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ORIGINAL ARTICLE

Serum Heat Shock Protein 70, as a Potential Biomarker for Small Cell Lung Cancer

Madaras Balázs[1] · Horváth Zsolt[2] · Gráf László[3] · Gálffy Gabriella[4] · Tamási Lilla[4] · Ostoros Gyula[5] · Döme Balázs[5] · Mórocz Éva[6] · Bártfai Zoltán[7] · Prohászka Zoltán[3] · Kocsis Judit[2,3]

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Abstract The 70 kDa heat shock protein (Hsp70) is a highly conservative molecular chaperone, that has important role in cell integrity. Recently considerable amount of data are accumulating on the potential role of Hsp70 in carcinogenesis and tumor progression. Most papers are focusing on intracellular or membrane bound protein, however very limited data exist on serum Hsp70, that can also induce innate and adaptive immune response. Previously we have published data on the correlation between coloretal cancer progression and serum Hsp70 concentration. The objective of this study was to compare the serum Hsp70 level in patients with small cell lung cancer (SCLC n = 70) and age matched healthy controlls (n = 121) and correlate Hsp70 level with other known serum biomarkers (LDH and NSE) of the disease. We found that the serum level of Hsp70 was significantly higher in SCLC patients compared to control subjects (mean value 6.91 vs 2.47 ng/ml, p = 0.001). The highest Hsp70 concentration was measured in stage IV advanced SCLC (Stage IV versus Stage I-III disease: 9.91 vs 4.38 ng/ml, p = 0.003). The serum Hsp70 level correlated with serum LDH (r = 0.426, p < 0,001) and NSE level (r = 0.455, p < 0,001). We found that high serum Hsp70 level predicted unfavorable survival, risk of death within 1 year was more than 3 times higher in patients with high baseline Hsp70 level (HR:3.509, CI: 1.066–11.562; p = 0.039). Our observations indicate that serum Hsp70 could be a valuable diagnostic and prognostic marker in small cell lung cancer.

Keywords Small cell lung cancer · Hsp70 · Biomarker · Prognostic marker · Target

Introduction

Heat shock proteins are conservative intracellular proteins, with molecular chaperone funcion. Their main physiologic role is maintance of cell homeostasis. 70 kDa heath shock protein (Hsp70) is a well investigated member of the group, with many intra- and extracellular functions. [1] More evidences are accumulating on the role of Hsp70 in tumorigenesis, however these data are contradictory. Increased production may provide survival advantage for malignant cells [2, 3], while on the other hand large amount of extracellular Hsp70 can induce either antitumor immunity or immune suppression [4, 5].

Many data support that Hsp70 expression is misregulated in cancer cells. Increased production can be induced by several factors, like heat shock transcription factor 1 (HSF1) [6, 7, 8], loss of p53 function and increased expression of some proto-oncogens like HER2

Drs Madaras and Horváth contributed equally to this article

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✉ Kocsis Judit
kocsis.judit@med.unideb.hu; kocsisjucidr@gmail.com

1 National Institute of Oncology, Budapest, Hungary

2 Institute of Oncology, University of Debrecen, Debrecen, Hungary

3 3rd Department of Internal Medicine, Semmelweis University Budapest, Budapest, Hungary

4 Department of Pulmonology, Semmelweis University Budapest, Budapest, Hungary

5 Department of Tumor Biology, National Koranyi Institute of Pulmonology, Budapest, Hungary

6 Pulmonology Hospital, Törökbálint, Hungary

7 Department of Pulmonology, Elizabeth Teaching Hospital and Rehabilitation Institute Sopron, Sopron, Hungary

and c-Myc. [9] Tumor cells may avoid apoptosis through Hsp70 interaction with multiple components of the caspase dependent and independent apoptotic pathways, and TNF alpha induced cell death. [6, 10, 11] Like other chaperones Hsp70 slows down senescence by assembling telomerase enzyme in tumor cells. [12].

There are numerous publications focusing on Hsp70's prognostic and predictive value in various cancer types. High Hsp70 expression in tumor tissue, predicts a failed response to chemo-, radiotherapy and hyperthermia and indicates worse prognosis. [13–19].

Besides intracellular Hsp70, the extracellular form also influences tumor progression and survival, as extracellular Hsp70 (with other Hsps) plays a complex role in tumor immunity. It can activate both arms of the immune system, innate and adaptive immune response [20]. Due to these properties Hsp70 is considered a potential target of new antitumor therapies. [21, 22].

However, very limited number of papers are focusing on the diagnostic and prognostic value of the serum Hsp70 in cancer, compared to tissue (intacellular or membrane bound) Hsp70. Although heat shock proteins are intracellular, cytosolic or membrane binded proteins, they can be released into the circulation by active, exocytotic trafficing or via cell disintegration. Measuring serum Hsp70 level could provide diagnostic or prognostic information about the tumor without invasive sampling. It is known from our previous work that elevated level of Hsp70 in colorectal cancer predicts higher mortality rate [23]. Similar observation was published regarding chronic myeloid leukemia (CML) [24], and squamous cell carcinoma of the head and neck (SCCHN) [25]. To our best knowledge, however, no data are available on serum levels of soluble Hsp70 in patients with small cell lung cancer (SCLC).

In our present work, we measured serum Hsp70 level in 70 patients with different stages of SCLC, and 121 healthy subjects. Than we tested whether the serum level of the protein correlates with tumor burden (eg. stage of the disease) and other clinical factors, such as known biomarkers of SCLC (serum LDH and NSE levels) and survival.

Methods

Patients and Controls

Serum samples of SCLC patients were collected consecutively after signed consent at five dedicated lung cancer centers and oncology centers of Hungary. Between June 2011 and September 2012 seventy (n = 70) patients diagnosed with different stages of SCLC gave permission for serum sample and clinical data collection. The cohort of age-matched control subjects consisted of onehundred twenty one (n = 121) healthy individuals, who had no malignant disease in their medical history and underwent a recent screening procedure, excluding manifest malignancy. The study was approved by the Medical Research Council Scientific and Research Committee of Hungary. Basic clinical characteristics of the patients and controls are summarized in Table 1.

All patients received adequate therapy for small cell lung cancer, according to their tumor stage. All patients received first line chemotherapy that consisted of platinum (cisplatin or carboplatin) plus etoposid combination in 4–6 chemotherapy cycles. Patients with limited stage disease received thoracic radiotherapy as well. In the majority of patients (80 %) serum sample collection was performed at their first presentation. In 14 cases serum and data collection was started during the first line chemotherapy courses. Serum samples were stored at -20C. Clinical data were collected at the same time (age, gender, tumor stage), than patients were followed for survival.

Serum Hsp70 Analysis

Soluble Hsp70 level was measured by using R&D Systems (Minneapolis, MN, USA, Catalogue No. DYC1663E) enzyme-linked immunosorbent assay kit. Ninety-six-well microtitre plates were coated with mouse anti-human Hsp70 capture antibody (100 μl; 2 μg/ml) in carbonate buffer (pH 9.5) overnight at 4 °C. Plates were washed with phosphate-buffered saline (PBS) containing 0.1 % Tween 20 three times and nonspecific binding sites blocked by incubation with 200 μl of PBS containing 0.5 % gelatinec and Tween 20 for 1 h at room temperature. After washing, 100 μl of the

Table 1 Clinical characteristics of 70 patients with small cell lung cancer (SCLC) and 121 healthy controls. Tumor staging is according to TNM Classification of Malignant Tumors verion 7

	SCLC (n = 70)	Control (n = 121)	P-value
age (mean ± SD, range)	62.60 ± 7.71 (51–85)	61.05 ± 16.42 (19–89)	0.331 (Mann-Whitney test)
sex (female/male)	34/36	66/55	NS (χ2-test)
Stage of the disease (I/II/II/IV)	1/6/31/32	NA	NA
Localised vs advanced disease*	38/32	NA	NA

NA not applicable

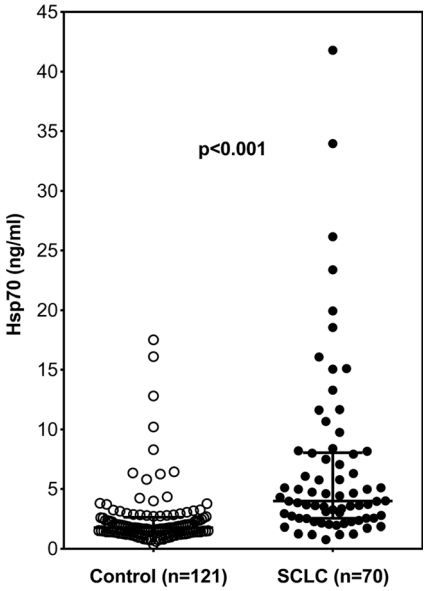

Fig. 1 Comparison of serum Hsp70 levels between healthy contols and patients with small cell lung cancer. Mann-Whitney test

reference preparation (recombinant human Hsp70, 0–10 μg/ml) or samples (1:1) were added and the plates were incubated for 2 h at room temperature. Plates were subsequently washed and Hsp70 binding was determined using biotinylated rabbit anti-human antibody (100 μl; 0.5 μg/ml) in PBS gelatine. After 1.5 h at room temperature, plates were washed and incubated with streptavidin–horseradish–peroxidase (1:200) in PBS gelatine for 20 min at room temperature. Plates were washed and 100 μl of o-phenylene-diamine (Sigma, St Louis, MO, USA) in citrate buffer was added. The optical density was measured at λ=490 nm (reference at λ=620 nm).

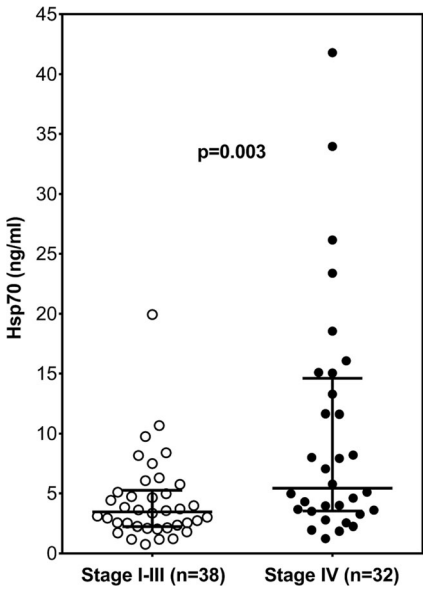

Fig. 2 Comparison of serum Hsp70 levels between patients with advanced (Stage IV) ad localised (stage I-III) small cell lung cancer. Mann-Whitney test

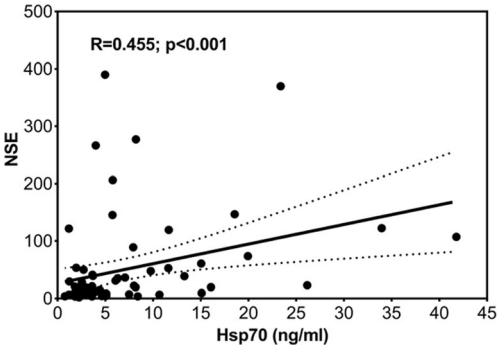

Fig. 3 Correlation berween serum Hsp70 and NSE levels in patients with small cell lung cancer

The detection range of the assay was 0.05–10 ng/ml, the intra/inter-assay variability <10/<16 %, respectively.

Serum LDH level was measured with the Roche Cobas system as recommended by IFCC. Serum NSE level was measured with Roche Cobas sytem using Electrochemiluminescence immunoassay.

Statistical Analysis

Data are given as mean ± standard deviation (SD) or median and interquartile range (IQR) if data were not Gaussian distributed.

The differences between groups were evaluated with the Mann-Whitney test. Correlations between the variables were expressed using the non-parametric Spearman's correlation coefficients. Correlation between serum protein levels and survival was analysed with Cox regression. Survival was calculated according to the Kaplan-Meier method. Receiver operating characteristic (ROC) curve analysis was used to determine the optimal cut-off value of Hsp70. We selected one year survival as an end point for this trial, since median survival of patiens with newly diagnosed small cell lung cancer is poor, only 6–12 month in extensive disease and 16–24 month in limited disease. The curves were compared for statistical significance using log-rank testing. All tests were two-tailed, *p*-values of <0.05 were accepted as statistically significant.

Statistical analysis was performed using the GraphPad Prism v6.01 (GraphPad Software Inc., San Diego, CA, www.graphpad.com) and SPSS v22 (SPSS Inc., Chicago, IL) software.

Results

Comparison of Serum Hsp70 Concentration in Patients with Small Cell Lung Cancer and Healthy Subjects

We studied whether baseline serum concentration of soluble Hsp70 is different between patients with SCLC and controls. Highly elevated levels of Hsp70 were found in SCLC patients,

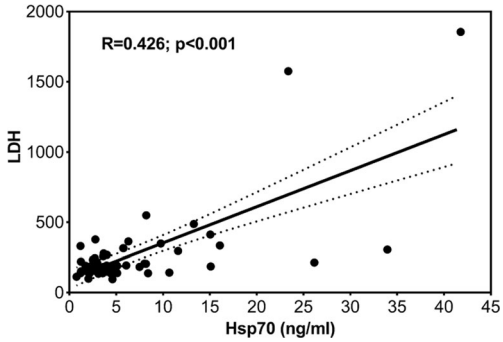

Fig. 4 Correlation berween serum Hsp70 and LDH levels in patients with small cell lung cancer

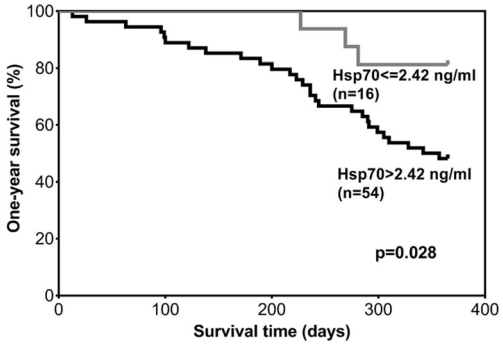

Fig. 5 One year survival depending on baseline serum Hsp70 level

compared to healthy subjects: mean Hsp70 value was 6.91 ng/ml (SD: 7.58) in patients with small cell lung cancer and 2.47 (SD: 2,56) in healthy subjects (Fig. 1). The difference was statisically significant ($p < 0{,}001$).

Comparison of Serum Hsp70 Concentration in Different Stages of SCLC

We compared Hsp70 levels between different tumor stages. It was found that serum Hsp70 level consequently increases with tumor burden, the highest sHsp70 concentrations were detected in the metastatic patient popuplation. Mean Hsp70 level was 9.91 ng/ml (SD: 9.78) in stage IV disease, whereas the mean detected value was 4.38 ng/ml (SD: 3.54) in patients with stage I-III disease ($p = 0.003$, Fig. 2).

Correlation of Soluble Hsp70 Level with LDH and NSE Levels and Survival

NSE and LDH are unspecific tumor markers of SCLC. We could measure the serum level of NSE in all patients and the level of LDH in 65 patients. Positive and significant correlation was found between serum Hsp70 and NSE ($r = 0.455$, $p < 0{,}001$, Fig. 3.) or LDH level ($r = 0.426$ $p < 0{,}001$, Fig. 4).

Mean overall survival of patients was 15,9 month (CI: 13.0–18.8). We performed a univariate Cox regression to test wether there is any connection between survival of patients and baseline biomarker (LDH, NSE) and Hsp70 level. As Table 2 shows, all the three examined serum protein concentration had a negative correlation with survival. The correlation was significant in the case of NSE and LDH, and was nearly significant (trend) with Hsp70 level.

Using the ROC curve the cut-off value of Hsp70 was 2.42 ng/ml. Values ≤2.42 ng/ml were considered to be favorable and values >2.42 as unfavorable prognostic marker. The risk of death within 1 year was more than 3 times higher in the unfavorable group (HR:3.509, CI: 1.066–11.562; $p = 0.039$, Fig. 5).

Table 2 Examination of clinical characteristics and biomarkers as prognostic factors for overall survival in patients with small cell lung cancer. Univariate Cox regression

	HR (hazard ratio)	95 % CI	p
Age	1.009	0.971–1.048	0.663
Sex (male)	1.572	0.901–2.742	0.111
Stage IV	1.493	0.865–2.577	0.150
Hsp70	1.028	0.997–1.060	0.077
LDH	1.001	1.000–1.002	0.048
NSE	1.006	1.003–1.010	<0.001

Discussion

In this study we evaluated the serum level of Hsp70 in SCLC patients and compared it to those of healthy subjects. We found significantly higher level of Hsp70 in sera of SCLC patients, compared to healthy controls. The elevated level of Hsp70 increases with tumor stage, much higher levels were detected in patients with advanced disease (stage IV) than in earlier stages (stage I-III). High Hsp70 level (>2.42 ng/ml cut off value) predicts unfavorable prognosis with a 3.5 times higher risk of death during the first year. We could also establish, that serum Hsp70 level significantly correlates with LDH and NSE, known biomarkers of small cell lung cancer.

This is the first consistent and comparative report on elevated serum Hsp70 level in a malignant disease on a relatively large patient population, in which we used a well established and validated ELISA method. Data are sparse regarding serum Hsp70 levels in other tumors. The Hsp70 level we detected in SCLC patients are almost 3 times higher, than in healthy controls (6.91 versus 2.47 ng/ml) or in patients with colorectal cancer as we published earlier [26]. Dutta et al. reported [27] on highly elevated Hsp70 levels in pancreatic patients, raising the possibility that Hsp70 could be a potential biomarker for early detection of pancreatic cancer. However their study is limited due to the small sample size ($n = 27$ cancer patients and $n = 10$ controls). Gehrmann and his coworkers found elevated serum Hsp70 level in patients with SCCHN

(n = 21) compared to healthy controls and serum level correlated with Hsp70 membrane expression as well [25]. Sato et al. [28] investigated Hsp27 and Hsp70 content of sera (n = 8) and bile acid (n = 10) of patients with cholangiocarcinoma. They found increased heat shock protein levels in patients with cancer, which was significantly higher in the bile, but not in the serum. In patients with non small cell lung cancer (NSCLC) Zimmermann et al. [29] found elevated Hsp27 and Hsp70 levels. Comparing to healthy controls (n = 33) and chronic obstructive pulmonary disease (COPD) patients (n = 34) they found that the serum level of Hsp70 (and a co-chaperon Hsp27) was significantly higher in NSCLC patients (n = 109). In the latter study Hsp70 elevation was independent of the disease stage, the magnitude of elevation was greater than two fold.

The origin of extracelluar Hsp70 is not fully understood. One possible explanation is that in high grade, agressive, rapidly proliferating tumors such as SCLC, the protein enters the circulation passively, as tumor cell apoptosis and lysis occurs permanently. Another probable mechanism is active release of Hsp70 from cancer cells to their microenvironment. [30, 31].

Many studies are focusing on prognostic and predictive markers in SCLC. Results are conflicting, however clinical stage of the disease, ECOG performance status, baseline white blood cell count (WBC), presence of brain metastasis seem to be the most important independent prognostic factors. [32] LDH has both prognostic and predictive value; high serum levels of LDH predicts poor outcome. Studies confirm that serum LDH correlates positively with tumor mass and tumor agressivity [33]. The origin of elevated serum LDH is supposed to be tissue injury, cell infarction or necrosis. [34] Many oncologist use LDH level for monitoring tumor response and disease acitivity together with serum NSE [32, 35].

Moreover classic tumour markers, such as NSE and Cyfra 21–1 are used for a long time as diagnostic and independent prognostic tools in SCLC. [36–38] Both of them have negative prognostic value. Patients with elevated NSE and Cyfra 21–1 levels have even worse disease outcome. Our study showed that there is a strong correlation between Hsp70 and LDH and NSE levels. There are some data from the literature, including our previous publications proposing that elevated serum Hsp70 is a negative prognostic factor in various tumour types, like colorectal, breast, endometrial and bladder cancer. [23, 39, 40] Taking into account the negative prognostic value of LDH and NSE it is tempting to speculate that Hsp70 could be a valuable additional prognostic biomarker in SCLC. The unfavourable survival data that we observed among patients with high Hsp70 levels confirms this hypothesis.

Beside its potential biomarker and prognostic role, Hsp70 could be a potential target of new antitumor therapies. It is known that intense Hsp70 production in tumour tissue enhances cell protection, that can lead to chemotherapy resistance. [14–16, 41] On the other hand, cell membrane bound and extracellular Hsp70 induces specific and non-specific immune response, [41, 42] Hsp70 can activate both native and adaptive immune response. Hsp70 as a chaperone presents tumor specific peptides to antigen presenting cells, through which activation of specific cytotoxic T cell response is induced. Hsp70 per se can provide activatory signals for the innate immune system. [43] It can bind to Toll- like receptors of antigen presenting cells, initiating inflammation and other non specific immune activation. Tumor membrane bound Hsp70 is able to induce NK cell activation. [44].

Interestingly, Hsp70 is a druggable target compared to other heat shock proteins, since it's expression and activation is regulated differently [45] Therefore, targeting of Hsp70 is an attractive anticancer strategy [20]. Several compounds are able to inhibit HSF1, the heat shock transcription factor responsible for Hsp70 expression, including quercetin, triptolide, diterpenetriperoxid [45].

Nowadays there are some preclinical data on the use of Hsp70 targeted therapies [46], and early clinical trials are still in progress. [22] Based on our results it can be proposed that small cell lung cancer patients should be included in early phase clinical trials targeting Hsp70.

Our observations indicate that serum Hsp70 could be a valuable diagnostic and prognostic marker in small cell lung cancer as well as a new potential antitumor target.

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