Circulating heat shock protein 70 and progression in patients with chronic myeloid leukemia

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Abstract

We evaluated the association of circulating levels of heat shock protein 70 (Hsp70) in plasma with clinical behavior and progression in 139 chronic myeloid leukemia (CML) patients. Circulating Hsp70 levels did not differ significantly between CML patients in the chronic phase ($n=93$; median 33.24 ng/mL, range 3.89–128.2 ng/mL) and those in the accelerated/blast phase ($n=46$; median 26.57 ng/mL, range 4.5–114.7 ng/mL). However, overall CML patients had significantly higher levels of Hsp70 than healthy subjects ($n=95$, median 4.17 ng/mL, range 1.75–24.7 ng/mL) ($P<0.001$). In chronic phase CML patients, Hsp70 levels above the median were associated with a higher rate of progression to the accelerated/blast phase and a tendency toward shorter survival. Plasma Hsp70 thus could be a potential marker for predicting disease progression in patients with chronic phase CML.

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1. Introduction

Heat shock proteins (Hsps) are one of the most conserved proteins ubiquitously existed in all organisms, from prokaryotes, yeasts and plants, to eucaryotes. Hsps maintain cellular homeostasis and function as molecular chaperones that assist in protein folding and translocation. They prevent misfolding and aggregation of proteins, and facilitate refolding of denatured proteins under physiological and stress conditions [1–3]. Expression of Hsps can be induced in response to a range of stimuli including fever, heavy metals, oxidative stress, alcohol, inflammation and infection [4–6]. They are also associated with cell protection mechanisms through interaction with key apoptosome components such as apoptosis-inducing factor (AIF) and

apoptosis signal-inducing kinase-1, or inhibiting lysosomal membrane permeabilization, thereby preventing caspase activation and apoptosis [7–11].

Among Hsps, Hsp70 has been found abundantly expressed in malignant human tumors of various origins, whereas in normal cells, its expression is mainly stress inducible [12–14]. Ectopic overexpression or induced endogenous levels of Hsp70 not only potently inhibits apoptosis [15,16], but also enhances cell tumorigenic potential *in vitro* and *in vivo* [17–19]. In human primary breast tumors, Hsp70 upregulation correlates with increased cell proliferation, poor differentiation, lymph node metastases, and poor therapeutic outcome [20–23]. Conversely, knockdown of Hsp70 expression has been shown to induce apoptosis [24,25]. Together, these findings highlight the role of Hsp70 in mediating malignant transformation and tumor cell resistance to chemotherapy-induced apoptosis, and eventually leading to the invasive phenotype.

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In addition Hsp70 has been demonstrated to enter the blood stream and travel throughout the body stimulating various cellular processes, particularly stimulating the immune system and potentially triggering anti-cancer immune response [26].

Recently, Bcr–Abl-expressing leukemia cells have been found to display high levels of Hsp70 and are resistant to apoptosis induced by several chemotherapeutic agents including imatinib mesylate (Gleevec/Glivec, or STI571), a relatively specific, ATP-binding site antagonist of Bcr–Abl [27–29]. The development of imatinib resistance in patients with chronic myeloid leukemia (CML) has been a daunting concern, and overcoming this obstacle remains a challenge for successful treatment of CML, particularly in the advanced phases [30,31]. CML is characterized by an acquired reciprocal chromosomal translocation *t* [9,22] that links *c-abl* gene from chromosome 9 to the breakpoint cluster region (*bcr*) on chromosome 22, known as the Philadelphia (Ph) chromosome [32,33]. In 95% of CML cases, this positioning effect results in the generation of the 210-kDa chimeric oncoprotein, Bcr–Abl, with constitutive tyrosine kinase activity [34,35]. Among the underlying mechanisms identified for imatinib resistance from both *in vitro* and *in vivo* studies are mutations within the kinase domain of Bcr–Abl, progressive Bcr–Abl gene amplification, additional chromosomal aberrations [36–38] or dysregulation of Bcr–Abl diverse signaling pathways leading to antagonize apoptosis at multiple points, e.g., activation of Src family kinase, Bcl-x_L, NF-κB and Hsp70 [15,16,39,40]. Although multiple mechanisms of Hsp70-mediated anti-apoptotic pathways or immune-modulated are well described and could explain imatinib resistance, direct clinical correlation between Hsp70 expression and the prognosis of CML and the mechanistic links to imatinib resistance are still lacking.

Our present study with large number of clinical samples demonstrates, for the first time, that circulating Hsp70 levels from Ph + CML patients are significantly higher than those of healthy donors, and the elevation of Hsp70 is significantly correlated with disease progression/resistance to imatinib therapy.

2. Materials and methods

2.1. Patients

The study was conducted with approval by an Institutional Review Board. Plasma samples from patients with the diagnosis of CML were collected prior to initiating imatinib therapy and stored at -70°C until analysis. All patients were treated at M.D. Anderson Cancer Center. Diagnosis was confirmed by cytogenetic and FISH studies. Progression and transformation to accelerated/blast phase was confirmed by complete marrow evaluation and flow cytometry.

Patients were treated with standard imatinib therapy. All patients were followed at M.D. Anderson Cancer Center and the incidences

of disease progression were recorded. Normal control samples were recruited from healthy individuals with similar male:female ratio.

2.2. Measurement of plasma Hsp70

Plasma was separated from EDTA peripheral blood samples by centrifuging at $1000 \times g$ for 10 min. Samples were stored at -70°C until analyzed.

Plasma levels of Hsp70 (the inducible form) were measured using MA2400 total Hsp70 whole cell lysate kits (Meso Scale Discovery, Gaithersburg, MD) according to the manufacturer's instruction. Briefly, 25 μL of plasma was applied onto MSD Single-Spot 96-well plates that were pre-coated with anti-Hsp70 monoclonal antibody and incubated for 1 h at room temperature. The plates were washed four times with Wash buffer and then incubated with 5 nM of Sulfo-Tag detection antibody for another hour at room temperature. After final washing, 150 μL of Read buffer T was added to each well, and plates were analyzed by a MSD SECTOR Imager 2400 reader (Meso Scale Discovery). Each assay included 7-point standards with known amounts of human recombinant Hsp70, ranging from 625 to 40,000 pg/mL (Epoch Biolabs, Sugar Land, TX). Three positive plasma (high, medium, low) and two negative controls (normal plasma and blank) were also run in parallel. Sensitivity of this assay as determined by limit of detection (LOD) and limit of quantitation (LOQ) are 76 and 625 pg/mL, respectively. Specificity of this assay was determined based on mixing recombinant Hsp70 with various plasma samples and was determined to be at 97% based on the recovery and background. Numerous experiments were performed to confirm specificity of antibodies by exchanging specific antibody with isotypic control and demonstrating no significant detectable level.

2.3. Statistical analysis

Descriptive statistics were analyzed and univariate analyses were performed using the chi-square or Kruskal–Wallis test for categorical data and *t* test for continuous data. Estimates of survival curves were calculated according to Kaplan–Meier product-limit method. Survival times were compared by means of the log-rank test. Clinical and biological characteristics were analyzed for their associations with survival using Cox proportional hazards models.

3. Results

3.1. Higher levels of Hsp70 in patients with CML

Of the 139 patients enrolled in the study, 93 were in the chronic phase of disease and 46 were in the accelerated or blast phase (Table 1). The age was similar between patients in the chronic group and in the accelerated/blast phase. The major differences, as expected, were in blast count, basophil count, and LDH levels. Patients with CML had significantly ($P < 0.001$) higher levels of Hsp70 than did healthy donors (Table 2). High Hsp70 were detected in all CML patients, whether they were in the chronic or the accelerated/blast phase, and did not differ significantly between phases ($P = 0.08$).

Table 1

Baseline clinical characteristics of 139 patients with chronic myeloid leukemia, by phase of disease

Characteristic	Chronic phase (<i>N</i> = 93), median (range)	Accelerated/blast phase (<i>N</i> = 46), median (range)
Age (year)	51 (21–85)	52 (25–79)
Hemoglobin (g/dL)	12.2 (7.9–16.7)	11.1 (7.5–16.6)
Platelets ($\times 10^9/L$)	330 (100–1178)	192 (6–1159)
White blood cells ($\times 10^9/L$)	18.0 (1.6–192.5)	18.2 (4.5–249)
Eosinophils (%)	2 (0–5)	2 (0–10)
Basophils (%)	2.3 (0–15)	6 (7–86)
Albumin (g/dL)	4.0 (3–4.9)	3.9 (2.5–5.3)
Creatinine (mg/dL)	0.9 (0.6–1.6)	0.9 (0.6–0.16)
LDH (U/L)	694 (326–3162)	1044 (27–5041)
SGOT (U/L)	27 (10–69)	28 (10–93)
BM cellularity (%)	90 (10–100)	90 (10–100)
BM blasts (%)	2 (0–13)	4 (0–89)
BM promyelocytes (%)	1 (0–19)	1 (0–8)
BM myelocytes (%)	14 (1–37)	11 (1–44)
BM basophils (%)	2 (0–13)	2 (0–31)
Splenomegaly (%)	30	55

LDH—lactate dehydrogenase (soluble), SGOT—aspartate aminotransferase, BM—bone marrow.

3.2. Clinical correlations

Hsp70 levels in patients in the chronic phase correlated positively with platelet count, white blood cell count, percentage of basophils, percentage of Philadelphia-positive cells, and percentage of blast cells (Table 3). Among patients in the accelerated/blast phase, Hsp70 levels were also significantly and positively correlated with platelet count and percentage of basophils, and negatively correlated with albumin, creatinine, and bilirubin (Table 3); plasma Hsp70 levels were also *negatively* correlated with the percentage of blasts in this group, in contrast to the findings for patients in the chronic phase.

Follow-up data were available on all patients, and the median follow-up was 49 months. During follow-up, 15% of the patients in the chronic phase progressed to the accelerated or blast phase. In addition, death occurred in 11% of the patients. Patients in the chronic phase and with baseline levels of Hsp70 above the median had a significantly ($P = 0.02$) higher rate of progression to the accelerated or blast phase while on imatinib therapy (23 vs. 7%; Fig. 1). These patients

also had shorter survival, although not statistically significant ($P = 0.07$) (Fig. 2). Baseline plasma Hsp70 levels were not correlated with survival in patients with accelerated/blast phase either.

4. Discussion

In this study, we showed that baseline plasma levels of Hsp70 were significantly higher in patients with CML than in healthy individuals. Further, there is a strong clinical correlation between plasma Hsp70 levels and progression to the accelerated/blast phase in CML patients ($P = 0.02$). This finding supports the involvement of Hsp70 expression in the development of imatinib resistance. Although a larger clinical trial is needed to confirm our observation, the data here suggest that plasma Hsp70 could be a predictive marker for developing imatinib resistance in patients with chronic phase CML. Therefore, inhibitors against the Hsps may help overcome drug resistance, and clinical trials exploring the use of Hsp antagonists in patients with high plasma Hsp70 values should be considered.

Our data also implicate an association between high levels of plasma Hsp70 and shorter survival in patients with chronic phase of CML. The lack of correlation between plasma Hsp70 and clinical outcome in patients with accelerated/blast phase suggests that Hsp70 may not be critical in CML following the transformation and dominance of blast cells. In line with these findings, a close correlation was observed between Hsp70 levels and the percentage of blasts in patients with chronic phase but not those with accelerated/blast phase. Again, our data point out a clinically relevant role of Hsp70 during CML disease progression.

In addition to its role in maintaining cellular homeostasis and in promoting cell survival in response to stressful conditions, tumor-specific membrane-bound Hsp70 released either as a result of cellular apoptosis/necrosis or via a stress-induced exocytotic pathway. The released Hsp70 may also act as a potent danger signal to activate the adaptive and innate immune system, e.g., binding to LOX-1 receptors on dendritic cells or association with CD94 on natural killer (NK) cells [41–44]. This property of Hsp70 is of clinical rele-

Table 2

Baseline plasma levels of Hsp70 in 139 patients with chronic myeloid leukemia and 95 healthy controls

Statistic	Plasma heat shock protein 70 (ng/mL)		
	Healthy controls (<i>n</i> = 95)	Chronic myeloid leukemia	
		Chronic phase (<i>n</i> = 93)	Blast phase (<i>n</i> = 46)
Mean	4.93	38.34	33.52
Median	4.17	33.24	26.57
Minimum	1.75	3.89	4.50
Maximum	24.68	128.17	114.75

All values for healthy controls differed significantly ($P < 0.001$) from those of patients; values did not differ significantly by disease phase ($P = 0.08$).

Table 3

Correlations between plasma Hsp70 levels and laboratory characteristics in 139 patients with chronic myeloid leukemia, by disease phase

Characteristic	Blast phase (n = 46)		Chronic phase (n = 93)	
	Spearman's rho	P	Spearman's rho	P
Age	−0.11	0.48	−0.10	0.35
Hemoglobin	−0.049	0.74	0.04	0.70
Platelets	0.73	<0.001	0.54	<0.001
White blood cell count	0.24	0.11	0.32	0.001
% Blasts	−0.39	0.04	0.29	0.03
% Promyelocytes	0.015	0.94	0.06	0.66
% Myelocytes–metamyelocytes	−0.19	0.25	0.16	0.17
% Neutrophils	−0.08	0.59	0.02	0.81
% Lymphocytes	−0.12	0.43	−0.26	0.01
% Eosinophils	0.03	0.87	−0.13	0.24
% Basophils	0.50	0.001	0.31	0.003
% Monocytes	−0.09	0.55	−0.15	0.16
Albumin	−0.32	0.02	0.22	0.03
Creatinine	−0.33	0.02	−0.10	0.34
Bilirubin	−0.34	0.02	−0.07	0.48
LDH	0.07	0.64	0.27	0.009
SGOT	−0.04	0.78	0.03	0.80
% Ph+ cells	0.12	0.43	0.30	0.003

LDH = lactate dehydrogenase (soluble), SGOT = aspartate aminotransferase, BM = bone marrow, Ph+ = Philadelphia-positive cells.

vance in tumor immunotherapy as anti-tumor responses can be elicited by Hsp70-chaperoned tumor-specific antigenic peptide complexes on antigen-presenting cells [45,46]. In this regard, elevated levels of circulating Hsp70 could reflect heavier tumor loads and predict higher immune responses.

Recombinant Hsp70 protein and Hsp70 peptides, which mimic surface-bound Hsp70, have been shown to activate NK cells directly in some systems [42]. Further, reports on the use of tumor-derived Hsp70 as a prophylactic and therapeutic cancer vaccine have been documented [46]. Therefore, NK cell-mediated immune responses elicited by high levels of circulating Hsp70 could occur

in our study. Indeed, high levels of circulating Hsp70 were found to correlate with higher platelet and white blood cell counts, and correlate with basophil counts in both the accelerated/blast and chronic phases, suggesting a robust immunologic reaction in these CML patients.

In conclusion, our findings suggest that plasma levels of Hsp70 can be used as a biomarker of disease progression in patients with CML and support the concept that Hsp70 may be crucial in developing resistance to imatinib. Determination of circulating Hsp70 content in plasma may further help stratifying CML patients for therapy with imatinib, newer kinase inhibitors or stem cell transplantation protocol. The

Fig. 1. Time to progression to the accelerated/blast phase of disease among patients in the chronic phase of chronic myeloid leukemia, with baseline Hsp70 values below (top or blue line) and above the median (bottom or red line). (Abbreviation: E, number of patients had an event; N, total number).

Fig. 2. Time to death among patients in the chronic phase of chronic myeloid leukemia, with baseline Hsp70 values below (top or blue line) and above the median (bottom or red line). (Abbreviation: E, number of patients had an event; N, total number).

role of plasma Hsp70 in other types of leukemias should also be explored.

Conflicts of interest

There are no conflicts of interests.

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