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ORIGINAL ARTICLE

Stress protein Hsp27 expression predicts the outcome in operated small cell lung carcinoma and large cell neuroendocrine carcinoma patients

D. M. Marinova[1], Y. G. Slavova[2], N. Trifonova[3], D. Kostadinov[1], V. Maksimov[1], D. Petrov[4]

[1]Department of Pneumology, [2]Department of Pathology, and [4]Department of Thoracic Surgery, University Hospital for Pulmonary Diseases "Saint Sofia", Medical University of Sofia; [3]Department of Biology, Medical Faculty, Medical University of Sofia, Sofia, Bulgaria

Summary

Purpose: *Heat shock protein (Hsp)27 is overexpressed in a range of human cancers and is implicated in tumor cell proliferation, differentiation, invasion, metastasis, and survival. The aim of the present study was to determine the prognostic significance of Hsp27 expression in small cell lung carcinoma (SCLC) and large cell neuroendocrine carcinoma (LCNEC).*

Methods: *Surgically resected SCLCs (N=51) and LCNECs (N=15) were studied. The Hsp27 expression was detected immunohistochemically.*

Results: *Hsp27 positive immunoreaction in the cytoplasm was observed in 45 (88%) SCLCs and 14 (93%) LCNECs. A combination of cytoplasmic with nuclear Hsp27 expression was observed in 28 (62%) SCLCs and 14 (100%) LCNECs. There was a correlation between Hsp27 cytoplasmic overexpression and Hsp27 nuclear expression with patient survival confirmed by Cox multivariate analysis.*

Conclusion: *We conclude that the higher Hsp27 cytoplasmic expression and nuclear expression may represent favorable prognostic factors in SCLC and LCNEC.*

Key words: *heat shock proteins, Hsp27, immunohistochemistry, LCNEC, prognosis, small cell lung cancer*

Introduction

SCLC is the third most common type of lung cancer (after adenocarcinoma and squamous cell carcinoma), representing 15-20% of the cases [1]. The prognosis for patients with SCLC is poor, with only 5-9% of patients sur¬viving 5 years [2]. The application of multimodal combined therapy, including surgery, significantly prolongs 5-year survival from 20 to 52% [3].

LCNEC is a rare tumor, representing 3% of patients operated for lung cancer. The prognosis of patients with LCNEC is poor, with 15-57% of patients surviving 5 years [4].

In recent years, increasing evidence shows that SCLC and LCNEC are quite similar in both biological characteristics and behavior 5. Even survival of patients with SCLC or LCNEC is not significantly different [6].

In normal cells heat shock proteins expression increase in response to stressors and these proteins protect the cells from the damaging effects of stress by playing a role in cell recovery helping renaturation of partially denaturated proteins [7].

Hsp27 is a major small molecular weight Hsp which is found overexpressed in different human tumors and is believed to be implicated in tumor cell proliferation, differentiation, invasion, metastasis, and apoptosis [8].

Elevated levels of expression of Hsp27 counteract the apoptotic cell death induced by various stimuli in normal cells and stimulate the antioxidant defences of the normal cells; yet, Hsp27 is found in a wide range of tumors resistant to therapy. It has tumorigenic and metastatic potential thus designating this protein as potential target

Correspondence to: Dora Marinova, MD, PhD student. Department of Pneumonology, University Hospital for Pulmonary Diseases "Saint Sofia", 19 Acad. Ivan Geshov blvd, Medical University of Sofia, Sofia, Bulgaria. Tel: +359 888027907, E-mail: dmusic@abv.bg
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Table 1. Distribution of 66 patients by clinicopathological parameters and expression of Hsp27

Parameters	*N (%)*
Histology	
SCLC	51 (77)
LCNEC	15 (23)
Sex	
Men	51 (77)
Women	15 (23)
Age, years (mean±SD)	59±7
Size, cm (mean±SD)	5.1±2.9
T stage	
T1	14 (21.3)
T2	32 (48.5)
T3	16 (24.2)
T4	4 (6.1)
N stage	
N0	42 (63.6)
N1	7 (10.6)
N2	16 (24.2)
N3	1 (1.5)
M stage	
M0	62 (93.9)
M1	4 (6.1)
pTNM stage	
I	23 (34.9)
II	19 (28.8)
IIIA	16 (24.2)
IIIB+IV	8 (12.2)
Hsp27, Cytoplasmic	
Mean±SD	57±39
Median	65
Negative	7 (10.6)
<25%	14 (21.2)
26-75%	18 (27.3)
>75%	27 (40.9)
Hsp27, Nuclear	
Mean±SD	3±4
Median	1
Negative	24 (36.4)
Positive (<25%)	42 (63.6)

SCLC: small cell lung carcinoma, LCNEC: large cell neuroendocrine carcinoma, SD: standard deviation

for future anticancer therapeutic strategies [9].

The prognostic significance of Hsp27 has been studied in various cancers with contradictory results – in some cancers Hsp27 overexpression predicts a favorable prognosis but in others a poor prognosis [8].

To the best of our knowledge, there are no reports on the prognostic significance of Hsp27 expression in SCLCs and LCNECs, although there are some studies about its expression and prognosis in non-small cell lung carcinomas (NSCLCs) [10,11].

In this report, we present the relationship of altered expression of Hsp27 with survival of patients operated for SCLC and LCNEC.

Methods

Patients and tissue samples

Sixty-six cases of high grade neuroendocrine tumors of the lung – 51 SCLCs and 15 LCNECs - were selected from the files of the Department of Pathology (University Hospital for Pulmonary Diseases "St. Sofia"). The histological diagnoses were revised according to the World Health Organization histological criteria (immunohistochemical verification of the diagnoses was made with positivity of chromogranin or synaptophysin) [12]. All included patients were operated from 2000 to 2011 at the Department of Thoracic Surgery (same hospital). None of the patients received chemo- or radiotherapy prior to surgery. Fifty-one men (77%) and 15 women (23%) were included in the study with mean age 59 ± 7 years, ranging from 42 to 76 years. T stage, N stage, and pTNM stage were revised (for the cases before 2010) and determined according to the TNM classification of the revised International System for Staging of Lung Cancer [13]. T2 stage had 48.5% of the cases, N0 63.6%, M0 93.9%, and pTNM stage I 34.9% (Table 1).

Data on survival was available for all 66 patients (from Bulgarian National Cancer Registry). Since 2 patients died within the first month after surgery and thus they were excluded from the analysis, 64 patients were included in the survival analysis.

Immunohistochemistry

The immunohistochemical (IHC) staining was performed on formalin-fixed paraffin-embedded tissue sections (4 μm thickness, mounted on polylysine slides) with a polymer-based system - EnVision™ FLEX Mini Kit, High pH (Dako,code no K8024) according to the manufacturer's instructions [14]. The primary antibody used was Hsp27 (Santa Cruz Bio) – a mouse monoclonal antibody, clone F-4, in 1:300 dilution. First the slides were deparaffinised in xylene and hydrated in graded ethanol. Prior to IHC reaction, slides were subjected to heat-induced epitope retrieval with Dako PT link instrument according to the manufacturer's instructions (start t^{o} 65 ^{o}C, 20 min incubation time at 97 ^{o}C, finish to 65 ^{o}C). This procedure was followed by subsequent incubation with: 1) Peroxidase-blocking reagent for 5 min; 2) Hsp27 antibody for 20 min; 3) Horseradish peroxidase labeled polymer detection reagent for 20 min; 4) Diaminobenzidine (DAB) solution for 8 min. Finally a counterstaining with hematoxylin, followed by dehydration in graded ethanol and permanent mounting were done. In negative controls, normal mouse serum was used and primary antibody was omitted.

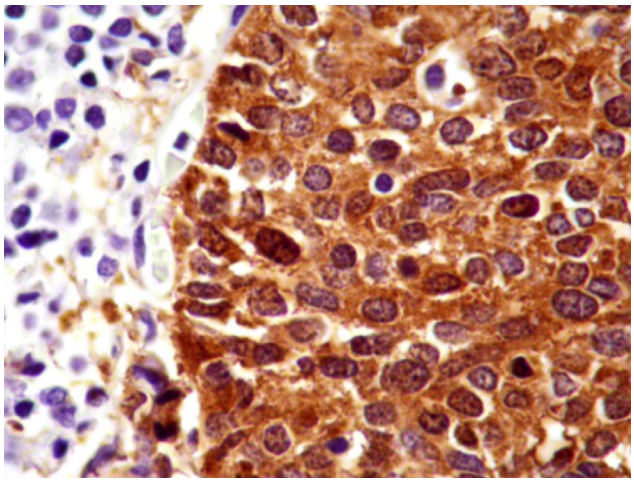

Figure 1. Immunohistochemical localization (cytoplasmic and nuclear) of Hsp27 in small cell lung carcinoma, x400.

Evaluation of immunohistochemical staining

The evaluation of IHC reactions was done by light microscopy (Carl Zeiss), at x400 magnification. Red-brown staining of the cytoplasm or the nucleus of the tumor cells was considered as positive reaction. Each tumor was classified in one of the 4 following categories according to the percent positive tumor cells (separately for cytoplasmic and nuclear expression) and the staining intensity: a) negative, when no tumor cells were stained; b) low, when positive cells ranged from 1-25% of the total; c) moderate, when stained cells ranged from 26–75% of tumor cells; d) high, when more than 76% of the cells were positive.

Statistics

Statistical analyses were performed by using the SPSS v.13.0 software, Windows Vista. Pearson's chi-square test was used to evaluate the relationships between clinicopathological parameters and Hsp27 expression. The Kaplan-Meier method was used for estimation of survival. Differences in survival were evaluated with log-rank test and Wilcoxon test. The potential prognostic significance of different factors was studied using the Cox regression multivariate analysis. A $p<0.05$ was considered statistically significant.

Results

Hsp27 cytoplasmic expression in SCLC and LCNEC (Table 1)

In normal lung the Hsp27 immunoreactivity (cytoplasmic staining) was observed only in bronchial epithelial cells adjacent to the basal membrane. Neither alveolar nor stromal cells were stained. A Hsp27 positive immunoreaction in the cytoplasm was observed in 59 (89%) cases – 45 (88%) SCLCs and 14 (93%) LCNECs (Pearson's x^2, $p=0.57$). The percent of the stained tumor cells was assessed as low in 14 cases (21%), as medium in 18 (27%) and as high in 27 (41%) cases. Figure 1 shows a microphotograph of a SCLC case with cytoplasmic and nuclear expression of Hsp27. There was no association between Hsp27 positive staining and most clinicopathological factors – histology, size, T stage, M stage and pTNM stage. Significant associations were noticed between the level of Hsp27 positivity in the cytoplasm and Hsp27 nuclear positivity (Pearson's x^2, $p=0.027$), and N status (Pearson's x^2, $p<0.001$).

Prognostic significance of Hsp27 cytoplasmic expression in SCLC and LCNEC

The 5-year overall survival and the median survival time were significantly higher/longer in cases with high Hsp27 cytoplasmic positivity compared to negative cases (Wilcoxon, $p=0.027$; log rank, $p=0.030$), and there was a similar tendency compared to cases with moderate rate Hsp27 cytoplasmic positivity (Wilcoxon, $p=0.067$; log rank test, $p=0.083$) (Figure 2, Table 2). Lack of Hsp27 protein

Table 2. Correlations between level of Hsp27 expression and survival

		Survival (%)		*Survival (months)*			
	N	*1-year*	*5-year*	*Median*	*95% CI*	*HR*	*Cox, p-value*
Hsp27 Cytoplasmic							
Negative	7	43	14	8.6	0-20	3.08	0.037
Low	15	80	39	23	13-32	1.57	0.31
Moderate	17	65	24	19	10-27	2.03	0.086
High	25	84	45	45.8	18-72	1	
≤65%	35	66	26	18	13-22	2	0.040
>65%	29	83	44	45.8	18-73	1	
Hsp27 Nuclear							
Negative	23	70	13	17.5	14-21	2.43	0.014
Positive	41	76	45	34.9	13-56	1	

SCLC: small cell lung carcinoma, LCNEC: large cell neuroendocrine carcinoma, CI: confidence interval, HR: hazard ratio

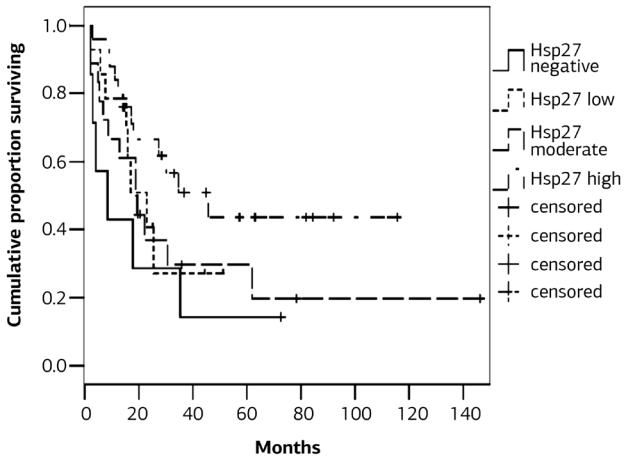

Figure 2. Kaplan-Meier survival curves of SCLC and LCNEC patients stratified by Hsp27 cytoplasmic expression (negative, low, moderate, high). Log rank, p=0.148 (overall); high vs negative, log rank, p=0.030; high vs moderate,log rank, p=0.083; high vs low, log rank, p=0.198; moderate vs low,log rank, p=0.683; moderate vs negative,log rank, p=0.438; low vs negative, log rank, p=0.399.

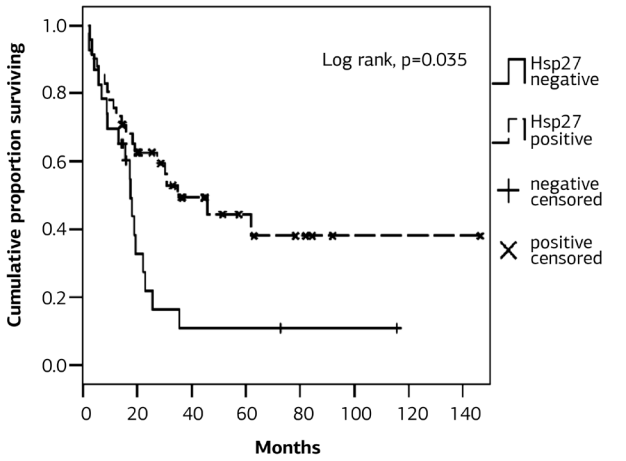

Figure 3. Kaplan-Meier survival curves of SCLC and LCNEC patients stratified by Hsp27 nuclear expression (negative/positive).

expression was associated with very poor outcome: 86% of patients with Hsp27 negative tumors died within 5 years after surgery.

The median Hsp27 cytoplasmic expression was 65% and we have used it to separate the cases into two groups: ≤65% and >65%. The 5-year overall survival rate and the median survival time were significantly higher/longer in cases with >65% Hsp27 cytoplasmic positivity compared to cases with ≤65% rate (Wilcoxon, p=0.040; log rank, p=0.035) (Table 2). Cox regression multivariate analysis (standardized by sex, age and histological type) showed a significant survival advantage for high-rate Hsp27 positive cases compared to negative ones (Cox regression, p=0.037) and a similar trend compared to moderate cases (Cox regression, p=0.086). The probability of death was two-fold (95% confidence interval 1-3.9), higher in cases with ≤65% Hsp27 positivity compared with >65% Hsp27 positive cases (Cox regression, p=0.040) (Table 2). There was an evident correlation between prognosis and Hsp27 cytoplasmic expression in SCLCs and LCNECs.

Hsp27 nuclear expression in SCLC and LCNEC (Table 1)

In 42 cases (71% of tumors with cytoplasmic expression) there was a combination of cytoplasmic with nuclear expression of Hsp27 – 28 SCLCs (62% of SCLCs cases with cytoplasmic expression) and 14 LCNECs (100% of LCNECs cases with cytoplasmic expression). In all studied cases the nuclear expression of Hsp27 was seen in low numbers of positive tumor cells. There was no association between Hsp27 positive nuclear staining and the clinicopathological factors – size, T stage, M stage, and pTNM stage. There was significant association between Hsp27 nuclear positivity and the histological type (p=0.031).

Prognostic significance of Hsp27 nuclear expression in SCLC and LCNEC

The median survival time was significantly longer in cases with Hsp27 nuclear positivity compared to negative cases (log rank test, p=0.035) (Figure 3, Table 2). Lack of Hsp27 nuclear expression was associated with very poor outcome: 87% of patients with Hsp27 nuclear negative tumors died within 5 years after surgery.

The Cox regression multivariate analysis showed a significant survival advantage for Hsp27 nuclear positive cases compared to negative cases (Cox regression, p=0.014). The probability of death was 2.4 times (95% confidence interval 1.2-4.9) higher in negative cases compared with nuclear Hsp27 positive cases (Cox regression, p=0.014) (Table 2). There was an evident correlation between prognosis and Hsp27 nuclear expression in SCLCs and LCNECs. Multivariate analysis showed that Hsp27 nuclear expression was an independent prognostic factor in all cases examined (independent of age, sex, histology, T stage, N stage and pTNM stage).

Discussion

To the best of our knowledge, there are no other published studies on the immunohistochemical detection of the Hsp27 protein in SCLC and LCNEC. Here we have shown that Hsp27 overexpression in the cytoplasm (independent of sex, age

and histological type) and positive expression in the nucleus (independent of sex, age, histological type, T stage, N stage and pTNM stage) are both associated with better survival. To the best of our knowledge, there is no other report analyzing the clinical outcome in SCLC and LCNEC in relation to Hsp27 expression. However, there are studies concerning the prognostic significance of Hsp27 in other types of cancer.

In some studies Hsp27 expression is associated with increased tumor cell differentiation [8] but SCLC and LCNEC are both poorly differentiated tumors [12]. So the positive correlation between high expression level of Hsp27 and good prognosis found in our study can not be explained by increased tumor cell differentiation. One possible explanation may be that Hsp27 expression is associated with inhibition of proliferation [10] and cytoskeletal stabilization as an actin capping protein [15]. Still, to prove this hypothesis, studies are needed to determine whether there exists an association between Hsp27 expression and proliferation levels in SCLC and LCNEC. Studies on other cancer types support the association between Hsp27 expression and inhibition of proliferation – for example in breast cancer [16]. Since the higher Hsp27 cytoplasmic expression is associated with better survival one can speculate that in SCLCs and LCNECs Hsp27 probably loses its antiapoptotic activity and its higher accumulation in the cytoplasm leads to cell death.

The prognostic significance of Hsp27 expression was studied in several human cancers. Higher Hsp27 expression was a favorable prognostic factor in NSCLC [10], endometrial carcinomas [17], ovarian cancer [18], oesophageal cancer [19] and oral cancer [20]. In contrast, Hsp27 expression was a poor prognostic factor in gastric, liver and prostate cancers, and osteosarcoma, while no significance was reported for head and neck cancer, bladder and renal cancer [8]. These results suggest that as the association between Hsp27 expression and prognosis may vary with cancer type, perhaps Hsp27 takes part in different pathways and has different fuctions dependent on the cell type.

In our study we have shown the favorable prognostic significance of Hsp27 expression in SCLC and LCNEC. Still, more studies are needed to validate this result and to reveal the mechanisms and functions of this protein in the context of SCLC and LCNEC.

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