Original paper

Clinical significance of nestin and its association with survival in neuroendocrine lung tumours

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Nestin is considered to be a cancer stem cell marker. Nestin expression in neuroendocrine tumours might be useful to predict prognosis and facilitate treatment planning. 88 patients with neuroendocrine lung tumours operated in the Department of Thoracic Surgery from 2007 to 2015 were included into the study. Immunohistochemical staining for nestin was performed. Clinicopathological and survival data were retrospectively analyzed. Nestin expression was detected in 15 (17%) specimens. Multivariate analysis showed that lymph node metastases ($p = 0.0001$; hazard ratio (HR) = 3.93; confidence interval (CI) 95%: 1.96-7.87), nestin expression ($p = 0.034$; HR = 2.30; CI 95%: 1.06-4.99) and patient's age ($p = 0.024$; HR = 1.04; CI 95%: 1.00-1.09) were independent negative prognostic factors. Nestin expression was significantly higher in large cell neuroendocrine carcinoma when compared with carcinoids ($p = 0.001$). Collected data support the thesis that nestin can be regarded as a biomarker in patients with neuroendocrine lung tumours.

Key words: neuroendocrine markers, neuroendocrine tumours, pulmonary pathology.

Introduction

Nestin is an intermediate filament class VI, a neural stem cell marker. During development, it is present in mammalian nervous system [1], also in skeletal muscles, hepatic cells and umbilical cord blood. After maturation, nestin is replaced by other proteins. In adults it might appear after nervous system injury, in Leydig or corticotroph cells [2]. In general, after embryogenesis, nestin expression is characteristic for immature cells of high plasticity or pathologic conditions [3]. Cancer stem cells (CSC) represent a small, multipotent population within tumour mass, with an increased proliferative capacity. They play major role in growth, migration and invasion of a neoplasm. CSC are identified by presence of specific markers [4]. Nestin was found to be co-expressed with typical CSC markers in several types of tumours [5]. Moreover, nestin expression occurs in various human malignancies. As anticipated, it was commonly observed in neuroectodermal neuroepithelial tumours [6]. This protein was also recognized in other types of solid neoplasms e.g. osteosarcoma [7], germinoma [8] or those originating from epithelial tissues, like pancreatic adenocarcinoma [9]. Additionally, recent research proved that nestin expression is associated with clinical course of malignant disease. Zhong

et al. disclosed poorer outcome among nestin-positive patients with esophageal squamous cell carcinoma [10]. In mucosal melanoma nestin expression was connected with worse prognosis, especially in advanced stages [11]. Meta-analysis concerning relationship between nestin and neoplasm stage revealed that expression of this protein is associated with higher stages of tumour, thus possibly with adverse outcome. Author especially emphasized this relation in lung tumours [12].

The group of pulmonary neuroendocrine tumours comprises entities varying from typical carcinoid (TC), atypical carcinoid (AC) to small cell lung cancer (SCLC) and large cell neuroendocrine carcinoma (LCNEC). Non-specific symptoms such as cough and dyspnea are very common. Carcinoid syndrome, Cushing syndrome or acromegaly occur rarely [13]. Although LCNEC and SCLC are clinically more aggressive and characterized by higher Ki-67 rate, they share common neuroendocrine features with carcinoids, such as morphology and expression of specific markers [14]. Moreover, the same pulmonary neuroendocrine cells probably give rise to all of these tumour types [15]. In the light of increasing incidence of neuroendocrine neoplasms [16], there is a great need for marker identification, which would be useful to predict prognosis and facilitate treatment. We hypothesized that nestin, due to its role in carcinogenesis, could be a potential biomarker in lung neuroendocrine tumours. In this study we analyzed association between nestin expression and survival in patients with lung neuroendocrine tumours.

Material and methods

We have performed retrospective chart review of all patients with neuroendocrine lung tumours, who were operated on in the Department of Thoracic Surgery in Poznań, Poland in the years 2007-2015. The diagnosis was established according to the 4th WHO classification [17]. For the study purpose all available specimens were re-analyzed to confirm previous diagnoses.

Patients were assessed according to 7th TNM classification [18]. We retrospectively evaluated multiple parameters, namely nodal involvement, nestin expression, and tumour vessel invasion. We also performed survival analysis. Overall survival time was determined as a time from operation to death or end of the follow-up.

Formalin-fixed tissue specimens were paraffin wax embedded and cut into 4-μm sections. Then we placed them on SuperFrost®Plus adhesive microscope slides (Menzel Gläser). Next steps were: heating for 50 minutes at 97°C in a water bath in low pH Dako EnVision FLEX Target Retrieval Solution (Glostrup, Denmark) to achieve paraffin, water removal, and heat-induced epitope retrieval. We ceased endogenous peroxidase action with Dako EnVision FLEX Peroxidase-Blocking Reagent (Glostrup, Denmark). Then, the tissues were kept in Leica Biosystems Novocastra Protein Block (Wetzlar, Germany) for 10 min. For the night, sections were left in humid chamber at 4°C with mouse monoclonal nestin Antibody (10c2) (Santa Cruz Biotechnology) (dilution 1 : 150) and monoclonal mouse Anti-Human Ki-67 Antigen antibody (Dako) (dilution 1 : 500). We diluted primary antibody in Dako EnVision FLEX Antibody Diluent (Glostrup, Denmark). Then, we conducted immunodetection with Novolink Polymer Detection System (Leica Biosystems), followed by visualization with 3,3`-diaminobenzidine tetrachloride (DAB, Leica Microsystems). As the last step, we counterstained tissues with Mayer's haematoxylin, removed water, cleared and placed them in DPX mountant.

Two experienced pathologists assessed specimens after immunohistochemical staining. The cytoplasmic nestin expression was considered positive when at least 5% of cells were stained. We evaluated nestin expression in comparison to vascular endothelial cells staining, as in previous studies [19, 20]. If reaction was negative, the score was 0, if it was present but weaker than in endothelial cells – 1. If the staining was similar to that of the endothelial cells, the score was 2, in case of more intensive reaction – score 3. We considered scores 2 and 3 as positive reaction.

Statistical analysis was performed by MedCalc Statistical Software version 16.4.3 (MedCalc Software bvba, Ostend, Belgium). Continuous variables were presented as median, minimal and maximal values, while categorical as numbers (%). p value less than 0.05 was considered significant. Cumulative survival was assessed by Kaplan-Meier method. To compare survival times between groups we used log-rank test. Cox proportional hazards regression model was performed to evaluate effects of chosen variables on survival. Into the final analysis following variables were included: nestin expression, nodal involvement and patient's age at the time of operation (entry level $p < 0.05$). Comparison between nestin expression in carcinoids and high grade neuroendocrine tumours was performed with χ^2 test.

The research protocol was approved by the local bioethical committee. All procedures performed were in accordance with the 1964 Helsinki declaration and its later amendments.

Results

Clinicopathological data are presented in Table I.

Nestin expression was positive in 15 (17%) cases including: 14 patients with LCNEC and one with typical carcinoid. Mean surveillance time for the entire group was 37 months. During the study 30 patients died due to neuroendocrine lung tumour. We registered 3 deaths due to other reasons. Nestin-negative patients were observed for 39 months on average, positive ones for 25 months. At the end of the study 9 nestin-positive patients (60.0%) died (all cancer-related deaths), while from nestin-negative group – 24 (32.9%) died. Survival was significantly shorter in nestin-positive patients when compared with negative ones ($p = 0.041$) (Fig. 1). In separate analysis of LCNEC, nestin expression did not affect survival length ($p = 0.698$), whereas lymph node metastases worsened prognosis ($p = 0.0042$) .

Patients with lymph node metastases were characterized by poorer outcome, than those without this feature ($p = 0.001$) (Fig. 2). Moreover, multivariate analysis showed that lymph node metastasis ($p = 0.0001$; HR = 3.93; confidence interval (CI) 95%: 1.96-7.87), nestin expression ($p = 0.034$; HR =2.30; CI 95%: 1.06-4.99) and patient's age at the time of surgery ($p = 0.024$; HR =1.04; CI 95%:1.00-1.09) were independent negative prognostic factors (Table II).

Nestin expression was significantly higher in LCNEC when compared with carcinoids ($p = 0.0010$). Figures 3 and 4 depict nestin expression.

Table I. Characteristics of patients with lung neuroendocrine tumours

LCNEC	48 (55%)
TC	32 (36%)
AC	8 (9%)
Female	46 (52%)
Male	42 (48%)
Mean observation period	37 months (0.6-100)
Mean age	59 years (28-78)
TNM stage	
Ia	27 (31%)
Ib	18 (20%)
IIa	24 (27%)
IIb	6 (7%)
IIIa	11 (12.5%)
IV	2 (2.5%)
Surgical procedure	
Pneumonectomy	7 (8%)
Lobectomy	77 (87.5%)
Segmentectomy	1 (1%)
Wedge resection	3 (3.5%)
Nestin	
Positive	15 (17%)
Negative	73 (83%)
Lymph node metastases	
Yes	30 (34%)
No	58 (66%)
Visceral Pleura involvement	
Yes	12 (14%)
No	76 (86%)
Parietal Pleura involvement	
Yes	3 (3.5%)
No	85 (96.5%)
Neoplastic clots in vessels	
Yes	14 (16%)
No	74 (84%)
Alive	55 (62.5%)
Cancer-related death	30 (34%)
Death due to other reasons	3 (3.5%)

LCNEC – large cell neuroendocrine carcinoma; TC – typical carcinoid; AC – atypical carcinoid; TNM – TNM Classification of Malignant Tumours

Discussion

LCNEC is a highly malignant neoplasm, constituting 3% of operated primary lung cancers. Five-year survival rate is very low and varies from 20 to 55%. LCNEC is characterized by high mitotic rates and large areas of necrosis [21]. On the contrary, carcinoids are located in the opposite side of lung neuroendocrine tumours spectrum. TC comprise 1-2%, while AC 0.1-0.2 % of lung tumours. Their mitotic rate is low, necrosis is present focally (AC) or absent (TC). Also, prognosis is much better with 5-year survival rates for TC about 90% and AC: 44-78%. Still, in the 4th edition of the World Health Organization (WHO) classification of tumours of the lungs, pleura, thymus and heart they are presented as a single group [22]. Taking into account, their common origin we chose to assess nestin expression in the whole group. Additionally, data concerning nestin and its possible role in neuroendocrine lung tumours are scarce.

We found that nestin expression was present in 15 cases. In our study nestin expression was associated with a 2-fold increase of the hazard of death. To our

Table II. Evaluation of chosen variables on survival. Cox proportional hazards regression model

	P	HR	95% CI
Nestin Positive vs. negative	0.0342	2.3058	1.0643-4.9957
Patient's age at operation time	0.0242	1.0477	1.0061-1.0911
Nodal involvement Positive vs. negative	0.0001	3.9347	1.9661-7.8743

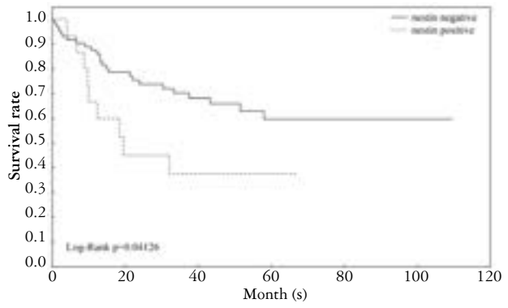

Fig. 1. Survival rates in nestin negative and positive groups. Kaplan-Meier plot

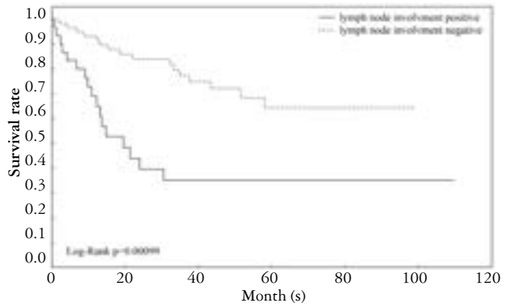

Fig. 2. Comparison of survival in patients with and without nodal metastases. Kaplan-Meier graph

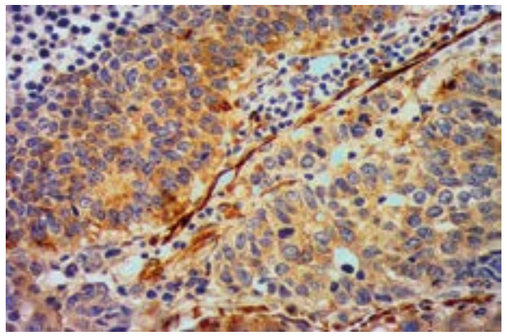

Fig. 3. Nestin expression in a case of LCNEC. Magnification 400×

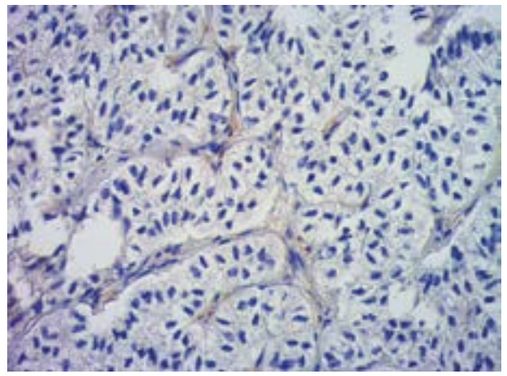

Fig. 4. Negative reaction in a case of typical carcinoid. Magnification 400×

knowledge, there was only one study evaluating this protein and survival in LCNEC. Nestin was detected in 8/30 patients and it posed an independent prognostic factor, increasing three times the hazard of death [19]. The limitations of the study were: small sample size and short follow-up time [19]. In the whole group we found nestin expression to be a negative prognostic factor shortening the survival length, though in a separate analysis of LCNEC, lymph node involvement had much stronger impact on the prognosis. So, nestin seems to have a limited role as a prognostic marker in LCNEC, but its increased expression rather simply reflects the high proliferative activity of LCNEC cells. On the other hand, from the spectrum of lung tumours, nestin expression in SCLC was examined by Takakuwo *et al.* They showed expression of this protein in 9/10 examined cell lines [23]. Additionally, when nestin was down-regulated, the cells exhibited lower proliferation and invasive potential [23]. When it comes to nestin and other lung malignancies, it was identified as CSC marker in non-small cell lung cancer (NSCLC) [24]. Narita *et al.* found nestin on immunohistochemical analysis performed on surgical specimens of NSCLC [25]. They also investigated several cell lines representing lung adenocarcinoma. Nestin expression in tumour samples positively correlated with tumour size, lymph node metastasis and there was negative association with survival. On cell lines, it corresponded with increased proliferation, migration and invasion capacities [25]. Basing on our results and presented studies, nestin detection may serve in the future, as a biomarker used to predict prognosis and facilitate patient-oriented approach to treatment.

With regard to carcinoids, in our study, nestin was positive in just one out of 40 cases. At the end of the observation the nestin-positive patient was alive. In a single published study, Ehrman *et al*. investigated only 9 carcinoid specimens, using different method, the histoscore. Nestin staining was weak and heterogeneous, thus confirming our results and pointing to a conclusion that it is of little use in carcinoids [26]. In our study, the nestin expression in LCNEC was significantly higher than in the carcinoid cells. As mentioned before, LCNEC is much more aggressive than carcinoids. Nestin is usually detected in more

advanced tumour stages and in very malignant neoplasms [27, 28]. Thus, presence of this protein in LCNEC might be associated with properties of nestin positive cells: high plasticity and increased proliferative potential. Confirmation of LCNEC diagnosis from small biopsy specimens prior to operation, is hard to obtain [29]. Nestin detection may assist to support diagnostic accuracy.

Additionally, we found that lymph node involvement and patient's age at the time of operation were additional independent prognostic factors. However, Yi Chen Yeh *et al*. did not disclose correlation between nodal status, TNM classification stage and survival in LCNEC and SCLC, it probably resulted from study limitations [30]. Adversely, Filloso *et al*. confirmed our findings, that lymph node metastases and older age are independent negative prognostic markers [31].

Nestin expression might be useful as a negative survival biomarker in a spectrum of neuroendocrine lung tumours. However, some authors suggested that low and high-grade neuroendocrine tumours do not represent the continuum of the same lesion [32]. In our study, in LCNEC patients lymph node status, but not nestin expression, was associated with shorter survival. On the other hand, we found significant nestin overexpression in LCNEC as compared to carcinoids. Thus, nestin expression might be helpful in certain cases to differentiate between LCNEC and carcinoids, in pathological examination.

The main limitation is that our study was conducted at a single institution on a relatively small sample. Nevertheless, it must be mentioned, that collecting a larger cohort is difficult since the incidence of these tumours is relatively low (lung tumours include 1-2.0% carcinoids and 3.0% LCNEC) [22].

To summarize, nestin potential role as a prognostic factor in neuroendocrine lung tumors needs further multi-institutional investigation as for any potential biomarker to be included into the routine usage. At present, nestin might be regarded as one of the markers helpful in pathological examination of bronchopulmonary neuroendocrine lung tumors.

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The authors declare no conflict of interest.

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