Abstract. Background: The expression pattern of stress (heat shock) proteins (HSPs) in cancer cells is frequently different from that observed in normal cells; most often some stress-inducible HSPs are constitutively and highly expressed. The objective of this study was to determine the prognostic significance of stress proteins HSP70i and HSP27 in non-small cell lung carcinoma (NSCLC). Materials and Methods: An immunohistochemical procedure that enables unambiguous detection of HSP70i protein was used. Results: Strong HSP70i staining showed a survival advantage, although multivariate analysis did not confirm this result. There was an evident correlation between HSP27 overexpression and survival of patients and the results were confirmed by multivariate analysis: 70% of patients with HSP27-negative tumors died within one year after the surgery. Conclusion: Our data suggest that HSP27 and HSP70i positivity may represent a favorable prognostic factor in NSCLC.

Lung cancer is the leading cause of death from malignant tumors in males and the second in the female population. Although great improvement has been made in diagnosis and treatment of lung cancer, the overall patients’ survival is still very low and does not exceed 15% (1). With the advent of adjuvant therapies, it has become increasingly important to discover biomarkers that will identify patients with the highest likelihood of recurrence. A substantial amount of clinical and basic science research has focused on the prognostic factors in lung cancer and a variety of molecular markers have been proposed that may have the potential to predict treatment outcomes. Such markers include cyclin E, cyclin D1, p21, p27, p16 and VEGF (reviewed in (2)). Nonetheless, no single marker has yet been shown to be satisfactory in predicting patient outcome.

The heat shock protein (HSP) family, is a group of molecular chaperones, which maintain protein homeostasis under physiological conditions and protect cells against damage in stressful conditions. The expression pattern of HSPs in cancer cells is frequently different from that observed in normal cells: most often some stress-inducible HSPs are constitutively and highly expressed. The accumulation of cytoplasmic HSPs at a physiological temperature in the nuclei or at the cell surface of cancer cells can also be observed (3). The molecular mechanism responsible for overexpression of HSPs encoded by heat shock factor (HSF)-inducible genes such as HSP70i and HSP27 in cancer cells seems to be multiple and has not yet been fully determined. Gene chip microarray studies of prostate cancer cells showed insignificant differences in HSF and HSP gene mRNA expression between the normal and malignant cells, which indicates that HSP expressions might be regulated at the post-transcriptional level in cancer cells (4). Conversely, in pancreatic cancer, HSP70 expression was elevated when compared to normal neighboring tissue from the same patient. There is still the question of whether HSP overexpressions are cancer-causative or if they only reflect increased tumor metabolic activity.

From the clinical point of view, stress inducible HSP70i and HSP27 can modulate some processes which are important for tumor development and response to therapy. An elevated level of HSP27 is associated with a diminished cell proliferation rate both in vitro and in vivo, while HSP70i overexpression correlates with increased cell proliferation rate (5,6). Both these proteins hamper apoptosis in many ways (7). This apoptosis-related effect is mainly responsible for the chemoresistance of HSP-overexpressing tumors to commonly used cytostatic drugs (reviewed in (8)). Overexpression of HSP70i also seems to be a factor facilitating aneuploidation of cancer cells (9).
The phenomenon of HSP70i and HSP27 overexpression was studied in relation to prognosis in various cancers, but the results were inconsistent (reviewed in (3)). Any correlation between the expression of stress proteins and prognosis in non-small cell lung carcinomas has not been evaluated to date. In our previous studies, we analyzed the expression of HSP27 and constitutively expressed cognate HSC70 proteins in primary non-small cell lung carcinomas (10). Here, we report our study of the expression of inducible HSP70i in the same cohort of patients using a method which enables us to discriminate between HSC70 and HSP70i. The relationship of altered expression of HSP70i and HSP27 to NSCLC patient survival is presented.

Patients and Methods

Patients. Examination of HSP70i immunoreactivity was performed in 103 NSCLC cases tested earlier for HSC70 and HSP27 (10). All patients were operated on between 1993-1995 at the Department of Thoracic Surgery of the Silesian Medical Academy. None of the patients received chemo- or radiotherapy prior to surgery. Squamous cell carcinoma (SCC) was diagnosed in 57 men and 6 women, adenocarcinoma (AC) in 21 men and 10 women, and in 9 cases large cell carcinoma was diagnosed (men only). Thirty cases were assessed as stage I, 18 as stage II and 53 as stage III. Stage was assessed according to the 1997 AJCC classification (11). The majority of patients were smokers (61/103) (Table I).

Data on survival were available for 97 patients. Since 21 patients died (9 cases) or were lost at follow-up (12 cases) within the first month after surgery and were thus excluded from the analysis, 76 patients were therefore submitted for final survival analysis.

Immunohistochemistry. Precise conditions of HSP70i detection in formalin-fixed paraffin-embedded tissues have been discussed in detail in our recent paper (12). Briefly, samples were fixed in 10% formalin in phosphate-buffered saline (PBS) for 24 h and embedded in paraffin. Mouse monoclonal antibody SPA810 (20 µg/ml; StressGen, Canada) and ABC Elite kit (Vector, USA) were used for HSP70i detection. Prior to immunohistochemical reaction, slides were subjected to microwave antigen retrieval in 0.05M boiling citric buffer pH 6.0. Reaction without addition of primary antibody was also performed as a negative control.

Assessment of HSP70i immunohistochemical reaction was performed according to the intensity of staining and number of positively stained cell nuclei. Staining was regarded as positive only if cell nuclei were stained. In the case of HSP27, staining was exclusively cytoplasmic with evaluation based on the staining intensity and the number of positive cells. In both cases, tumors were assigned to one of the four following categories: a) negative, when fewer than 10% of the cells were stained; b) weak, when distinctly stained cells accounted for 10-30% of the total, or more cells were weakly stained; c) moderate, when stained cells accounted for 30-70% of the total; d) strong, when more than 70% of the cells were positive.

Statistical and survival analysis. The relationship between HSP70i and HSP27 expression and clinicopathological data was examined by Chi-square test. The Kaplan-Meier method was applied for estimation of survival curves. Differences in survival distribution were evaluated by log-rank test. The potential prognostic significance of multiple markers was studied using the Cox regression model.

Results

HSP70i expression and prognostic significance. In normal lung, the HSP70i immunoreaction was observed only in the bronchial epithelium, where positively stained nuclei were localized in cells adjacent to the basal membrane. In the apical part of ciliated cells, weak cytoplasmic staining was visible. In dysplastic epithelium, a strong nuclear staining was found. Neither alveolar nor stromal cells were ever stained. An HSP70i positive immunoreaction was found in 63 cases (61%): in 17 tumors staining was assessed as weak,
in 33 as moderate and in 13 cases as strong (Figure 1). Tumors displaying the immunostaining in the cytoplasm only (frequent in adenocarcinomas) or in single cells in areas adjacent to necrosis were regarded as negative. All cases displaying strong HSP70i immunoreaction were squamous cell carcinomas. There was no association between HSP70i positivity and other clinical data.

Cases with moderate and strong HSP70i staining showed a survival advantage \( (p=0.006) \), with a diminished death hazard ratio as compared to HSP70i-negative or HSP70i-weak cases (Figure 2, Table II). Multivariate analysis did not confirm the HSP70i expression as an independent prognostic factor. There was also no correlation between HSP70i expression and the histopathological type of tumor.

**HSP27 expression and prognostic significance.** As we described earlier (10), 70% of the cases indicated a positive cytoplasmic HSP27 immunoreaction, with different intensities of staining. Here an evident correlation between prognosis and HSP27 overexpression was found. Survival of patients with HSP27 overexpression was significantly \( (p=0.003) \) longer than that of patients without it, with a death hazard ratio 3.2-fold higher for HSP27-negative patients.

As shown in Figure 3 and Table II survival of HSP27-positive patients depended strongly on the level of expression and was highest for patients with tumors showing a strong HSP27 immunoreaction. Lack of HSP27 protein was associated with very poor outcome: 70% of patients with HSP27-negative tumors died within one year after the surgery.

Separate analyses of patients with squamous cell carcinomas (SCC) and adenocarcinomas (AC) revealed that HSP27 retained its prognostic significance in SCC but not in AC. Multivariate analysis showed that HSP27 expression was an independent prognostic factor in all cases examined.

**Relationship between HSP expressions.** In some cases we observed concomitant upregulation of HSP70i and HSP27. There was a statistically significant positive association between HSP70i immunoreactivity and expression of previously tested HSP27. All HSP70i strong cases showed positive (strong or very strong) anti-HSP27 reaction.

**Discussion**

**Expression pattern of HSP70i protein.** Stress-inducible HSP70i protein and constitutively expressed HSC70 are highly related but differently regulated. There are also significant structural and functional differences between
these proteins. However, a number of studies describing HSP70i expression have been carried out with the use of antibodies which recognize both HSC70 and HSP70i. In our previous study (12) we worked out a reliable method of specific detection of HSP70i.

We found strong, HSP70i-specific immunostaining only in SCC samples. Thus, in contrast to Bonay et al. (13), we found a significant association between the intensity of HSP70i-specific immunostaining and the histological type of the tumor. Concordantly to their results in normal lung, bronchial epithelial cells were HSP70i-positive while pneumocytes in alveolar epithelium negative. We also did not find any relationship between smoking and the expression pattern of HSP70i. To the best of our knowledge, there are no other published studies on immunohistochemical detection of the HSP70i protein in NSCLC with the use of a specific anti-HSP70i antibody. We do not compare our results to those obtained from studies using the antibody cross-reacting with HSC70.

**Prognostic significance of HSP70i protein.** We found that moderate and strong HSP70i staining correlated with increased survival as compared to HSP70i-negative or HSP70i-weak cases. At present our results cannot be compared to others because the only report describing the expression pattern of HSP70i in NSCLC did not include such analysis (13).

However, there are studies concerning the prognostic significance of HSP70i in other types of cancer. Our finding is consistent with results obtained for oesophageal (14, 15) and renal carcinoma (16). HSP70i also seems to be the predictive marker in osteosarcoma: HSP70i-positive patients were found to respond better to neoadjuvant chemotherapy (17). In contrast, the overexpression of HSP70i correlated with poor prognosis in breast cancer and endometrial cancer. HSP70i showed no correlation with prognosis in oral cancer (reviewed in (3)).

A positive correlation between overexpression of HSP70i and better survival of HSP70i-positive NSCLC patients can be explained, at least partially, by HSP70i-mediated immunological antitumour reactivity. It has been reported that HSP70i-associated autologous tumor antigens stimulate proliferation of peripheral lymphocytes in human lung carcinoma (18). Recently, the immunostimulatory capacity of HSP70i was confirmed in a clinical phase I trial. Krause et al. showed that HSP70i could activate NK cells ex vivo, increasing their tumor-specific cytotoxicity (19).

**Prognostic significance of HSP27 protein.** Survival analysis presented now is based on the expression pattern of the HSP27 determined in our earlier study (10). Here we show that HSP27 overexpression is associated with better survival, independent of other clinical factors. To the best of our knowledge, there is only one report which analyzes the clinical outcome in NSCLC in relation to HSP27 expression (20). Although the protein studied by these authors is referred to as p29 estrogen receptor-related protein, it has been shown that p29 is identical to HSP27 protein (21). In contrast to our results Vargas et al. showed that survival of NSCLC patients was not significantly associated with the p29 score (20). They report, however, the gender-related influence of p29 expression on survival. In our studies, the influence of HSP27 on the prognosis was not gender dependent. It is difficult to compare the results obtained by us and by Vargas et al. due to different methods of tissue processing and use of different antibodies. Moreover, Vargas et al. detected p29 immunoreactivity in almost all (98%) NSCLC samples examined, while we found HSP27 overexpression in 70% of tumor tissue samples (10). Due to these discrepancies, more studies are needed in order to draw any final conclusions as to the prognostic significance of HSP27 in NSCLC.

The positive correlation between a high expression level of HSP27 and a good prognosis found in our study can be explained by the fact that HSP27 expression is associated with increased tumor cell differentiation and inhibition of proliferation. In our previous paper, we showed that HSP27 staining was stronger in suprabasal, non-proliferating cells in dysplasias and in foci of highly differentiated SCC (10). This observation was consistent with the results of in vitro studies. Overexpression of hsp25 (rodent homologue of human HSP27) inhibited the growth of Erlich ascites tumor cells (22). Overexpression of the human HSP27 gene transfected into breast carcinoma and epidermal cell lines resulted in inhibition of the proliferation and increased differentiation of cells (6, 23). Recently, overexpression of HSP27 was also reported to lower the metastatic potential of human melanoma cells in vitro (24).

Implications of HSP27 on tumor differentiation and proliferation have also been studied in primary tumors. A statistically significant inverse relationship between HSP27 expression and proliferation was shown in human breast cancer biopsy samples in the cell-by-cell study (5). In oral squamous cell carcinoma, HSP27 immunolabelling was down-regulated in poorly differentiated and up-regulated in highly differentiated areas (25). This protein has also been proposed as a marker of cell differentiation in carcinomas of the endometrium and cervix (26). A direct correlation of the protein level with cell differentiation was also found in canine squamous cell carcinoma of the skin (27). In contrast, there are also studies showing increased HSP27 expression in poorly differentiated carcinomas (28, 29).

The relation of HSP27 overexpression to survival was studied in several human tumors. Favorable prognosis was reported for endometrial carcinomas (30), oesophageal cancer (14, 15), malignant fibrous histiocytoma (31), oral cancer (32) and ovarian cancer (33). In contrast, HSP27
expression has been associated with poor prognosis in gastric, liver and prostate cancers, while no significance was reported for head and neck squamous cancer, bladder and renal cancer (reviewed in (3)). Data concerning breast cancer are contradictory.

These data suggest that the prognostic significance of HSPs may depend on the molecular contexts of cancer cells. One can speculate that also the mechanism behind either favorable or poor prognosis related to heat shock protein overexpressions could be cancer type specific. If this conclusion is true, for each particular type of cancer an extensive study of the pattern of expression and post-translational modification, mechanism of regulation of gene activity and the role of HSPs in cancer cells is necessary.

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