

The level of Hsp27 in lymphocytes is negatively associated with a higher risk of lung cancer

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Abstract Heat shock proteins (Hsps) can protect cells, organs, and whole organisms against damage caused by abnormal environmental hazards. Some studies have reported that lymphocyte Hsps may serve as biomarkers for evaluating disease status and exposure to environmental stresses; however, few epidemiologic studies have examined the associations between lymphocyte Hsps levels and lung cancer risk. We examined lymphocyte levels of Hsp27 and Hsp70 in 263 lung cancer cases and age- and gender-matched cancer-free

controls by flow cytometry. Multivariate logistic regression models were used to estimate the association between lymphocyte Hsps levels and lung cancer risk. Our results showed that Hsp27 levels were significantly lower in lung cancer cases than in controls (16.5 vs 17.8 mean fluorescence intensity, $P < 0.001$). This was not observed for Hsp70 levels. Further stratification analysis revealed that lymphocyte Hsp27 levels were negatively associated with lung cancer risk especially in males and heavy smokers. There was a statistical trend of low odd ratios (95% confidence intervals) and upper tertile levels of Hsp27 [1.000, 0.904 (0.566–1.444) and 0.382 (0.221–0.658, $P_{\text{trend}} = 0.001$) in males and 1.000, 0.9207 (0.465–1.822) and 0.419 (0.195–0.897, $P_{\text{trend}} = 0.036$) in heavy smokers] after adjustment for confounding factors. These results suggest that lower lymphocyte Hsp27 levels might be associated with an increased risk of lung cancer. Our findings need to be validated in a large prospective study.

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Abbreviations

BSA bovine serum albumin
CI confidence intervals
Hsp27 heat shock protein 27
Hsp70 heat shock protein 70
Hsps heat shock proteins
MFI mean fluorescence intensity
OR odds ratios
PBS phosphate-buffered saline

Introduction

Heat shock proteins (Hsps) are highly conserved and can be induced by many environmental stresses resulting from

physiological (heat, ultraviolet radiation) and chemical factors (carbon monoxide, heavy metals, free radicals, etc.; Lindquist and Craig 1988; Morimoto et al. 1994; Wu and Tanguay 2006). Many of these are common in human working and living environments and are risk factors for lung cancer. Intracellular Hsps have been shown to protect cells, and organs, e.g., heart, brain, and lung (Currie et al. 1993; Marber et al. 1995; Radford et al. 1996; Plumier et al. 1997; Wong and Wispe 1997), as well as whole organisms against damages caused by such abnormal environmental factors. Intracellular Hsps act as molecular chaperones, participating in the proper folding of nascent proteins and their transport to cell compartments, in refolding and solubilization of misfolded or damaged proteins, and in protecting cells against protein aggregation. Hsps can also sequester damaged proteins to avoid aggregate formation and target severely damaged proteins to degradation pathways, such as the proteasome (Jindal 1996; Nollen et al. 1999; Jolly and Morimoto 2000; Young et al. 2004). Intracellular Hsps have also been shown to be involved in cell differentiation, apoptosis, tissue development, and hormonal stimulation (Sarto et al. 2000).

Hsp27 and Hsp70 are the most studied Hsps members. They have cytoprotective effects in cultured cells, organs, and whole animal models (Beck et al. 2000; Garrido et al. 2006). They have also been associated with damages resulting from exposure to environmental hazards (Xiao et al. 2002, 2003), some of which are important risk factors for lung cancer (Alberg and Samet 2003). A previous study from our lab suggested that high lymphocyte Hsp70 levels might provide protection or serve as response biomarkers in workers exposed to coke oven emissions, which are known occupational stressors. Thus, high lymphocyte Hsp70 levels were found to be associated with a dose-dependent risk of cancer in various organs, including lung, skin, and bladder in humans (Yang et al. 2007). Some studies have also reported that Hsp levels are associated with aging, which is associated with an increased incidence of cancer. Age-related decreases in lymphocyte Hsp27 and Hsp70 have been observed (Jin et al. 2004a; Njemini et al. 2006), and high levels of DNA and protein damage are associated with reduced Hsp70 expression in aged brains in humans (Li et al. 2005).

Lymphocytes are frequently used as surrogate tissues to investigate the association between protein expression levels and risk of diseases (Bonassi and Au 2002). A number of recent studies have suggested the possible significance of lymphocyte Hsp measurements for better understanding of the mechanisms of pathogenesis, diagnosis, and prognosis in many diseases (Njemini et al. 2002; Xiao et al. 2002, 2003; Jin et al. 2004a, b; Njemini et al. 2006, 2007; Tan et al. 2007; Tanguay and Wu 2006). However, the association between lymphocyte Hsp levels and lung cancer risk remains largely unknown. We therefore determined lymphocyte Hsp27 and Hsp70 levels in a case-control study of 263 lung cancer

cases and 263 cancer-free controls by flow cytometry and investigated their associations with the risk of lung cancer.

Subjects and methods

Subjects

This analysis included 263 lung cancer patients and 263 cancer-free controls. Patients were diagnosed with lung cancer for the first time between 1 July 2004 and 30 September 2005 at the Zhongnan Hospital, Hubei Cancer Hospital and Wugang Staff-Worker Hospital in Wuhan without restrictions of age or gender. The diagnoses of lung cancer were confirmed by histology or cytology. Cancer-free controls were randomly selected from 1,075 individuals who participated in a community-based health examination in Wuhan city, Hubei province during the same period in which the cases were recruited. These control subjects had no history of cancer and were frequency-matched to the cases on age (± 5 years) and gender.

Each participant was scheduled for an interview after a written informed consent was obtained, and a structured questionnaire was administered by interviewers to collect information on demographic data, lifestyle factors, and tobacco smoking. Those who had smoked less than one cigarette per day and less than 1 year in their lifetime were defined as non-smokers; otherwise, they were considered as smokers. Those smokers who quit for >1 year were considered former smokers. Family history of cancer was defined as any reported cancer in first-degree relatives (parents, siblings, or children). All procedures and the study were approved by the institutional review board of Tongji Medical College of Huazhong University of Science and Technology.

Blood sampling

To isolate lymphocytes, venous blood (~5 ml) was drawn from each subject after overnight fasting and before any treatment. The blood was drawn into a heparinized tube for isolating lymphocytes using Ficoll-Hypaque (Biochemical Reagent, Shanghai, China) as previously described (Xiao et al. 2002). The collected lymphocytes were washed twice with phosphate-buffered saline (PBS) and counted. The number of lymphocytes was adjusted to 5,000 / μ l with PBS. Aliquots of 200 μ l cell suspension were centrifuged for 3 min at 900 \times g and then fixed in 500 μ l PBS containing 4% paraformaldehyde (Biochemical Reagent) at room temperature. The washed lymphocytes were stored at 4°C for further analysis.

Detection of Hsps

Cellular staining of Hsps for flow cytometry analysis was performed as described previously (Xiao et al. 2003) with

minor modifications. Lymphocytes were suspended in 100 μ l of diluted antibodies (1:300 dilution of rabbit-anti-human Hsp27, 1:500 dilution of rabbit-anti-human Hsp70; SPA-803E, anti-Hsp27 antibody; SPA-812E, anti-Hsp72 antibody; Stressgen Bioreagents, Victoria, BC, Canada) in a permeabilization solution (PBS containing 1% BSA and 0.04% Triton X-100). Then, the cells were incubated with 100 μ l of a fluorescein-isothiocyanate-labeled anti-rabbit immunoglobulin G (IgG, diluted 1:100; Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). The stained cells were analyzed using a flow cytometer (FACS CALIBUR, Becton Dickinson Company, San José, CA, USA). A total of 10,000 cells were counted, and the mean fluorescence intensity (MFI) was measured at 525 nm.

Statistical analyses

The cumulative smoking dose (pack-years) was defined as the number of packs of cigarettes smoked per day multiplied by the number of smoking years. Smokers were further dichotomized by the cumulative dose of 29 pack-years according to the distribution of controls. Gender, smoking status, and family history of cancer were compared between cases and controls using χ^2 test. Age was analyzed by independent sample Student's *t* tests. Wilcoxon rank test was used to compare Hsp levels between cases and controls. Odds ratios (OR) and 95% confidence intervals (CI) were computed by conditional logistic regression analysis for estimating lung cancer risk. ORs were computed according to tertile level in controls of lymphocyte Hsps. Statistical inference was based on a significance level of $P < 0.05$. All analyses were done using Statistical Package for social Sciences software (version 12.0) for Windows (SPSS, Chicago, IL, USA).

Results

Baseline characteristics of lung cancer cases and controls

The characteristics of lung cancer cases and controls are summarized in Table 1. Cases and controls were well matched for age and gender. However, the controls were more likely to be non-smokers (40.3%) than were the cases (23.2%); there were more heavy smokers (54.0%) in cases than in controls (26.2%; $P < 0.001$). A family history of cancer was also significantly more common in cases than in controls ($P = 0.026$), although only 13 cases and four controls reported a positive family history of cancer.

Lymphocyte Hsps levels in lung cancer cases and controls

The levels of two Hsps, Hsp27, and Hsp70 were next measured in lymphocytes by FACS. Hsp27 levels were significantly lower in lung cancer cases than in the matched controls

Table 1 Characteristics of lung cancer cases and cancer-free controls

Variables	Controls (<i>n</i> =263)	Cases (<i>n</i> =263)	<i>P</i> value
Age (years, mean \pm SD)	59.8 \pm 10.1	59.6 \pm 10.6	0.712 ^a
Gender (<i>n</i> , %)			1.000 ^b
Male	216 (82.1)	216 (82.1)	
Female	47 (17.9)	47 (17.9)	
Smoking status (<i>n</i> , %)			<0.0001 ^b
Non-smoker	106 (40.3)	61 (23.2)	
Light smoker	88 (33.5)	60 (22.8)	
Heavy smoker	69 (26.2)	142 (54.0)	
Family history of cancer (<i>n</i> , %)			0.026 ^b
No	259 (98.5)	250 (95.1)	
Yes	4 (1.5)	13 (4.9)	

^a Student's *t* test

^b Chi-square test

(16.5 vs 17.8 MFI, $P < 0.001$). In contrast, Hsp70 levels were similar in lung cancer cases and in controls (Table 2). As shown in Table 3, there were no differences in lymphocyte Hsp27 and Hsp70 levels among different histological tumor types of cancer cases.

We also analyzed Hsp levels among in relation with the smoking status and gender in the cancer-free controls. Results showed that Hsp27 and Hsp70 levels were significantly different among non-smokers and smokers ($P = 0.036$ and $P = 0.004$, respectively). Hsp27 and 70 levels of smokers were higher than non-smokers, especially for light smokers. Hsp70 levels were significantly higher in males than in females (Table 4).

Association of Hsp27 and Hsp70 levels with lung cancer risk

On the basis of tertile Hsp27 levels in controls, cases were next divided into three subgroups. As shown in Table 5, ORs (95% CIs) across the tertile Hsp27 levels were 1.000, 1.111 (0.728–1.695) and 0.452 (0.279–0.732; $P_{\text{trend}} = 0.003$) with adjustment for age, gender, smoking status, and family history of cancer. For further analyses, we performed stratified analysis by smoking status and gender because Hsp27 and 70 levels were different according to the smoking status and gender of normal individuals. Results showed that the negative association between the Hsp27 levels and lung cancer patients was only significant in males and heavy smokers. ORs (95% CIs) across the tertile Hsp27 levels were 1.000, 0.904 (0.566–1.444) and 0.382 (0.221–0.658; $P_{\text{trend}} = 0.001$) in males and 1.000, 0.920 (0.465–1.822), and 0.419 (0.195–0.897; $P_{\text{trend}} = 0.036$) in heavy smokers with adjustment for

Table 2 Lymphocyte Hsp27 and Hsp70 levels between 263 lung cancer cases and 263 cancer-free controls

Variables	Controls ($n=263$), median (25th–75th percentile)	Cases ($n=263$), median (25th–75th percentile)	P value ^a
Hsp27	17.8 (15.2–21.0)	16.5 (14.8–18.7)	<0.001
Hsp70	17.2 (15.4–22.3)	17.3 (15.1–20.3)	0.165

^a Wilcoxon rank test

other confounded factors. A borderline negative association ($P_{\text{trend}}=0.057$) was observed in light smokers.

A similar analysis was performed with lymphocyte Hsp70 levels, and only a borderline significantly negative association ($P_{\text{trend}}=0.063$) between Hsp70 and lung cancer patients in heavy smokers was found (data was not shown).

Discussion

Lung cancer is one of the most common malignancies in humans (Ferrigno et al. 1994). The accumulation of damage at different molecular and cellular levels is the underlying mechanism of lung carcinogenesis (Finkel et al. 2007). The damage may partly result from a constant exposure to environmental stresses that trigger the stress responses. Under stress conditions, elevated Hsp levels allow cells to cope with increased concentrations of unfolded or denatured proteins (Nollen et al. 1999; Garrido et al. 2006). Hsp27 and Hsp70 are inducible proteins that have a low level of expression under normal conditions but show rapid overexpression after a wide range of stresses, and play among other things, an important role in the protection against oxidative damage (Bellmann et al. 2000; Arrigo et al. 2005b). In bronchial epithelial cells, Hsp27 can protect against oxidative stress-mediated apoptosis (Merendino et al. 2002). Interestingly, Hsp27 and Hsp70 expression is often deregulated in cancer with high levels observed in lung cancer and other tumors (Ciocca and Calderwood 2005; Mosser and Morimoto 2004). Their abnormal expression is ascribed to the high demand in the proliferation of tumor cells and their anti-apoptotic properties which can be associated with poor prognosis and resistance to therapy in breast cancer and endometrial cancer (Ciocca and Calderwood 2005).

In the present study, we have observed an association between increased lung cancer risk and a decreased lymphocyte Hsp27 level in male cases only. It is well known that Hsp27 has a protective activity against oxidative stress (Rogalla et al. 1999) and can be used to protect cells against cytotoxic effects induced by oxidative stress (Arrigo et al. 2005a). This protection conferred by Hsp27 may result from a decrease in the level of reactive oxygen species concomitant with an increase in glutathione. Overexpressed Hsp27 confers resistance against oxidative stress efficiently in L929 cells and rat neuronal cells (Mehlen et al. 1997). Furthermore, the upregulation of Hsp27 can moderately elevate the removal capacity of ultraviolet C-induced DNA damage in UV-sensitive human R5a cells (Wano et al. 2004). In animal models, overexpression of Hsp27 can protect hearts from oxidative damage induced by ischemia–reperfusion injury (Hollander et al. 2004). These results suggest that the cellular and/or DNA damage caused by oxidative stress would not be eliminated completely when the expression of Hsp27 is decreased or insufficient. As a result, the damage may accumulate in cells and cause mutations or even cell death. Therefore, the lower level of Hsp27 may participate in the initiation of lung cancer.

Tobacco smoke can activate heat shock factor and induce the expression of Hsps (Vayssier et al. 1998). Smoking has been shown to be correlated with greater expression of Hsps in cell cultures and in exposed tissues from smokers (Vayssier-Taussat et al. 2001; Ryder et al. 2004). We found that lymphocytic Hsp27 and 70 of smokers were higher than non-smokers in cancer-free controls. For lung cancer patients, Hsp27 expression was aberrant in smokers. This suggests that lung cancer cases may have an altered response to environmental stresses.

In female subjects, no association was observed between lymphocyte Hsp27 and Hsp70 levels and lung cancer risk in the present study. It is known that sex hormones play a role in

Table 3 Lymphocyte Hsp27 and Hsp70 levels among lung cancer cases

Histology	Number (%)	Hsp27 median (25th–75th percentile)	Hsp70 median (25th–75th percentile)
Adenocarcinoma	90 (34.2)	17.1 (14.9–19.1)	17.0 (15.0–20.3)
Squamous cell carcinoma	131 (49.8)	16.2 (14.6–18.5)	16.9 (15.1–19.8)
Small cell lung cancer	20 (7.6)	17.6 (16.1–19.3)	19.0 (15.3–21.7)
Other types	22 (8.4)	16.0 (14.9–18.2)	19.7 (15.7–22.5)
P value ^a		0.189	0.152

^a Wilcoxon rank test

Table 4 Lymphocyte Hsp27 and Hsp70 levels among cancer-free controls

Variables	Number (%)	Hsp27 median (25th–75th percentile)	Hsp70 median (25th–75th percentile)
Smoking status (<i>n</i> , %)			
Non-smoker	106 (40.3)	16.8 (14.9–20.6)	16.3 (14.8–21.1)
Light smoker	88 (33.5)	18.9 (15.7–24.5)	18.9 (15.9–26.9)
Heavy smoker	69 (26.2)	17.6 (15.1–20.8)	18.2 (15.6–21.7)
<i>P</i> value ^a		0.036	0.004
Gender (<i>n</i> , %)			
Male	216 (82.1)	17.9 (15.2–21.6)	18.0 (15.6–23.3)
Female	47 (17.9)	17.6 (15.1–20.6)	15.7 (14.6–19.5)
<i>P</i> value ^a		0.355	0.031

^a Wilcoxon rank test

the heat shock response. *Hsp27* is an estrogen-responsive gene. *Hsp27* and *Hsp70* expression may be different between males and females (Dunn et al. 1993; Vargas et al. 1998; Voss et al. 2003; Milne and Noble 2008). However, because the number of female lung cancer cases was very small in the present study, the analysis lacked adequate statistical power, and we only found that *Hsp70* level was

different between normal males and females. These results should be examined in future large studies.

As a major stress response protein, *Hsp70* has been reported to protect cells, tissues, and organisms against damage from a wide variety of stressful stimuli. In our previous study, overexpressed *Hsp70* could protect A549 cells against DNA damage (Niu et al. 2006). An aberrant expression of *Hsp70*

Table 5 ORs and 95% CIs for lung cancer risk by lymphocyte Hsp27 levels

Hsp27	Tertiles			<i>P</i> _{trend} ^a
	First	Second	Third	
Overall				
Range	≤15.9	15.9–19.7	>19.7	
Control/case	89/106	85/112	89/45	
OR (95% CI) ^b	1.000	1.111 (0.728–1.695)	0.452 (0.279–0.732)	0.003
Male				
Range	≤16.0	16.0–21.1	>21.1	
Control/case	73/97	72/85	71/34	
OR (95% CI) ^c	1.000	0.904 (0.566–1.444)	0.382 (0.221–0.658)	0.001
Female				
Range	≤15.3	15.3–19.3	>19.3	
Control/case	18/14	14/26	15/7	
OR (95% CI) ^c	1.000	2.699 (0.959–7.593)	0.654 (0.200–2.135)	0.618
Non-smoker				
Range	≤15.3	15.3–19.0	>19.0	
Control/case	37/17	34/31	35/13	
OR (95% CI) ^d	1.000	1.939 (0.878–4.278)	0.783 (0.322–1.904)	0.632
Light smoker				
Range	≤16.3	16.3–21.0	>21.0	
Control/case	30/26	30/25	28/9	
OR (95% CI) ^d	1.000	0.991 (0.454–2.165)	0.372 (0.144–0.958)	0.057
Heavy smoker				
Range	≤15.7	15.7–19.5	>19.5	
Control/case	23/62	24/57	22/23	
OR (95% CI) ^d	1.000	0.920 (0.465–1.822)	0.419 (0.195–0.897)	0.036

^a Conditional logistic regression for the trend of lung cancer risk with lymphocyte Hsp27 levels^b Adjusted for age, sex, smoking status, and family history of cancer^c Adjusted for age, smoking status, and family history of cancer^d Adjusted for age, gender, and family history of cancer

has also been reported in a wide range of tumors (lung cancer, breast cancer, liver cancer, and leukemia, etc.; Ciocca and Calderwood 2005). However, we did not find altered lymphocyte Hsp70 expression in lymphocytes of lung cancer cases.

Hsp expression is under complex regulation, operating at both transcriptional and translational levels (Morimoto 1998; Shi et al. 1998). Variation in Hsp gene expression might affect individual response to stressors (Favatiere et al. 1997; Dierick et al. 2007). On the other hand, decreased expression of Hsp27 may be due to alteration of stress tolerance in long-term exposure to environmental hazards. However, it remains unclear why Hsp27 and Hsp70 show a different response in lymphocytes in lung cancer cases.

We recognize that the case-control study design of the present study may have limitation, because the blood samples were collected after the occurrence of the lung cancer events. Thus, our findings would need to be confirmed in a larger group and prospective studies in the future. Another limitation is that environmental exposure and occupational information were not available for further analysis because the cases came from hospitals and it was not possible to get this information. Although we used regular method to separate lymphocyte, we cannot be absolutely sure that the Hsp27 levels measured were constitutive or inducible, since the PBS buffer different from plasma might induce osmotic stress of lymphocytes. However, since lymphocytes from both groups were isolated the same way, we think that the data are valid. In addition, because lymphocytes are used as surrogate tissues, their relevance to lung tissues remains to be determined in future studies.

In summary, we found that decreased lymphocyte Hsp27 levels are associated with a higher risk of lung cancer. However, this result needs to be validated in large prospective studies. More work will also be necessary to establish the underlying molecular mechanism of Hsp27 in lung carcinogenesis.

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