotic mechanisms of cancer cells, making thus worth studying these biological compounds in cancer [13-18].

HSPs are molecular chaperones and they have been classified according to their molecular weight [13]. Recent studies have revealed that the

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major HSP, Hsp70, has several fundamental functions related to intracellular protein homeostasis, such as protein transportation and degradation [19-21]. But the reason of the great interest in HSP70 in cancer studies is its ability to direct inhibition of apoptosis which makes it an attractive potential targeted therapy for cancer. Hsp70, is an effective inhibitor of apoptosis and often constitutively overexpressed in tumors [17-22].

HSP70 is produced at low or undetectable levels in unstressed, healthy cells (including molecularly normal colon) [23], but enhanced expression has been shown in stressful conditions, such as heat, hypoxia, neoplasia and viral infection [24]. In contrast to normal cells, tumors could have Hsp70 in the cytosol, on their plasma membrane [25,26] and/or they can actively (exosomal) and/or passively (by dying tumor cells) release it [26-29].

Previous studies have shown that HSP70 is overexpressed in human colorectal cancer cells [3,22]. Also, in animal model studies a correlation between serum Hsp70 levels and tumor volume has been indicated [30].

In this study, we aimed to evaluate the association of serum levels of HSP70 with CRC and its relation with the patient clinicopathological features. Also, we tried to determine its importance according to anatomic localization of disease and its predictive value in CRC detection and diagnosis.

Methods

This clinical trial was conducted at the Department of Medical Oncology of our hospital. The study was approved by our hospital's local ethics committee and all of the patients signed informed consent prior to enrollment in the study. Although a total of 53 patients with CRC were initially selected, in the end 33 patients were eligible for inclusion according to inclusion criteria of this study.

Inclusion criteria

Inclusion criteria were: patients ≥18 years of age with histologically confirmed CRC; patients with diagnosis made or confirmed in our hospital; patients who did not have any surgical intervention or chemotherapy or radiation therapy before obtaining blood samples; patients who did not have any acute or chronic disease other than CRC at the time of blood collection; patients who signed informed consent prior to enrollment in the study.

Thirty three CRC patients and 31 healthy controls matched by age and gender were finally enrolled. Patients divided into subgroups with respect to ECOG performance status score, presence or absence of comorbidities, LVI and/or PNI, tumor histological grade, depth of tumor invasion, presence or absence of lymph node metastasis, liver or metastasis to other distant organs, localization (colon or rectum), presence or absence of leukocytosis, thrombocytosis, anemia, level of serum albumin (normal or low), level of serum lactate dehydrogenase (LDH) (high or normal) and level of serum CEA (high or normal).

Blood sample collection

Blood samples of patients were drawn before initiating chemo(radio)therapy and/or surgical tumor resection. After overnight fasting, venous blood samples were obtained from the antecubital vein of the participants. All samples were collected in blood collection tubes and centrifuged at 4,000 g (10 min) to remove the serum. Serum samples were kept at -80 °C until the analysis of HSP70 levels.

Measurement of HSP70

Enzyme-linked immunosorbent assay (ELISA) technique was used to assess the serum level of HSP70. Serum HSP70 was analysed using Human Heat Shock Protein 70 (HSP70) ELISA Kit (Cat no:20,959), purchased from Bio Medical Assay (BMASSAY, Beijing, China), following the manufacturer's instructions. Its level was expressed in ng/ml. The detection and quantification limit was set at <0.05 ng/ml for HSP70. The intra-assay coefficient variations (CV) of HSP70 was 8.2%.

Other variables

Complete blood count was determined in a Coulter LH 750 autoanalyser (Beckman Coulter, CA, USA). Serum carcinoembryonic antigen (CEA) levels were determined by Beckman Coulter AU 5800 chemistry auto-analyser and DXI 800 systems by using commercial kits (Beckman Coulter, CA, USA). Reference range: CEA: 0-3.3 U/ml.

Statistics

Statistical analyses were done by NCSS (Number Cruncher Statistical System) 2007&PASS (Power Analysis and Sample Size), 2008 Statistical Software (Utah, USA). During the evaluation of the study variables, descriptive statistical methods (mean, standard error, median, rate and ratio) were used. For comparison of variables of normal distribution, the t-test for independent samples was used and the Kruskal-Wallis test and the Mann-Whitney U test were used for comparison of variables with non-normal distribution. Receiver operating characteristic (ROC) analysis was used for the determination of the possible use of the markers for clinical differentiation between the patient and the control groups. When the area under the curve (AUC) was significant, the cut-off values were determined and

Table 1. Comparison of gender, age and serum HSP70 levels of patients with colorectal cancer and controls

		Patients N=33	*Control N=31*	*p value*
Gender, N (%)	Male	21 (63.6)	13 (41.9)	0.137[a]
	Female	12 (36.4)	18 (58.1)	
Age (years)	Mean ± SD	62.28 ± 14.77	60.77 ± 6.43	0.600[b]
	Min - Max (median)	23 - 84 (64)	48 - 82 (60)	
HSP 70 (ng/ml)	Mean ± SD	4.52 ± 1.83	1.22 ± 0.48	0.001[c]
	Min-Max (median)	2.05 - 8.21 (4.28)	0.21 - 2.36 -1.23	

[a]Chi square test, [b]Student's t-test, [c]Mann-Whitney U test, HSP70: Heat shock protein 70, SD: standard deviation

sensitivity and specificity for that particular cut-off point were calculated as well. Results were evaluated in confidence intervals (CI). Statistical significance was set at $p<0.05$ in all tests.

Results

A total of 64 subjects were enrolled in this study and they were divided into 2 groups; group 1 consisted of 33 CRC patients (female/male 12/21), and group 2 consisted of 31 healthy controls (female/male 18/13). There was no significant difference in the mean age of the groups (62.28±14.77 vs 60.77±6.43 years respectively; p=0.60). Serum HSP70 levels were significantly higher in patients than in controls (4.52±1.83 vs 1.22±0.48 ng/mL respectively; p=0.001) (Table 1).

Multiple comparisons were performed to determine whether there was any difference in serum HSP70 levels among subgroups. Comparison of patients with and without comorbidity showed that those with comorbidity had significantly higher serum HSP70 levels than those without (5.08±1.87 vs 3.84±1.57 ng/mL, respectively; p=0.048) (Table 2). Also, patients with rectal cancer had higher HSP70 levels than patients with colon cancer (5.76±1.63 vs 4.06±1.7, respectively; p=0.010) (Table 2). The rest of the subgroup comparisons showed no statistical differences ($p \geq 0.05$) (Table 2).

After noticing a significant difference in HSP70 levels between CRC patients and healthy controls we tried to find an optimal cut-off value for HSP70 to detect CRC. The area under a ROC curve for HSP70 to predict CRC was determined at ≥ 2.25 ng/ml. This cut-off value had sensitivity of 96.96%, specifity of 96.77%, and positive and negative predictive values of 96.97 and 96.77% respectively [AUC: 0.998 (95% CI, 0.993-1.003), p=0.001] (Figure 1).

Discussion

HSPs family resides mainly in the cytosol, where they maintain protein homeostasis by supporting nascent polypeptides (folding, refolding, and assembly), preventing protein aggregation, and assisting the transport of proteins across membranes [31]. HSP70 production is found at low or undetectable levels in healthy cells [23], whereas, in contrast to normal cells, tumors frequently overexpress HSP70. This protein can be found in the cytosol, plasma membrane and/or it can be actively (exosomal) and/or passively (by dying tumor cells) released [26-29]. Although there is no direct evidence to support the neoplastic origin of high soluble HSP70 concentration in the blood of patients with cancer, an alternative explanation could be that the increase in HSP70 levels results from injury and necrosis of tumor cells or its secretion by tumor cells into the extracellular space. Releasing of HSP70 into the extracellular space makes itself detectable in the blood and measurement of its level may provide clinically important information about the tumor.

It has been shown that colorectal tumors also display HSP70 overexpression [23,32].

In principle, increased membranous expression of HSP70 can be beneficial for the patients because it can promote lysis of these tumor cells by natural killer cells [33,34]. In CRC, Hwang et al. [23] found that HSP70 expression was elevated in highly metastatic colorectal cell lines. According to recent studies by Pfister et al. [32], HSP70 membranous expression correlated significantly with improved overall survival in patients with colon cancers, whereas a significant negative association was found in those with lower rectal cancer.

Although overexpression of HSP70 has been shown in various types of cancer, including also CRC cell lines, there are only few data on the

Table 2. Comparison of clinicopathologic features and serum HSP70 level in patients with colorectal cancer

Features		*HSP70*			
		%	*Mean ± SD*	*Median*	*[a]p value*
Performance score	0		4.56 ± 1.93	3.51	[a]0.903
	1	15.20	4.29 ± 1.22	4.64	
Comorbidity	(+)	54.5	5.08 ± 1.87	5.11	[a]0.048
	(-)		3.84 ± 1.57	3.44	
Lymphovascular invasion	(+)	75.8	4.63 ± 1.98	4.28	[a]0.789
	(-)		4.19 ± 1.29	3.89	
Perineural invasion	(+)	75.8	4.72 ± 1.96	4.64	[a]0.420
	(-)		3.9 ± 1.23	3.45	
Grade	1	27.3	4.5 ± 2.15	3.45	[a]0.695
	2	66.7	4.71 ± 1.7	4.71	
	3	6.1	2.59 ± 0.76	2.59	
Depth of tumor invasion	2	14.8	3.83 ± 1.09	3.33	[b]0.621
	3	74.1	4.7 ± 1.93	4.31	
	4	11.1	3.64 ± 0.87	3.15	
Lymph node metastasis	(-)	37	3.72 ± 1.26	3.33	[a]0.103
	(+)	63	4.87 ± 1.9	4.33	
Localization	Rectum	27.3	5.76 ± 1.63	6.33	[a]0.010
	Colon	72.7	4.06 ± 1.7	3.34	
Liver metastasis	(+)	27.3	4.5 ± 1.9	4.77	[a]0.953
	(-)		4.53 ± 1.84	3.87	
Metastasis	(+)		4.62 ± 1.99	4.95	[a]0.984
	(-)		4.49 ± 1.81	3.56	
Stage	2	33.3	4.28 ± 1.72	3.87	[b]0.598
	3	36.4	4.96 ± 2.02	5	
	4	30.3	4.35 ± 1.85	4.17	
Leukocytosis	(-)		4.63 ± 1.86	4.28	-
	(+)	9.4	3.12 ± 1.15	2.99	
Anemia	(-)		3.91 ± 1.36	3.44	[a]0.584
	(+)	83.9	4.68 ± 1.91	4.49	
Thrombocytosis	(-)		4.57 ± 1.91	4.1	[a]0.717
	(+)	25	4.26 ± 1.73	3.86	
Albumin	Normal		4.62 ± 1.79	4.28	[a]0.680
	Low	43.3	4.36 ± 1.98	3.56	
LDH	Normal		4.66 ± 1.82	4.31	[a]0.580
	High	37.9	4.38 ± 2	3.56	
CEA	Normal		4.48 ± 1.68	4.31	[a]1.000
	High	50	4.51 ± 2.05	3.51	

HSP70: Heat shock protein 70, SD: standard deviation, LDH: lactate dehydrogenase, CEA: carcinoembryonic antigen, [a]Mann-Whitney U test, [b]Kruskal-Wallis test

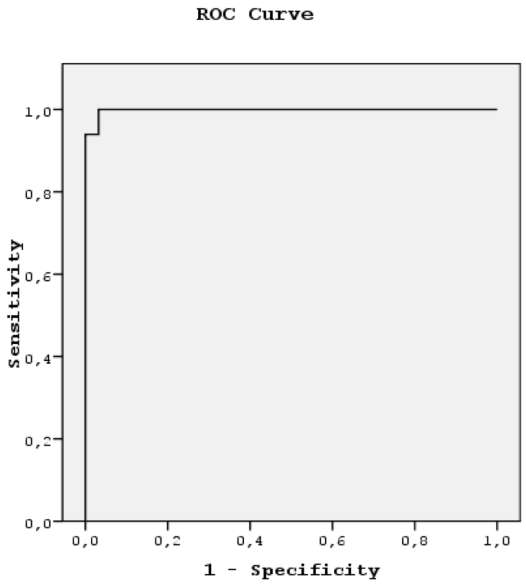

Figure 1. Receiver operating characteristics (ROC) curve for serum heat shock protein70 for the diagnosis of colorectal cancer. The area under the curve (AUC) for HSP70 is 0.998 with p=0.001.

measurement of serum HSP70 in cancer [3,22]. Kocsis et al. have found that serum HSP70 level was associated with high mortality in patients with CRC without distant metastasis [34]. Suzuki et al. has determined that HSP70 was associated with increased risk of lung cancer [35]. In pancreatic cancer patients, serum HSP70 level was significantly increased and Dutta et al. have suggested that it could be useful as an additional biomarker for the detection of pancreatic cancer [36]. In our previous study, the HSP70 level was found significantly higher in patients with breast cancer than in healthy controls [37].

Based on these studies we intended to determine the serum levels of HSP70 in CRC patients. In the present study serum HSP70 levels were found significantly higher in patients with CRC than in healthy subjects. Comparison of patients with and without comorbidity showed that those with comorbidity had significantly higher serum HSP70 levels than those without. This can be explained by the fact that elevated serum HSP70 levels had been shown in various non-neoplastic conditions, such as diabetes, heart failure, etc. [38,39]. Also patients with rectal cancer had higher serum HSP70 levels than patients with colon cancer. However, in our patient study group, the number of patients with comorbidity was not statistically different between rectal cancer group and colon cancer group (44.4 vs 58.3%, respectively; p≥0.05). Because we did not study the cellular expression of HSP70 in the pathology specimens of CRC patients, we could not determine the exact source of this different levels of serum HSP70 between rectal and colon cancer patients. Nevertheless, this difference may support the argument of Pfister et al. who proposed HSP70 membrane expression by CRC may be related with the different routes of metastasis of rectal and colon cancer [32]. In another study which showed that CEA and CA 19-9 levels were related with prognosis and survival, the difference in HSP70 levels between colon and rectum might also be an indicator of prognosis and/or survival; however, further studies are needed in this field [40].

To the best of our knowledge, this is the first study that showed the different levels of serum HSP70 between rectal and colon cancer patients. Certainly, more studies are needed to elucidate this field.

Next, we tried to determine the best cut-off value as we found a significant difference in serum HSP70 levels between CRC patients and healthy controls. A serum HSP70 cut-off value for predicting CRC was found as ≥2.25 ng/ml. To the best of our knowledge, this is the first study to provide a cut-off value of serum HSP70 in patients with CRC. The cut-off value of serum HSP70 has been investigated in some types of cancer. The cut-off point for serum HSP70 levels in patients with localized untreated prostate cancer was found as 1.15 ng/ml, and in cholangiocarcinomas it was 5.67 ng/ml [41,42]. In our previous study, the cut-off value for serum HSP70 level in patients with breast cancer was determined as >2.41 ng/ml, which is also close to the value of CRC [37].

As a result, in our study CRC patients had higher serum HSP70 values than healthy controls. Also patients with rectal cancer had higher values of serum HSP70 than colon cancer patients. As expected, patients with comorbidities had significantly higher serum HSP70 levels than those without. We determined a cut-off value for HSP70 for predicting CRC and we believe this is the first study to provide this information for CRC patients. This information may encourage the use of this non-invasive and inexpensive marker in the clinic and contribute to the broad field of cancer screening and diagnosis. Further studies are needed to define the importance of serum HSP70 in CRC patients, especially the different levels between rectal and colon cancer. Due to the limited size of the study population, our present work needs to be confirmed by subsequent studies con-

ducted on larger cohorts of patients.

The present study has some limitations. We have not made a sample size method in order to reach the necessary number for our group of patients. As the number of patients and the duration of follow-up were not large enough for accurate statistical calculation, the relation between HSP70 and prognosis could not be determined. Also, we did not examine the cellular expression of HSP70 in pathology specimens.

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