

**EVALUATION OF THE EXPRESSION OF THE LOW
WEIGHT HEAT SHOCK PROTEIN α B-CRYSTALLIN
IN PULMONARY NEUROENDOCRINE TUMOURS**

Dora M. Marinova, Yanina G. Slavova*, Nedka B. Trifonova,
Vladimir N. Maksimov, Dimitar T. Kostadinov, Danail B. Petrov*****

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Abstract

α B-crystallin is a major heat-inducible small heat shock protein, which is supposed to be implicated in tumour cell proliferation, differentiation, invasion, metastasis and apoptosis. Still the expression pattern of the phosphorylated on serin 59 form of α B-crystallin in lung cancer is not yet investigated.

The aim of the present work is to study this pattern in different histological subtypes of pulmonary neuroendocrine tumours (pNETs).

Surgically resected specimens from 143 patients with pNETs were studied. The histological subtype, pathological Tumour-Node-Metastasis stage and the immunohistochemical expression of phosphorylated α B-crystallin were evaluated.

We demonstrated that the nucleus was the major localization site of the protein although there were some rare cases showing also diffuse cytoplasmic expression. High grade pNETs (small cell lung carcinoma and large cell neuroendocrine carcinoma) showed nuclear expression in all cases but most frequently it was in less than 50% of the tumour cells; cytoplasmic expression was shown in single cases. Just the opposite was observed in low and intermediate grade pNETs (typical carcinoids and atypical carcinoids) – nuclear expression was not seen in all of the cases, but when it was present it was in almost all tumour cells; the cases expressing phosphorylated α B-crystallin also in the cytoplasm were mainly typical carcinoids. We demonstrated that cytoplasmic

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expression was associated with earlier stage and absence of metastases, which may determine it as a potential marker for a favourable prognosis in patients.

We conclude that phosphorylated α B-crystallin probably plays a major role in pNETs biology, it may be associated with tumour grade, it may have a prognostic value, and it may be used as future target for therapy.

Key words: heat shock proteins, α B-crystallin, pulmonary neuroendocrine tumours

1. Introduction. The lung cancer clinical course is determined by its histological and biological features [1]. The prognosis in lung cancer patients is associated with certain clinicopathological features as histological type, tumour size and invasion of adjacent structures (T-status), lymph node metastases (N-status) and pTNM (pathological Tumour–Node–Metastasis) stage [2, 3]. These features are not always sufficient for complete and objective assessment of lung tumours, which requires in-depth studies of immunohistochemical and genetic indicators that could serve as better markers for determining the biological behaviour of these tumours [4].

Adenocarcinoma and squamous cell carcinoma are the most common lung tumours (85% of all lung cancers) and thus they are the most actively studied group. A more special and rare group are the pulmonary neuroendocrine tumours (pNETs) comprising very different tumours regarding biological behaviour and prognosis – typical carcinoids (TCs), atypical carcinoids (AtCs), small cell lung carcinomas (SCLCs) and large-cell neuroendocrine carcinomas (LCNECs) [4]. The carcinoid tumours are abruptly different from SCLCs and LCNECs by clinical, epidemiological and genetic features and by methods of treatment [5].

Various factors (such as hyperthermia, hypoxia, heavy metals, ethanol, free radicals, infectious agents, pH changes, lack of glucoses and growth factors) lead to stress of the normal cells [6]. The result is unfolding and aggregation of various cellular proteins [7], activation of the stress genes [8] and heat shock proteins (Hsps) synthesis. The concentration of proteins, products of these genes, is increased in response to stressors, thus protecting the cells from the damaging effects of stress and accelerating recovery by helping renaturation of partially-denatured proteins [9].

The Hsps family consists of proteins with common function as molecular chaperons – proteins which have in common the property of modifying the structures and interactions of other proteins [10]. α B-crystallin is a major heat-inducible small Hsp (sHsp) with dynamic phosphorylation and oligomeric properties suggesting the existence of different functional forms. According to a review by ARRIGOS [11], elevated levels of expression of α B-crystallin counteract the apoptotic cell death induced by various stimuli. Arrigos [11] concludes that the high expression level of α B-crystallin in a wide range of tumours resistant to therapy as well as its tumourigenic and metastatic potential designate this protein as potential target for future anti-cancer therapeutic strategies. CIOCCA

and CALDERWOOD [10] suggest that the expression of α B-crystallin is implicated in tumour cell proliferation, differentiation, invasion, metastasis and apoptosis.

The expression and the role of α B-crystallin in lung tumours are poorly investigated. CHERNEVA et al. [12] investigate the expression profile of α B-crystallin in non-small cell lung cancers (NSCLCs). In their study, cytoplasmic and nuclear expression is detected. The cytoplasmic expression of α B-crystallin in the tumours is related to the local invasion, while nuclear expression is more commonly detected in advanced stages, and α B-crystallin is determined as biomarker of aggressive tumour biology. They conclude that α B-crystallin plays an essential role in NSCLCs biology and its nuclear staining is an independent factor of poor survival [12].

MORRISON et al. [13] show that the phosphorylated form of α B-crystallin (on Ser-59) is necessary to confer maximal cytoprotection from ischemia-induced apoptosis in cardiomyocytes. Still the expression pattern (presence and localization) of the phosphorylated on serin 59 form of α B-crystallin in lung cancer is not yet investigated.

We have already studied the expression pattern of another sHsp – Hsp27 – in pNETs. The results of this study are shown elsewhere [14].

Because of the above-stated reasons, we aimed to investigate the expression pattern of the phosphorylated on serin 59 α B-crystallin in different histological subtypes of pulmonary neuroendocrine tumours and to evaluate the presence of associations with known prognostic clinicopathological parameters.

2. Material and methods. 2.1. Patients and tissue samples. Surgically resected specimens from 143 patients who underwent surgical treatment for pNETs in the University Hospital for Pulmonary Diseases “St. Sofia”, from April 2000 to December 2011, were investigated. 86 men (60%) and 57 women (40%) were included in the study with mean age 53.5 ± 12.8 years, ranging from 17 to 76 years. The histological type of the tumours was determined according to the World Health Organization criteria [4] as: typical carcinoids (TCs), $n = 64$; atypical carcinoids (AtCs), $n = 13$; small cell lung carcinomas (SCLCs), $n = 51$; and large cell neuroendocrine carcinomas (LCNECs), $n = 15$. The T-status (tumour size and presence/absence of invasion of adjacent structures), the N-status (involvement of regional lymph nodes) and the pTNM stage were determined according to the TNM classification of the revised International System for Staging of Lung Cancer [15].

2.2. Immunohistochemistry. The immunohistochemical staining was performed on formalin-fixed, paraffin-embedded tissue sections (4 μ m) with the polymer-based system EnVisionTM FLEX Mini Kit, High pH (Dako, code No K8024) by protocol of the producer [16]. The primary antibody used was phosphorylated α B-crystallin (hSer 59)-R (Santa Cruz Bio) – a rabbit polyclonal antibody, dilution 1:300. 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 by protocol of the producer (starting $t^\circ = 65^\circ\text{C}$, 20 min incubation time at 97°C , finishing $t^\circ = 65^\circ\text{C}$). This procedure was followed by subsequent incubation with: 1) Peroxidase-blocking reagent for 5 min; 2) Primary antibody for 20 min; 3) Horseradish peroxidase labelled polymer detection reagent for 20 min; 4) Diaminobenzidine (DAB) solution for 8 min. Finally, a counterstaining with hematoxylin, followed by dehydration in grading ethanol and permanent mounting, were done. Negative controls were present in each series of reactions by omitting the primary antibody and by using normal rabbit serum instead.

2.3. Evaluation of immunohistochemical staining. The evaluation of immunohistochemical reactions was done by light microscopy (Carl Zeiss), magnification $\times 200$. The presence of red-brown staining of certain cell sites (cytoplasm, nucleus) was considered as a positive reaction. The expression of phosphorylated αB -crystallin was evaluated (nuclear and cytoplasmic) by counting an average number of 500 tumour cells for each section. Each tumour was classified in one of the five categories, according to the percentage of positive tumour cells: 0 – negative, 1–25% – a small number (low rate) of positive cells; 26–50% and 51–75% – average categories; 76–100% – a large number (high rate) of positive cells. The staining intensity was disregarded because of its heterogeneous character. Almost every tumour has shown various intensity ranging from weak to intensive in different tumour cells.

2.4. Statistical analyses. Statistical analyses were performed by using the SPSS v.13.0, Windows Vista. Chi-square test and bivariate correlations were used to evaluate the associations between clinicopathological parameters and phosphorylated αB -crystallin expression. A $P < 0.05$ was considered statistically significant.

3. Results. 3.1. Clinicopathological parameters (tumour size, T-, N-, M-status and pTNM stage) in different pNETs (Table 1). There are some remarkable differences between the different histological subtypes of pNETs. According to the size of the tumour, TCs and AtCs are statistically significant, smaller than SCLCs and LCNECs ($P = 0.002$). According to T-status, SCLCs and LCNECs are more locally advanced than TCs and AtCs ($P = 0.019$). The metastases in lymph nodes are more frequent in SCLCs and LCNECs than in TCs and AtCs ($P = 0.002$). According to the pTNM stage category, TCs and AtCs are more frequently at earlier stages, opposite to SCLCs and LCNECs usually presenting at advanced stages of the disease ($P < 0.001$).

3.2. Phosphorylated αB -crystallin expression pattern in different pNETs (Table 1). Nuclear expression of phosphorylated αB -crystallin is observed in 136 (95.1%) tumours. In 19 (14% of tumours with nuclear expression) cases, there is a combination with cytoplasmic expression of the marker. The phosphorylated αB -crystallin expression in typical carcinoid, atypical carcinoid, SCLC and LCNEC is shown in Figs 1, 2, 3, 4 respectively.

T a b l e 1

Distribution of different pNETs cases by size, T-status, N-status, M-status, pTNM-stage, expression of phosphorylated α B-crystallin, number, percentage. $P < 0.05$ is statistically significant

| | | Histology | | | | | Chi-square <i>P</i> |
|--|---------------|---------------|---------------|---------------|---------------|---------------|------------------------|
| | | TC (n/%) | AtC (n/%) | SCLC (n/%) | LCNEC (n/%) | Total (n/%) | |
| Size, mean \pm SD, cm | | 2.9 \pm 1.2 | 3.1 \pm 1.5 | 4.8 \pm 2.8 | 5.8 \pm 2.9 | – | 0.002 |
| Size, range, cm | | 0.5–6.0 | 1.0–5.3 | 1.0–13.5 | 2.5–14.0 | – | |
| T-status | T1 | 20/31.3% | 2/15.4% | 11/21.6% | 3/20.0% | 36/25.2% | 0.019 |
| | T2 | 38/59.4% | 9/69.2% | 24/47.1% | 8/53.3% | 79/55.2% | |
| | T3 + T4 | 6/9.4% | 2/15.4% | 16/31.4% | 4/26.7% | 28/19.6% | |
| N-status | N0 | 57/89.1% | 11/84.6% | 30/58.8% | 12/80.0% | 110/76.9% | 0.002* |
| | N1 | 2/3.1% | 0 | 6/11.8% | 1/6.7% | 9/6.3% | |
| | N2 | 5/7.8% | 2/15.4% | 14/27.5% | 2/13.3% | 23/16.1% | |
| | N3 | 0 | 0 | 1/2.0% | 0 | 1/0.7% | |
| M-status | M0 | 64/100.0% | 12/92.3% | 47/92.2% | 15/100.0% | 138/96.5% | 0.094 |
| | M1 | 0 | 1/7.7% | 4/7.8% | 0 | 5/3.5% | |
| pTNM-stage | I | 50/78.1% | 7/53.8% | 18/35.3% | 5/33.3% | 80/55.9% | < 0.001 |
| | II | 9/14.1% | 4/30.8% | 11/21.6% | 8/53.3% | 32/22.4% | |
| | III + IV | 5/7.8% | 2/15.4% | 22/43.1% | 2/13.3% | 31/21.7% | |
| p-alphaB-crystallin nuclear expression | negative | 6/9.4% | 1/7.7% | 0 | 0 | 7/4.9% | 0.005 |
| | Mean \pm SD | 64% \pm 39% | 68% \pm 38% | 62% \pm 27% | 59% \pm 30% | 63% \pm 34% | |
| | < 25% | 12/18.8% | 2/15.4% | 6/11.8% | 2/13.3% | 22/15.4% | |
| | 26–50% | 2/3.1% | 0 | 14/27.5% | 5/33.3% | 21/14.7% | |
| | 51–75% | 9/14.1% | 3/23.1% | 12/23.5% | 3/20.0% | 27/18.9% | |
| | > 75% | 35/54.7% | 7/53.8% | 19/37.3% | 5/33.3% | 66/46.2% | |
| p-alphaB-crystallin cytoplasmic expression | negative | 50/78.1% | 11/84.6% | 49/96.1% | 14/93.3% | 124/86.7% | 0.023** |
| | Mean \pm SD | 8% \pm 21% | 7% \pm 23% | 4% \pm 19% | 6% \pm 25% | 4% \pm 16% | |
| | < 25% | 7/10.9% | 1/7.7% | 2/3.9% | 1/6.7% | 11/7.7% | |
| | 26–50% | 3/4.7% | 0 | 0 | 0 | 3/2.1% | |
| | 51–75% | 0 | 0 | 0 | 0 | 0 | |
| | > 75% | 4/6.3% | 1/7.7% | 0 | 0 | 5/3.5% | |

* for N0 vs N1, N2&N3;

** for Tcs&AtCs vs SCLCs&LCNECs.

Abbreviations: TC – typical carcinoid; AtC – atypical carcinoid; SCLC – small cell lung carcinoma; LCNEC – large cell neuroendocrine carcinoma; SD – standard deviation

In our study, the different pNETs show positive nuclear expression of phosphorylated α B-crystallin in different percentage of cases – TCs – 90.6%, AtCs – 92.3%, SCLCs – 100%, LCNECs – 100%. We found a statistically significant difference between high grade pNETs (SCLCs and LCNECs) and low and intermediate grade pNETs (TCs and AtCs). In the majority of cases, the nuclear

expression of phosphorylated α B-crystallin is observed in almost all the tumour cells of carcinoids contrary to SCLCs and LCNECs – often in high grade pNETs, the percentage expressing tumour cells is below 50% ($P = 0.005$).

With regard to cytoplasmic expression, it is observed more often in TCs – 22% of the cases with nuclear expression and AtCs – 15.4%, and rarely in SCLCs – 4% and LCNECs – 6.6% ($P = 0.023$). Phosphorylated α B-crystallin is seldom expressed in the cytoplasm and is often in a low percentage (below 25%) of tumour cells.

Though there are statistically significant associations between the cytoplasmic expression of phosphorylated α B-crystallin and the absence of lymph node metastases (N0-status) ($P = 0.01$), and earlier pTNM stage ($P = 0.03$), there are no associations between the clinicopathological characteristics and the nuclear expression of phosphorylated α B-crystallin.

4. Discussion. According to a review by Ciocca and Calderwood [10], the sHsps have a major role in cancer progression and could serve as suitable targets for therapy, can show the grade of differentiation and the aggressiveness in some cancers. There is ongoing research with an aim to obtain more information about the exact role of sHsps in tumour growth, invasion and metastasis [10].

Overexpression of α B-crystallin is found in glioma, kidney tumours, breast cancer and ductal carcinoma in situ [17]. Recently it has also been shown in non-small cell lung carcinomas [12].

According to cell cycle and cellular status, α B-crystallin has dynamic phosphorylation and oligomeric properties suggesting the existence of different functional forms [11].

Morrison et al. [13] show that the phosphorylated form of α B-crystallin (on Ser-59) is necessary and sufficient to confer maximal cytoprotection from ischemic-induced apoptosis in cardiomyocytes and they suggest the important role of this form for the cellular survival. DEN ENGELSMAN et al. [18] demonstrate that α B-crystallin is imported into the nucleus dependent on the phosphorylation of Ser-59. In our study, the nucleus was the major localization site of the expression of phosphorylated α B-crystallin in the studied tumours – we found it in 95% of pNETs. We have also observed in some cases (14%), in a small percentage of tumour cells, a diffuse cytoplasmic expression of phosphorylated α B-crystallin.

Regarding the nuclear expression of phosphorylated α B-crystallin, the following differences between groups of pNETs were observed: 1) All cases of high grade pNETs (SCLCs & LCNECs) were expressing it, unlike low and intermediate grade pNETs (TCs & AtCs); 2) In the majority of cases, the nuclear expression of phosphorylated α B-crystallin was observed in almost all tumour cells of carcinoids, contrary to SCLCs and LCNECs – often in high grade pNETs the percentage expressing tumour cells was below 50% ($P = 0.005$). Cytoplasmic expression was observed in decreasing degree of % positive cases from low grade pNETs to

high grade pNETs ($P = 0.023$). These data suggest that perhaps the expression of phosphorylated α B-crystallin is associated with the grade of differentiation.

Though there was a statistically significant association between the cytoplasmic expression of phosphorylated α B-crystallin and the absence of lymph node metastases (N0-status) ($P = 0.01$) and earlier pTNM stage ($P = 0.03$), there were no associations between the nuclear expression of phosphorylated α B-crystallin and the clinicopathological characteristics. This may be due to the relatively small number of cases in each group.

Arrigos [11] has stated that the high expression level of α B-crystallin is associated with resistance to cytostatic therapy in a wide range of tumours, thus designating this protein as potential target for future anti-cancer therapeutic strategies. In our study, we have demonstrated the high level of expression of phosphorylated α B-crystallin in all four groups of pNETs. It is well known that carcinoid tumours are in general resistant to chemotherapy [5], thus we suppose that the expression of phosphorylated α B-crystallin can be implicated in their resistance, suggesting eventual future therapeutic strategy based on downregulation of phosphorylated α B-crystallin. To prove this thesis, more research is needed.

In our study, the expression pattern of the sHsp phosphorylated α B-crystallin in pNETs was investigated. We demonstrated that the nucleus was the major localization site of the protein although there were some rare cases with a diffuse cytoplasmic expression. We observed distinct nuclear and cytoplasmic expression patterns in different pNETs and this may be associated with tumour grade. We presented that cytoplasmic expression was associated with earlier pTNM stage and absence of metastases, which may determine it as a potential marker for a favourable prognosis in pNETs patients.

We conclude that phosphorylated α B-crystallin probably plays a major role in pNETs biology, it may be associated with tumour grade, it may have a prognostic value, and it may be used as future target for therapy.

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*Department of Pneumology
St Sofia University Hospital
for Pulmonary Diseases
Medical University of Sofia
19, Ivan Geshov Blvd
1431 Sofia, Bulgaria
e-mail: dmusic@abv.bg
dimko@mail.bg*

**Department of Pathology
St Sofia University Hospital
for Pulmonary Diseases
Medical University of Sofia
19, Ivan Geshov Blvd
1431 Sofia, Bulgaria
e-mail: yaninaslavova@abv.bg*

***Department of Biology
Medical Faculty
Medical University of Sofia
2, Zdrave Str.
1431 Sofia, Bulgaria
e-mail: trifonovitn@yahoo.com*

****Department of Thoracic Surgery
St Sofia University Hospital
for Pulmonary Diseases
Medical University of Sofia
19, Ivan Geshov Blvd
1431 Sofia, Bulgaria
e-mail: danail_petrov@hotmail.com*