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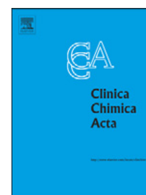

Discrimination of clinical stages in non-small cell lung cancer patients by serum HSP27 and HSP70: A multi-institutional case–control study

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ABSTRACT

Introduction: Lung cancer represents a major healthcare problem. Accordingly, there is an urgent need to identify serum biomarkers for early diagnosis of lung pathology. We have recently described that patients with manifest COPD evidence elevated levels of heat shock proteins (HSPs). Based on these data, we speculated whether HSPs are also increased in patients with diagnosed lung cancer.
Methods: Serum levels of HSP27, phospho-HSP27 (pHSP27) and HSP70 in patients with non-small cell lung cancer (NSCLC) diagnosed at an early (stages I–II, $n=37$) or advanced (stages IIIA–IV, $n=72$) stage were determined by using ELISA. Healthy smokers ($n=24$), healthy never-smoker volunteers ($n=33$) and COPD patients ($n=34$) according to GOLD classification served as control population.
Results: Serum levels of HSP27 were elevated in patients with NSCLC diagnosed at an early or advanced stage when compared with both healthy control groups ($P<0.005$ and $P<0.0001$ respectively). Statistically significant differences were furthermore found between the groups of patients with early vs. advanced stage NSCLC ($P=0.0021$). Serum levels of HSP70 were also significantly elevated in patients with NSCLC diagnosed at an early or at an advanced stage when compared with either healthy control groups ($P=0.0028$ and $P<0.0001$ respectively). In univariate logistic regression models including healthy subjects and patients with NSCLC, HSP70 had an area under the curve (AUC) of 0.779 ($P<0.0001$) and HSP27 showed an AUC of 0.870 ($P<0.0001$).
Conclusion: Our data suggest that serum HSP27 levels might serve as a possible tool to discriminate between early and advanced stages NSCLC.

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1. Introduction

The relationship between smoking, lung cancer and airflow obstruction is well recognized [1]. It is currently well accepted that the presence of chronic airway inflammation is a significant risk factor for the development of lung cancer [2,3]. Cross-sectional studies have evidenced that the prevalence of chronic obstructive pulmonary disease (COPD) is 40–70% among those diagnosed with lung cancer [4]. Petty hypothesized in an editorial [5] that COPD and lung cancer could have common origins based on the same inflammatory disease process that include genetic predisposition and environmental risk factors. Other authors suggested that interindividual differences between genes that control genomic integrity and those that control tissue injury may distinguish between lung cancer and COPD outcomes in response to inflammation caused by smoking [6]. Intuitively, this hypothesis pertains to airflow obstruction and emphysema, two overlapping manifestations of COPD that are related to cigarette smoking.

COPD is characterized by a largely irreversible obstruction of the small airways due to aberrant inflammatory response and airway remodeling [7]. Chronic bronchitis and lung emphysema are pathologic characteristics of COPD and both conditions result from progressive inflammatory destruction of the lung parenchyma. Recently, COPD was accepted as a disease entity featuring immunological alterations seen in autoimmune disease. These reports demonstrated alterations in CD8+ and CD4+ T cells as a part of the adaptive immune system [8–10]. We have recently demonstrated that levels of systemic CD4+ CD28null T cells, a cell population described in various rheumatologic

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Table 1
Characteristics of the study subjects. *P*-values were determined using one-way ANOVA or chi^2 test for categorical variables (*$P<0.001$, **$P<0.0001$). Abbreviations: FVC, forced vital capacity; FEV1, forced expiratory volume in 1 s; AC, adenocarcinoma; SCC, squamous cell carcinoma; NSCLC-NOS, NSCLC not otherwise specified.

	Healthy		COPD	NSCLC		Total	*P*
	Non-smokers	Smokers	GOLD I–IV	Early stage	Advanced stage		
n	33	24	34	37	72	200	-
M/F %	48.5/51.5	37.5/62.5	60.0/40	59.5/40.5	56.9/43.1	52.5/47.5	*n.s.*
Age (years)	55.8 ± 7.8	56.3 ± 7.0	59.9 ± 8.4	59.9 ± 6.2	57.8 ± 6.9	57.9 ± 7.2	*n.s.*
Smoking history %							
Current/Ex	0	100	100	73.0	63.9	67.4	-
Never	100	0	0	13.5	7.0	24.1	-
No details	0	0	0	13.5	29.1	8.5	-
Lung function							
FVC(L)	3.73 ± 0.98	3.52 ± 0.85	2.80 ± 1.08	3.50 ± 0.81	3.18 ± 0.91	3.3 ± 0.99	*
FEV1(L)	2.96 ± 0.73	2.71 ± 0.67	1.63 ± 0.80	2.46 ± 0.74	2.12 ± 0.75	2.3 ± 0.88	**
FEV1%	99.4 ± 9.5	92.1 ± 13.9	52.76 ± 23.7	79.2 ± 20.8	71.3 ± 22.5	76.2 ± 25.1	**
FEV1/VC	0.80 ± 0.06	0.77 ± 0.06	0.57 ± 0.14	0.70 ± 0.11	0.66 ± 0.13	0.69 ± 0.14	**
Histological classification							
AC	-	-	-	24	52	76	*n.s.*
SCC	-	-	-	11	15	26	*n.s.*
NSCLC NOS	-	-	-	1	5	6	-
Others	-	-	-	1	0	1	-

diseases, were increased in COPD patients and correlated with severity of COPD GOLD (Global Initiative for Obstructive Lung Disease) classification [11]. Moreover, we were able to evidence that manifest COPD is associated with increased systemic release of apoptosis-specific proteins as markers for increased cellular turnover as compared to controls [12]. Remarkably, increased serum levels of heat shock protein (HSP) 27 and HSP70 evidenced a high sensitivity and specificity as diagnostic marker for manifest COPD [13]. In a further attempt, we were able to show that increased levels of HSP27 positively correlate with the presence of air trapping and emphysema in subjectively healthy smokers with normal lung function [14].

Recently, CT-based screening programs have clearly shown that emphysema and chronic airway inflammation are associated with the risk of lung cancer [15].

Lung cancer was not only the most commonly diagnosed malignant disease but also the leading cause of cancer-related deaths in men in 2008 worldwide. In women, it was the fourth most frequently diagnosed malignancy and the second leading cause of cancer-related deaths, worldwide.

NSCLC therapy has included surgery and (chemo)radiotherapy; more recently, targeted drugs have been incorporated into therapeutic protocols. However, the overall prognosis of NSCLC patients remains poor: the five-year survival rate has been in a plateau of 15% for three decades [16]. Given the poor prognosis for patients who present in an advanced stage, there has been great interest in screening for lung cancer [17]. Investigators of the National Lung Screening Trial (NLST) observed recently that screening with low-dose CT significantly reduces mortality from lung cancer among current or ex-smokers [18]. Nevertheless, as the authors of this study themselves acknowledge, circulating molecular markers are also needed to identify individuals who are best suited for CT screening and/or to help confirm positive CT screening results. Some lung-specific serum tumor-markers, such as CEA, CA-125, CYFRA21-1, SCC, NSE, proGRP, chromogranine, and TPA have been evaluated in patients with NSCLC, as well as with small cell lung cancer. Despite extensive studies, however, few have turned out to be useful in clinic [19,20].

Based on the obvious relationship between COPD pathogenesis and lung cancer immunology, we hypothesized that HSP27 and HSP70 levels are increased in patients with manifest NSCLC. To test this hypothesis, peripheral blood HSP27 and HSP70 levels of early (IA–IIB) and advanced (IIIA–IV) stages NSCLC patients were compared to those of age-, sex-, and smoking status-matched controls by ELISA.

2. Materials and methods

2.1. Study subjects

The study protocol has been approved by the Ethics Committee of the Medical University of Vienna, Austria (EC-No.: 091/2006 respectively 449/2008). Informed and written consent was obtained from each subject included in the study and all clinical and laboratory tests were performed in accordance with the Declaration of Helsinki and the guidelines for Good Clinical Practice of the Medical University of Vienna.

109 NSCLC patients and 57 healthy controls were included in this case–control study (Table 1). Healthy smoker volunteers without any clinical signs of cancer ($n=24$), healthy volunteers without any smoking history nor any clinical signs of cancer ($n=33$), patients with NSCLC diagnosed at an early stage ($n=37$) and at an advanced stage ($n=72$) were evaluated in four study groups. To prove that levels of serum HSP27 are also augmented in patients with NSCLC but without impairment of lung function we performed a subgroup analysis by differentiating those NSCLC patients with ($n=41$) and without ($n=41$) COPD classified by GOLD. In order to relate these NSCLC study group evaluations we further included a patient cohort with manifest COPD staged according to the GOLD classification ($n=34$).

Diagnosis of NSCLC was confirmed through histological specimens in all cases. The cases were staged according to operative and pathologic findings based on seventh edition of the TNM staging system (2009) of the International Association for the Study of Lung Cancer (IASCL) [21]. Patients with adenocarcinoma (AC), squamous cell carcinoma (SCC), NSCLC not otherwise specified (NSCLC-NOS) and large cell carcinoma were included in this study. Characteristics of the study subjects are depicted in Table 1. After informed and written consent, all study subjects were asked to answer a questionnaire regarding their smoking habits, and pulmonary function parameters (forced vital capacity [FVC], forced expiratory volume in 1 s [FEV1], and FEV1/FVC ratio) were obtained by spirometry. Measurements were made before and – if criteria for airflow obstruction were met – 15–30 min after inhaling of 200 μg salbutamol. Blood samples were collected at the time of first admission to the Department of Thoracic Surgery, serum was obtained after centrifugation and aliquots were stored at −80 °C until further testing. Exclusion criteria were any other known malignant or inflammatory diseases, autoimmune diseases and alpha1-antitrypsin deficiency.

2.2. Quantification of serum HSP27

Serum levels of HSP27 were determined using adapted enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA). Ninety-six well microtitration plates were coated overnight at room temperature with a capture antibody against human HSP27 at a concentration of 1 μg/ml. Plates were then washed and blocked with block buffer for 2 h. Following another washing step, samples and standard protein in different concentrations were added to the wells. After a washing step, a biotin-labeled antibody was added to each well and incubated for 2 h. After another washing step, horseradish–peroxidase-conjugate (HRP) was applied for 20 min. Wells were washed, and color reaction was achieved using tetramethylbenzidine (TMB) (Sigma-Aldrich Corp, St. Louis, MO, USA) and the reaction was stopped by an acid stop solution. Color development was then monitored using a Wallac Multilabel Counter 1420 (PerkinElmer, Waltham, MA, USA). The optical density (OD) values obtained at 450 nm were compared to the standard curve calculated from OD values of standards with known concentrations of antigen. Specificity was demonstrated by the manufacturer by Western blot analysis of the protein bound by the capture antibody supplied in the kit. HSP70 cross reactivity was 0.23%.

2.3. Quantification of serum pHSP27 (S78/S82)

Serum levels of phosphor-HSP27 were determined using adapted enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA). Ninety-six well microtitration plates were coated overnight at room temperature with a capture antibody against human pHSP27 at a concentration of 2 μg/ml. Plates were then washed and blocked with block buffer for 2 h. Following another washing step, samples and standard protein in different concentrations were added to the wells. After a washing step, a biotin-labeled antibody was added to each well and incubated for 2 h. After another washing step, horseradish–peroxidase-conjugate (HRP) was applied for 20 min. Wells were washed, and color reaction was achieved using tetramethylbenzidine (TMB) (Sigma-Aldrich Corp, St. Louis, MO, USA) and the reaction was stopped by an acid stop solution. Color development was then monitored using a Wallac Multilabel Counter 1420 (PerkinElmer, Waltham, MA, USA). The optical density (OD) values obtained at 450 nm were compared to the standard curve calculated from OD values of standards with known concentrations of antigen.

2.4. Quantification of serum HSP70

Serum levels of HSP70 were determined using adapted ELISA kits (R&D Systems, Minneapolis, MN, USA). Ninety-six well microtitration plates were coated overnight with a capture antibody against human HSP70 at a concentration of 2 μg/ml. Plates were then washed and blocked with block buffer for 2 h. Following another washing step, samples and standard protein in different concentrations were added to the wells. After a washing step, a biotin-labeled antibody was added to each well and incubated for 2 h. After another washing step, horseradish–peroxidase-conjugate was applied for 20 min. Wells were washed, and color reaction was achieved using TMB (Sigma-Aldrich Corp, St. Louis, MO, USA) and the reaction was stopped by an acid stop solution. Color development was then monitored using a Wallac Multilabel Counter 1420 (PerkinElmer, Waltham, MA, USA). The OD values obtained at 450 nm were compared to the standard curve calculated from OD values of standards with known concentrations of antigen.

2.5. Statistical methods

Statistical analysis was performed using SPSS software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism5 (GraphPad Software, La Jolla, CA, USA) was used for data visualization. Data are given as mean ± standard deviation (SD) or median and interquartile range (IQR) if data were not Gaussian distributed. To determine Gaussian distribution, Shapiro–Wilk test was used. Pair-wise comparisons between groups were performed using Student's *T* test. Either one-way ANOVA or, if data were not Gaussian distributed, Kruskal–Wallis tests were used to determine statistical significance between more than two study groups. Categorical variables were compared using chi^2 test. Univariate logistic regression models were calculated for HSP27, pHSP27 and HSP70. Receiver operating characteristics (ROC) curves with area under the curve (AUC) were plotted to demonstrate sensitivity and specificity of the evaluated serum proteins. *P*-values <0.05 were considered statistically significant.

3. Results

3.1. Heat shock protein 27

Serum levels of HSP27 [mean] were 1648 ± 777 pg/ml in healthy non-smoker controls, 2346 ± 1080 pg/ml in healthy smokers, 3647 ± 1613 pg/ml in patients with early stage NSCLC, and 5364 ± 2679 pg/ml in patients with advanced stage NSCLC. Statistically significant differences were found between all four groups (healthy vs. early stage, healthy vs. advanced stage, and early vs. advanced stage: in each case $P<0.001$) (Fig. 1A). Assessment of HSP27 levels indicated no significant differences between the groups of COPD-free and COPD patients either in the early or in the advanced NSCLC patient groups (Fig. 1B). Furthermore no significant differences could be detected between the two main histological subtypes (AC vs. SCC, $P=0.116$). Detailed results (IASCL stages I to IV) of HSP27 serum levels are given in Table 2.

3.2. Phospho heat shock protein 27 (S78/S82)

Serum levels of pHSP27 [median] were 315 (Q1 = 172, Q3 = 527) pg/ml in patients with early stage NSCLC and 447 (Q1 = 229, Q3 = 1733) pg/ml in patients with advanced disease. Statistically significant differences were found between the two groups (early vs. advanced stage: $P=0.015$).

3.3. Heat shock protein 70

Mean serum levels of HSP70 [mean] were 305 ± 212 pg/ml in healthy non-smokers, 321 ± 316 pg/ml in healthy smoker individuals, 603 ± 386 pg/ml in patients with early stage NSCLC, and 793 ±545 pg/ml in patients with advanced disease. Statistically significant differences were found between healthy non-smoker controls and patients with NSCLC diagnosed at an early or an advanced stage ($P=0.0028$ and $P<0.0001$ respectively), between healthy smoker controls and patients diagnosed at an early or an advanced stage ($P=0.006$ and $P<0.0001$ respectively), but not between the two NSCLC groups (Fig. 1C). HSP70 levels of patients with or without COPD did not differ from each other significantly in either NSCLC groups, nor between the two main histological subtypes (AC vs. SCC, $P=0.852$). Detailed results (IASCL stages I to IV) of HSP70 serum levels are depicted in Table 2.

3.4. Regression models

In univariate logistic regression models including healthy volunteers and patients with NSCLC, HSP70 had an area under the curve (AUC) in the receiver operating characteristic (ROC) curve of 0.779 (0.707–0.851 95% confidence interval; $P<0.0001$), pHSP27 an AUC of 0.682 (0.580–0.783 95% confidence interval; $P=0.002$); and HSP27 showed an AUC of 0.870 (0.817–0.923 95% confidence interval; $P<0.0001$) (Fig. 2).

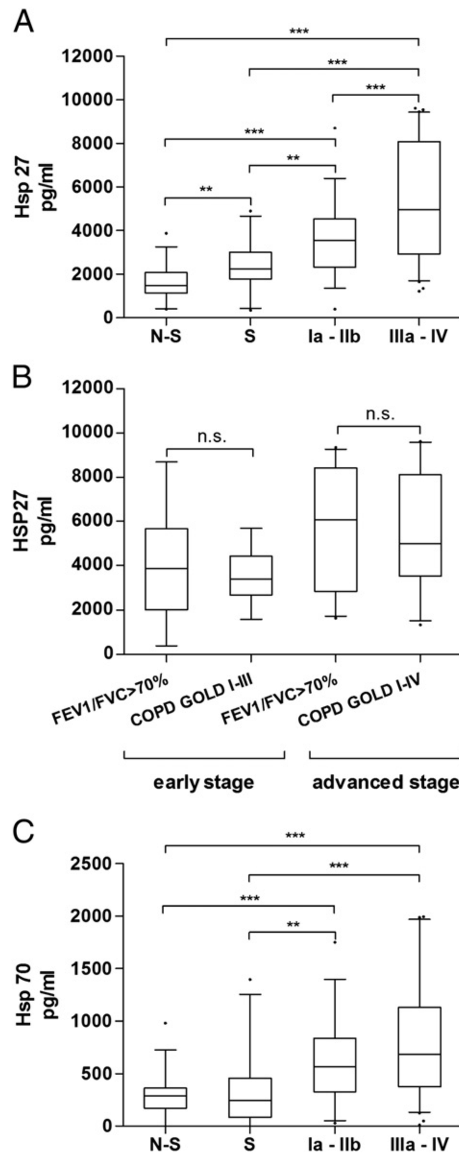

Fig. 1. Box plots showing mean (inner lines), inter-quartile ranges (boxes), minimum/maximum levels (whiskers) and outside values (dots) of HSP27, pHSP27 and HSP70 serum levels. A. Serum HSP27 levels are significantly elevated in patients with NSCLC diagnosed at an early (IA–IIB) or at an advanced stage (IIIA–IV) when compared with either healthy control groups (**$P<0.01$, ***$P<0.001$). Between early and advanced stage NSCLC patients, the difference is also statistically significant (***$P<0.001$). N-S, healthy never-smokers; S, healthy smokers. B. Serum levels of HSP27 do not show any statistically significant differences between patients with and without COPD within the two NSCLC groups. C. Serum levels of HSP70 are elevated in patients with NSCLC diagnosed at an early (IA–IIB) or at an advanced stage (IIIA–IV) when compared with either healthy control groups, (**$P<0.01$, ***$P<0.001$). However, there is no statistically significant difference between the two NSCLC patient groups.

4. Discussion

In the present study, we found that serum HSP27 and HSP70 levels were significantly increased in a population of NSCLC patients as compared to our sample of community based smoker and non-smoker controls. In addition, serum levels of HSP27 were significantly indicative for presence of early vs. advanced NSCLC. Furthermore impaired lung function parameters were significantly correlated with early and advanced stage NSCLC. This finding corroborates recently published studies that tied CT verified lung pathology with impaired lung function parameters [4]. We believe that this study is the first case–control study that proves that HSP27 is a potential discriminator of early and advanced NSCLC patients.

Table 2
Detailed results of HSP27 and HSP70 serum levels in NSCLC patients (IASCL stages I to IV respectively with and without COPD), COPD patients (GOLD stages I to IV) and in healthy controls.

		HSP27		HSP70	
	n	Median	IQR	Median	IQR
Healthy non-smokers	33	1482	1136–2071	285	166–345
Healthy smokers	24	2242	1787–3009	244	82–456
COPD GOLD I	5	3106	2740–4135	367	271–697
COPD GOLD II	15	3027	1741–3565	511	244–589
COPD GOLD III	5	4498	2568–4896	267	210–1464
COPD GOLD IV	9	4059	3002–4746	277	138–468
NSCLC I-IIb, without COPD	15	3876	2027–5665	557	353–943
NSCLC I-IIb, with COPD	14	3381	2670–4423	556	217–841
NSCLC IIIa-IV, without COPD	26	6073	2833–8428	672	429–1274
NSCLC IIIa-IV, with COPD	27	4985	3521–8112	740	284–1124
NSCLC stage Ia	10	3452	1823–4347	643	129–847
NSCLC stage Ib	15	3198	2469–4206	517	246–806
NSCLC stage IIa	3	2689	2258–4074	1014	58–1748
NSCLC stage IIb	9	4377	3105–5626	616	344–791
NSCLC stage IIIa	16	4023	3025–7355	452	282–1147
NSCLC stage IIIb	6	4339	3371–8620	825	318–1796
NSCLC stage IV	50	5558	2854–8125	719	432 – 1105

Heat shock proteins (HSPs) belong to a highly conserved protein family and normally act as intracellular molecular chaperones which maintain protein homeostasis. When cells are exposed to stressful conditions, HSP synthesis gets massively triggered in order to fold heat-denatured proteins and block caspase-dependent apoptosis, permitting repair and thwarting death [22]. However, HSPs can also be released into the circulation, where they are able to interact with the immune cells in a number of contexts. HSPs can act as proinflammatory mediators and lead to cytokine transcription and release. Through their ability to bind antigenic peptides during antigen procession, they can further act as stimulants of the adaptive immune response. Thus, anti-inflammatory and immunosuppressive patterns of HSPs are also described, depending on the biological microenvironment [23]. Especially HSP70, either as serum protein or cellular component, has been studied extensively in various inflammatory diseases [13,24].

There is growing evidence in literature that the expression of HSPs is increased in various human cancers. For example, HSP27 was found to be strongly expressed in breast cancer [25], hepatomas and well differentiated hepatocellular carcinomas, brain tumors [26], and prostatic carcinoma [27]. However, a growing list of contradictory data is emerging regarding expression patterns of HSPs in lung cancer tissues and serum/plasma samples of lung cancer patients. Michils and coworkers [28] evaluated the quantitative expression of low molecular weight (ubiquitin and HSP27) and high molecular weight (HSP60 and HSP70)

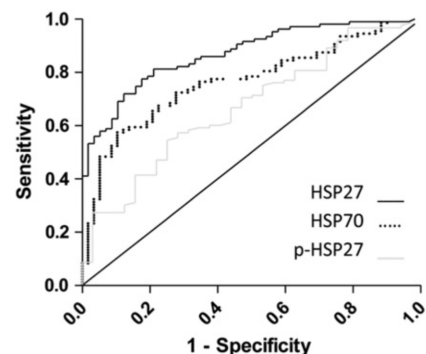

Fig. 2. ROC curve indicating sensitivity and specificity of HSP27 (AUC = 0.870), pHSP27 (AUC = 0.682) and HSP70 (AUC = 0.779) in the diagnosis of NSCLC.

HSPs in tumorous and healthy lung tissues. They concluded that HSP60 and HSP70, but not HSP27 expression, was increased in cell lysates of NSCLC tissue. In line with this conclusion is a publication authored by Huang et al. [29]. They have studied 60 NSCLC cancer patients and were able to demonstrate that clinicopathological features of NSCLC correlated with tissue expression of HSP70, but not with the expression of HSP27. No statistical significance was observed in histological types and gender with respect to both HSP70 and HSP27 expression.

In contrast, Malusecka et al. [30] reported that cytoplasmic immunostaining for HSP27 was positive in a high amount (70%) of samples obtained from patients with NSCLC. They further found a positive correlation between expression levels of HSP27 and HSP70, and a correlation between Ki-67 proliferation index and nuclear HSP70 staining. Another publication from these authors describes a significant survival advantage in patients overexpressing HSP27 in NSCLC tissue. Furthermore, a significantly decreased survival was observed in those patients that were HSP70 tissue negative [31]. Several years before, Volm and coworkers [32] described a wide range of HSP70 levels in human NSCLCs processed for immunostaining, possibly reflecting different biological stressors. Moreover, they found a strong correlation between the number of daily smoked cigarettes and HSP70 expression in NSCLC tissue. 75% of tumors from smoker patients showed high HSP70 expression, whereas only 57% of non-smokers presented with high HSP70 expression in the tumor samples [33]. The first attempt to investigate the relationship between serum HSP70 levels and lung cancer was by Susuki K et al. [34]. They detected a significant association between elevated serum HSP70 levels and increased lung cancer risk among Japanese males. However, no association between lung cancer risk and HSP70 levels in female subjects could be found. A recent study investigated the expression of HSP27 and HSP70 in coal-mine workers [35]. This group investigated the association between plasma levels of HSP27/70 in coal-mine dust exposed miners with or without lung cancer and in healthy controls. Interestingly, those miners exposed to coal dust without cancer evidenced a significant increment of plasma HSP27 as compared to control groups.

Concluding our results, we were able to show significantly elevated serum HSP27 and serum HSP70 levels in NSCLC patients compared with healthy age-, sex-, and smoking status-matched controls. Furthermore, we show that COPD patients also evidence increased HSP levels. However, we have also proven that NSCLC patients with normal lung function demonstrate increased HSP levels, indicating that both COPD and NSCLC are related to heightened serum levels of HSP27. If we interpret our data correctly, we feel justified to claim that COPD and NSCLC evidence similar immunological features as hypothesized previously [5,6]. Of utmost interest to us was the finding that in our highly specified group analysis HSP27 was highly appropriate to differentiate between early and advanced stage NSCLC: HSP27 serum levels showed a stage-dependent increase with 2- and 3-fold higher levels in early (IA–IIB) vs. advanced stage (IIIA–IV) NSCLC patients. These data indicate that augmented spillage of this stress protein into the systemic circulation occurs during disease progression, presumably caused by a continuous activation of the immune system. Further, HSP27 showed an excellent sensitivity and specificity in a regression model to distinguish between healthy subjects and NSCLC patients. With an overall area under the curve of 0.870, HSP27 is prone to serve as a possible diagnostic marker for NSCLC progression.

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