Expression profile of the small heat-shock protein alpha-B-crystallin in operated-on non-small-cell lung cancer patients: clinical implication

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Abstract

Objective: Alpha-B-crystallin, a small heat-shock protein, recently gained major interest because of its differential expression during tumourigenesis and metastasis in various epithelial tumours. The purpose of this study was to investigate the expression of alpha-B-crystallin and its biologic and prognostic significance in non-small-cell lung cancer (NSCLC). Methods: Immunohistochemical analysis was performed on a tissue microarray slide containing samples from 146 NSCLC patients who were operated on between 2004 and 2005. Results: Cytoplasmic and nuclear staining was detected. Squamous cell carcinomas and adenocarcinomas had a distinctive profile of expression. The cytoplasmic staining of the tumours, however, is related to the local invasion — T-factor (p = 0.044). Nuclear staining was more commonly detected in advanced stages, and was a biomarker of an aggressive tumour biology (p = 0.042). Kaplan—Meier analysis showed that patients with positive nuclear staining had shorter overall survival (log-rank p = 0.002). Using Cox’s proportional hazards model, we performed multivariate analyses to assess the independent prognostic value of nuclear staining. The variables used included age, histology, gender and stage. Alpha-B-crystallin was an independent negative prognostic factor of survival in addition to clinical stage. Conclusions: Alpha-B-crystallin plays an essential role in NSCLC biology and its nuclear staining is an independent factor of poor survival. Its clinical application in molecular biologic substaging of NSCLC patients needs further validation.

Keywords: Alpha-B-crystallin; NSCLC; Biomarker; Poor prognosis; Molecular substaging

1. Introduction

The ability to predict survival after lung cancer surgery is important because this information could help target therapies to those patients who would benefit the most. An underlying hypothesis of the modern era of cancer research has been that the prediction of a patient’s prognosis or response to therapy could be improved by combining standard clinical variables with intrinsic characteristics of the tumour. As no single marker could be perfect in predicting patients’ outcome, attention is focused on biomarkers or a panel of markers, primarily involved in one of the three major pathways of cancer — cell cycle regulation, apoptosis and angiogenesis.

Recently, alpha-B-crystallin has been reported to be widely expressed in many epithelial tumours — breast cancer, renal cancer and colorectal cancer [1,2]. In these tumours, it is assumed as a pathological factor, prognostic and predictive marker [1—3]. Alpha-B-crystallin is responsible for the control of the cell cycle, apoptosis, neoangiogenesis and cytoskeletal reorganisation during invasion and metastases [4—7]. These biological functions hint at the potential importance of this protein as an integral determinant for the hallmarks of cancer, described by Hanahan and Weinberg [8].

Because of the lack of data about the role of alpha-B-crystallin in lung tumour biology and its impact on prognosis, we decided to perform an explorative study of its expression, using immunohistochemical staining on one microarray block containing tissue samples of 146 NSCLC patients.

2. Materials and methods

2.1. Patients and tissue samples

A total of 155 consecutive lung cancer patients, operated on during the period between 2004 and 2005 at the Department of Thoracic Surgery, St. Sophia University
Hospital, Sofia, were included in the study. Most of them received resection defined as lobectomy or pneumonectomy. Only 17 (10.97%) patients underwent explorative thoracotomy because of locally advanced tumor. Complete medical records, follow-up data and adequate formalin-fixed, paraffin-embedded tissue blocks from the Department of Pathology were obtained for all of them. Our retrospective study design was reviewed and approved by the institutional ethics committee on human research. No patient received adjuvant chemotherapy or radiotherapy prior to surgery. Tissue sections (4 μm thick) from each tissue block, stained with haematoxylin and eosin and reviewed by a pathologist to confirm the diagnosis and help in the selection of the most representative part of the tumor, were also obtained. The histological type was determined according to the World Health Organization classification.

2.2. Patients’ characteristics

Nine of the patients from the study cohort were excluded because of established small-cell lung cancer. Therefore, a total of 146 NSCLC patients were investigated. This cohort included 20 (14%) female and 126 (86%) male patients with a median age of 58 years (range, 38–78 years). Twelve (9%) of them were non-smokers and 134 (91%) were smokers. The tumor histology in our study group was as follows: 96 (65.8%) squamous cell lung tumors, 10 (6.8%) adenocarcinomas, 35 (24%) adenosquamous carcinomas and five (3.4%) broncho-alveolar carcinomas. Thirty-seven patients (25.4%) were in stage I, 27 (18.5%) in stage II, 65 (44.5%) in stage III and 17 (11.6%) in stage IV. Twenty-nine of the tumors were well-differentiated, 56 had moderate and 36 had poor differentiation. For the rest of the patients, data about differentiation of the tumors were not available.

All patients were followed-up either until death or the end of the study (1 October 2007). The follow-up period ranged from 6 to 48 months.

2.3. Tissue microarray construction

Formalin-fixed, paraffin-embedded tissue samples from patients operated on lung cancer between 2004 and 2005 were obtained from the archives of the Department of Pathology at the St. Sophia Hospital, Sofia. TMA were constructed as previously described. Tissue cylinders with 0.6 mm diameter were obtained from each donor block and arrayed 0.8 mm apart into a recipient block by using microarray instrument (Beecher Instruments, Silver Spring, MD, USA). The specimens were sampled in triplicate core samples of tumor from each donor block. The TMA blocks were cut in 5-μm sections and placed on slide, using adhesive tape system and ultraviolet cross-linking. Twenty-five samples of normal tissue including bronchial epithelium, alveolar tissue and pneumosclerosis were used as a control group to detect alpha-B-crystallin expression in non-neoplastic tissue. One TMA block was constructed.

2.4. Immunohistochemistry

The slides were deparaffinised in xylene and hydrated through graded ethanol. The endogenous peroxidase activity was blocked by 5-min incubation in 3% hydrogen peroxide—methanol buffer. Subsequently, the slides were incubated with 2.5% normal horse serum/phosphate-buffered saline (PBS) for 30 min at room temperature to reduce non-specific background staining. A primary polyclonal anti-rabbit alpha-B-crystallin antibody (Calbiochem Cat No: 238702); Human (Rabbit) (diluted at 1:1000 in PBS), was applied for 24 h at 2–4°C. This was followed by a serial of rinses 3 × PBS. A subsequent incubation with secondary biotinylated anti-rabbit IgG antibody (diluted 1:400) was performed. After a series of 3 × PBS rinses, streptavidin—horseradish peroxidase Universal Elite ABC kit (Vectorstain, Burlingame, CA 94010, USA) was applied for 1 h at 37°C in the humidity chamber. Slides were again rinsed 3 × PBS and visualised by a 5-min incubation with liquid 3'3-diaminobezidin (DAB) in buffered substrate. Finally, slides were counterstained with haematoxylin and eosin. In negative controls, normal horse serum was used and primary antibodies were omitted.

2.5. Evaluation of immunohistochemical staining

The evaluation of TMA was done independently by two investigators. They examined the TMA by light microscopy (Nikon) (100 × 2.5) in a blinded manner with respect to the clinical data. In case of disagreement, both reached a consensus by jointly evaluating the TMA, using a multihed microscope.

Cytoplasmic immunostaining was classified according to its intensity as follows: 0 — no staining; 1 — weak staining; 2 — moderate staining and 3 — strong staining. Nuclear staining was classified as a lack of staining — 0; or positive staining — 1. In each spot (tissue cylinder) 100 cells were counted and the number of cells was described according to their nuclear and cytoplasmic staining. Since the model of triplicates was applied, we were able to detect the heterogeneity of the tumor. As a final result, the mean of the number of stained cells in the three spots was given. Again, this was done with regard to their cytoplasmic and nuclear staining. More than 35% of the cells of the same intensity of cytoplasmic staining (weak, moderate and strong) were accepted as definitive for the whole tumor. Tumor samples were classified as with positive nuclear staining when more than 10% of the cell nuclei showed staining.

2.6. Statistical analyses

Statistical analyses were performed by using the SPSS (13.0) software. Fisher’s exact test and chi-square test were used to evaluate the associations between clinicopathological parameters and alpha-B-crystallin expression. In summary, survival time was calculated from the date of surgery to the date of death from any cause. The Kaplan—Meier method was used to calculate survival curves, and they were examined using the log-rank test. Univariate and stepwise (forward LR) multivariate analyses were performed using Cox’s proportional hazards model to determine which independent factors might have a joint significant influence on survival. The predictive value of the following parameters was assessed — age, gender, histology, tumour grade and
clinical stage. A \( p < 0.05 \) was considered statistically significant.

3. Results

3.1. Immunohistochemical staining and clinicopathological characteristics

Immunohistochemical staining of TMA sections with alpha-B-crystallin antibody was performed successfully in 136 (93%) cases. Of the 146 cases included in the microarrays, 133 were interpretable for cytoplasmic or nuclear alpha-B-crystallin staining. Spots that were deemed uninterpretable had insufficient tumour cells, loss of tissue in the spot or an abundance of necrotic tissue.

Alpha-B-crystallin was not detected in the normal alveolar pneumocytes; a few of the peribronchial glands, however, stained faintly but only in the cytoplasm. Although partially, there were a few areas where basal epithelial cells of the normal ciliated bronchial epithelium also showed weak cytoplasmic staining and no nuclear staining. In contrast, the basal layer of the tumours showed intensive cytoplasmic staining and a lack of nuclear staining. Lymphoid cells infiltrating the tumour stroma as well as the macrophages showed no cytoplasmic staining, but the nuclear staining varied from intensive to a lack of staining. Apoptotic and necrotic cells had faint cytoplasmic and intensive nuclear staining. Intensive nuclear staining was also detected in cells undergoing mitosis.

Nuclear staining was detected in 133 tumours (95 squamous cell histology and 38 adenocarcinomas). Cytoplasmic staining was detected in 127 tumours (95 squamous cell histology and 32 adenocarcinomas). Only eight (6.3%) had weak cytoplasmic staining, 80 (62.9%) had moderate and 39 (30.8%) had intensive cytoplasmic staining (Fig. 1). Lack of nuclear staining was detected in 44 (33%) cases and intensive nuclear staining was observed in 89 (67%) (Fig. 1). A total of 26 tumours strongly expressed alpha-B-crystallin in both nucleus and cytoplasm. Most of the tumours showed homogeneous cytoplasmic staining; for example, more than 60% of the cells of the tumour had the same intensity of staining. The heterogeneity was detected up to the level of nuclear staining (Fig. 2). For accurate comparison, we tried to analyse the biologic role of alpha-B-crystallin in each histologic group. The cytoplasmic staining was not of statistical significance with histology. In contrast, the nuclear staining proved to be characteristic for the adenocarcinomas (Fisher’s exact test \( p < 0.001 \), Contigency Coeff Cramer 0.369).

Table 1 shows the relationship between clinicopathological characteristics and alpha-B-crystallin staining for the whole study group. There was a statistically significant

### Table 1

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association between alpha-B-crystallin cytoplasmic staining and the local tumour invasion (T-factor) (chi-square analysis $p = 0.044$). The nuclear staining was related to the stage of the disease (chi-square $p = 0.042$). No associations between lymph node involvement and alpha-B staining could be observed.

In squamous cell lung cancer, the cytoplasmic staining was significantly associated with the stage of the disease ($p = 0.034$). On the contrary, the nuclear staining showed no relationships with the clinicopathological parameters.

In adenocarcinomas, the nuclear staining, however, corresponded best to the stage of the disease (chi-square $p = 0.009$). The cytoplasmic staining remained statistically significantly associated with the stage of the disease (chi-square $p = 0.044$).

### 3.2 Immunohistochemical staining and survival

The Kaplan—Meier analysis gave no difference in overall survival between the groups with various intensity of cytoplasmic staining (log-rank test $p = 0.49$). In contrast, nuclear staining was related to a shorter overall survival (log-rank test $p = 0.002$) (Fig. 3). Using the univariate and multivariate Cox proportional hazards model (stepwise forward LR-analysis), we assessed the independent predictive value of the nuclear staining with alpha-B-crystallin. The variables used were age, histology, tumour...
grade and clinical stage. Cox’s regression model showed that alpha-B-crystallin is an independent prognostic factor (hazard ratio; HR = 2.18; 95% CI 1.28—3.72) in addition to clinical stage.

To characterise its role in molecular substaging, we applied Kaplan—Meier analyses in each stage. There was no statistical significance between overall survival in stages I and IV. Patients with nuclear staining in stage II (Fig. 4) and stage III (Fig. 5), however, had a significantly shorter overall survival ($p = 0.02$ and $p = 0.005$, respectively; log-rank test) in comparison to patients with no nuclear staining.

4. Discussion

In the present study, we demonstrated that alpha-B-crystallin is significantly over-expressed in NSCLC. In these tumours, we observed cytoplasmic as well as nuclear expression.

Alpha-B-crystallin is a major structural protein of human lenses that belongs to the family of small heat-shock proteins. It has autokinase activity and participates in intracellular architecture and membrane stabilisation [9,10]. It acts as molecular chaperone and stabilises proteins in large soluble aggregates in the cytoplasm [11]. The cytoplasmic expression of alpha-B-crystallin is also responsible for the regulation of cyclin-D1 ubiquitination and inhibition of pro-apoptotic proteins such as caspase-3, p53, Bax and Bcl-x$_S$ [12—14].

Alpha-B-crystallin cytoplasmic over-expression has been described in breast cancer, renal cancer and colorectal cancer. In these tumours, only membraneous and cytoplasmic expression has been observed. In breast cancer, alpha-B-crystallin expression was defined as oncogenic in cell cultures [15] and was associated with lymph-node involvement [16] and poor prognosis.

According to our results, the cytoplasmic expression of alpha-B-crystallin is statistically significantly related to the tumour size (T-factor). This might be due to the fact that alpha-B-crystallin has been reported to serve as a chaperone under stress conditions for other oncogenic molecules (beta-catenin, cyclin D1 and VEGF) [17] or is itself oncogenic.

In comparison to breast, renal and colorectal cancers, where only cytoplasmic and membraneous staining was reported, in our study we also confirmed nuclear staining. The nuclear re-localisation is a characteristic feature for the whole group of small heat-shock proteins and in most cases is triggered under stress conditions [18,19], but varies among...
cell types and species [20]. In the nucleus, alpha-B-crystallin is claimed to be responsible for the stabilisation of the speckled architecture of lamin A/C and is thus involved in the splicing factor compartment [20]. IJssel et al. [21] discuss that its fundamental role in the nucleus (transcription, splicing and genomic stability) is difficult to be discerned from its chaperone function in that cellular compartment.

The precise biologic function of both cytoplasmic and nuclear localisation of the protein is beyond the scope of our study and needs other approaches for elucidation. Moreover, the variability of cellular compartment expression is complicated by the fact that in many epithelial tumours the protein is down-regulated and lacks cytoplasmic expression – buccal cancer and head and neck cancer [22,23]. Ongoing studies with cell cultures and plasmid vectors for alpha-B-crystallin investigate its contribution to the malignant progression of NSCLC.

The importance of our study may be due to the fact that the nuclear staining was characteristic for adenocarcinoma histology and was significantly related to the tumour stage (p = 0.042). Patients whose tumours had nuclear staining had shorter overall survival time in comparison to those that lacked staining (log-rank test p = 0.002). This supports the hypothesis that the nuclear positivity of the tumours refers to a more aggressive tumour biology. According to our results, the nuclear positivity could also help stratify patients from II and III stage in risk subgroups. Keeping in mind that more than 75% of patients are diagnosed at stage III, the introduction and validation of prognostic markers at this stage would undoubtedly help in predicting recurrence and improving clinical prognosis. The selection of patients in early stages (I–II) that would mostly benefit from target therapies is also of importance. Ongoing studies are focussing on the validation and clinical significance of the nuclear positivity with alpha-B-crystallin on whole tissue sections by quantitative analyses of immunohistochemical staining, using appropriate software.

In conclusion, to the best of our knowledge, it is the first study describing the expression profile of alpha-B-crystallin in NSCLC. The preliminary results of our explorative study show that alpha-B-crystallin is expressed in both cytoplasm and nucleus of NSCLC. The nuclear expression might have clinical applications as a factor of poor prognosis that could stratify patients in risk subgroups. The role of the protein in lung cancer biology and its clinical usefulness should further be explored and validated.

References


Appendix A. Conference discussion

Dr R. Schmid (Berne, Switzerland): I am convinced, too, that biological staging will be the future. I think with TNM staging we are basically at the level of botanics in the medieval times where we describe this flower is growing here and this flower is growing there and we try to get something out of it or predict something out of it. Biological staging is the future. You present basically a negative study, and we know, of course, that there might be at least 200 genes involved in cancer progression. How do you want to expand your studies now? Will you add more markers? What are you planning in future?
Dr Cherneva: Some of the limitations of the study are that the group was very heterogeneous, first of all. What we studied was the non-phosphorylated form of the protein, which is mainly associated with its chaperoning function and it gives overall a very crude estimation of the biology of the tumour. At the time we gave our abstract for the presentation we had not done the study on the correlation between the clinical pathologic variables and the phosphorylated form of the alpha-B-crystallin, which is basically more important, because, as I said, it is responsible for the apoptotic control as it works different sides of the extrinsic and the intrinsic pathways of apoptosis, and it is also associated with the stabilisation of intermediate filaments, such as actin, desmin, and so on, which makes the phosphorylated form very much responsible for the cellular contact and for the mechanisms of metastasis. This is one of the limitations. The other is that we had not divided our study into examining the squamous cell histology group and the adenocarcinoma, which is basically a mistake, because, as you know, the two types of tumours have different biology. But the number of the studied patients, 146, is not a good number to make any statistical conclusions. We have to enlarge it in order to say yes, it’s true or it’s not true. It’s too early to say something determinant for this marker.

Dr Schmid: So you have to do a multicenter study in order to collect the tissue?

Dr Cherneva: Yes.

Dr G.A. Patterson (St. Louis, Missouri, USA): I may have missed it. Is there a difference between the markers that you studied in the primary tumour and metastatic disease? You had some stage III patients. You had some stage IV patients. Were all of these specimens taken from the primary tumour?

Dr Cherneva: The cytoplasmic staining could be seen in every stage, but the nuclear staining was more characteristic for patients with poor differentiation and with advanced tumour stage. These patients were characterised basically with cells that were of great proliferative index.