Analysis of the prognostic impact of nestin expression in non-small cell lung cancer

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Background. Nestin is an intermediary filament protein, expressed in progenitor cells of neural and muscle origin and in activated endothelium. The expression of this protein in tumours can be associated with degree of differentiation, biological potential and/or neoangiogenesis.

Aims. The aim of this study was to examine the immunohistochemical expression of nestin in primary non-small cell lung carcinomas (NSCLC) and to determine its prognostic significance.

Methods. Immunohistochemical detection of nestin was carried out on tissue microarrays constructed from 114 formalin-fixed and paraffin-embedded NSCLC samples. These included 78 squamous cell carcinomas and 37 adenocarcinomas. Expression of nestin was also analysed in 35 primary tumour independent NSCLC brain metastasis. The H-score and degree of nestin positive microvascularisation were determined. Both parameters correlated with the clinicopathological characteristics including disease-free and overall survival.

Results. We demonstrated that expression of nestin is not significantly higher in tumour cells of adenocarcinomas than in squamous cell carcinomas despite the fact that adenocarcinomas were more frequently positive ($P \leq 0.30$). On the other hand, nestin positivity and nestin positive neovascularisation were significantly more often found in stage IIIa tumours than tumours in stages I and II ($P \leq 0.04$, $P \leq 0.02$). Nestin expression was also significantly higher in brain metastases of squamous cell carcinomas than brain metastases of adenocarcinomas ($P \leq 0.003$). The expression of nestin, in general, did not significantly correspond to disease-free or overall survival.

Conclusion. Nestin expression in NSCLCs is associated with poorer prognosis and with greater nestin-positive microvessel density.

Key words: nestin, non-small cell lung cancer, brain metastasis, immunohistochemistry, prognosis

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INTRODUCTION

Nestin belongs to a class VI intermediate filament protein originally found in neuroepithelial stem cells and neural cells¹. Nestin forms intermediate filament bundles with vimentin by copolymerization in neuroepithelial cells². Nestin mRNA is expressed at a high level in the cerebrum of developing rat embryos on the 15th prenatal day, its level decreases toward the 12th postnatal day, and disappears from the 18th postnatal day when it reaches adult levels¹. High levels of nestin expression have been detected outside the nervous tissue in activated endothelium^{3,4}, the endothelium of pancreatic vessels, reactive astroglial cells, oligodendroglial cells, Sertoli's cells, hair follicle cells, stellate cells, pericytes, myoepithelial cells and odontoblasts⁵⁻¹².

Nestin is also present in skeletal muscle progenitors, cardiac muscle progenitors, infarcted myocardium, and a large number of neoplasms¹³⁻¹⁵.

Tumour angiogenesis is an important factor for the growth and metastasis of human neoplasms. The degree of tumour angiogenesis is associated with clinical outcome and with tumour aggressiveness in general¹⁶⁻¹⁸. Above all, tumour angiogenesis is essential for the establishment of metastases in distant organs¹⁹. A possible explanation of this metastatic mechanism is that an increase in the number of tumour vessels increases the chances for tumour cells to enter the circulation, and newly formed vessels or capillaries have leaky and weak basement membranes, through which tumour cells can penetrate more easily than through the basement membranes of normal mature vessels²⁰. Thus, patients with highly vascularized tumours are significantly more likely to die than those with less vascularized tumours. Angiogenesis in malignant neoplasms, as measured in terms of microvessel density (MVD), has been reported to correlate with clinicopathological status and survival of several carcinomas such as breast, ovarian, prostatic, oesophageal, gastric, colorectal, cervical carci-

noma and malignant melanoma^{16,17,21-29}. Despite studies on the prognostic relevance of microvessel density in NSCLC (ref.³⁰⁻³⁴), the significance of nestin expression in NSCLC vessels remains unclear.

The expression of nestin in tumour cells can be associated with the degree of tumour differentiation, the invasive and metastatic potential of the tumour and/or degree of neoangiogenesis. Immunohistochemical detection of nestin can therefore not only be important for classification of tumours, but also for their prognostic assessment¹⁵. Some studies suggest that nestin may play a role in the regulation of cell proliferation, survival and differentiation^{35,36}.

The expression of nestin in NSCLC cells was recently examined by Chen and co-workers³⁴, but, in our opinion, extrapolation of their results is controversial, since these authors did not report whether the studied patients underwent preoperative radiotherapy and/or chemotherapy, and since they did not stratify patients according to tumour stage. Another recent paper on nestin expression in NSCLC described significantly higher expression of nestin in lymph node metastasis, in squamous cell carcinomas, in carcinomas with poorer differentiation, with vascular and lymphatic invasion and poorer prognosis, as well as with increased probability to die³⁷. Thus, the aim of this study was to more precisely analyse immunohistochemical expression of nestin in cells of primary NSCLC, in intratumoral vessels and in brain metastasis in relation to important clinicopathologic characteristics such as sex, age, presence of metastasis, histological type, stage, grade of differentiation, disease-free and overall survival.

MATERIALS AND METHODS

Patients and tissues

Tissue microarrays were constructed from 114 formalin-fixed and paraffin-embedded non-small cell lung cancer samples from the tissue banks of the Department of Pathology, Palacky University and University Hospital, Olomouc and Israel. The cohort consisted of 78 squamous cell carcinomas and 37 adenocarcinomas. Adenocarcinomas and squamous cell carcinomas were further subdivided into poorly differentiated (grade 3) and well-differentiated (grade 1 and 2) subtypes according to World Health Organisation recommendations. Stage of tumours based on the TNM classification was determined according to the recommended guidelines. Lymph node metastases were found in 68 examined NSCLC patients. Expression of nestin was also determined in 35 primary NSCLC independent brain metastases. All tumour samples with the exception of brain metastases were obtained through surgery before the start of neoadjuvant or adjuvant chemotherapy. The study was approved by the local institutional ethics committees. Detailed characteristics of patient groups and their relation to nestin expression and nestin-positive vascularisation are shown in Tables 1, 2.

Immunohistochemistry

The detection of nestin was carried out by indirect immunohistochemistry on tissue microarray sections, utilising a commercially available anti-nestin monoclonal antibody (clone 10C2, cat. no MAB5326, Chemicon International, USA). After deparaffinization, endogenous peroxidase activity was blocked by incubation with 0.3% hydrogen peroxide in methanol for 30 min. Sections were pretreated in a microwave generator for 15 min in 10 mM citrate buffer (pH 6.0) and incubated with the anti-nestin antibody diluted 1:200 in PBS containing 1% BSA, for 60 min at room temperature. Nestin was visualized by a secondary staining system (EnVision, Dual Link System-HRP, cat. No. K4061, Dako North America Inc., USA) using diaminobenzidine tetrahydrochloride as substrate. The sections were counterstained with hematoxylin, dehydrated and mounted in Canadian balsam. Negative controls included sections where the staining with the primary antibody was omitted in the staining procedure. The positivity of blood vessel endothelium was considered to be a positive control.

Evaluation and statistical analysis

The nestin positivity was assessed using an H-score (0 – none positivity, 1 – weak positivity, 2 – moderate positivity and 3 – strong positivity multiplied by % of positive cells), and the degree of nestin positive vascularisation (determined semiquantitatively in areas of the most prominent vascularization – 1 – weak, 2 – moderate and 3 – strong) were determined according to the consensual methodology¹⁸. Both parameters were correlated with disease-free and overall survival and with other clinicopathological characteristics. The H-scores and grade of microvascularizations were calculated as an average of the values obtained by two independent experienced investigators. The data were analysed using the nonparametric Mann-Whitney test. Fisher's exact test was used to compare the number of positive and negative cases within individual groups. Associations of these parameters with disease free and overall survival were analysed by the Kaplan-Meier method. The data were processed with SPSS statistical software version 15 (SPSS Inc., Washington, USA). The values $P \leq 0.05$ were considered significant.

RESULTS

The results showed that the expression of nestin was not significantly higher in the tumour cells of adenocarcinomas than in squamous cell carcinomas despite the more frequently found nestin-positivity for adenocarcinomas. We used two thresholds of positivity: first where analysed tumours exhibited an H-index higher than 0 and second where the H-index was higher than 10. The differences in both cases were not significant ($P \leq 0.25$, resp. $P \leq 0.30$). These results are summarized in Table 3.

As it is shown in Table 4, the nestin positivity and nestin positive neovascularisation were demonstrated

Fig. 1. Comparison of typical nestin positive vascularisation in brain metastasis (a) and primary tumour (b).

Fig. 2. Comparison of nestin expression in metastasis of squamous cell carcinoma (a) and metastasis of adenocarcinoma (b).

Fig. 3. Comparison of typical nestin expression in primary squamous cell carcinoma (a) and brain metastasis of squamous cell carcinoma (b).

Fig. 4. Disease-free survival in patients with nestin positive and negative NSCLC.

Fig. 5. Overall survival in patients with nestin positive and negative NSCLC.

significantly more often in tumours of stage IIIa than in tumours of stages I and II ($P \leq 0.04$, $P \leq 0.02$). We found no significant differences between stages when mean and median H-indexes were compared.

We found a significantly higher expression of nestin in brain metastases of squamous cell carcinomas than in brain metastases of adenocarcinomas ($P = 0.003$, Table 5) and generally the mean neovascularisation in brain metastases was more intensive than in primary tumours (Fig. 1). Moreover, we observed a higher percentage of

nestin-positive cells surrounding tumour cell nests in the brain metastases of squamous cell carcinomas but not in the metastases of adenocarcinomas (Fig. 2,3).

Kaplan-Meier's curves of disease-free and overall survival showed no significant correlations with nestin expression in general, and/or with different histological subtypes, different degree of differentiation of primary tumours and brain metastases. However, some interesting trends were found. The disease-free interval was evidently longer for the first 18 months in patients with

Fig. 6. Disease-free survival in stage IIIA of nestin positive NSCLC.

Fig. 7. Disease-free survival with squamous cell nestin positive NSCLC.

nestin positive NSCLC. On the other hand, overall survival was shorter for patients with nestin positive NSCLC (Fig. 4,5). The disease-free interval was also longer for the first 18 months in patients with stage IIIa nestin-positive NSCLC (Fig. 6) and in patients with squamous cell nestin-positive carcinoma (Fig. 7).

DISCUSSION

We found no significantly different expression of nestin between histological subtypes of NSCLC, although

adenocarcinomas were more often nestin-positive. This result does not resolve the discordance between the published study of Chen and co-workers³⁴, who described significantly higher expression of nestin in adenocarcinomas, and Ryuge and co-workers³⁷ who reported higher positivity in squamous cell carcinoma. The differences could be explained by the use of different antibodies. The antibody used in our analysis was not identical either but it probably recognized a similar epitope of nestin as the antibody used by Ryuge and co-workers, because it produced only cytoplasmic immunostaining in tumour cells. The antibody used by Chen and co-workers stained

Table 1. Clinicopathological and histopathological characteristics of NSCLC patients and their relation to nestin expression (H-index) and nestin-positive vascularisation.

	Total	Nestin-positive cases (H-index>0)	%	Mean H-index	Number of cases with nestin-positive vascularisation	%
Number of patients	114	40	35.1	65.98	9	7.9
Male	92	31	33.7	66.52	8	8.7
Female	23	10	43.5	35.40	1	4.5
Squamous cell carcinoma	79	23	29.1	52.70	6	7.6
Grade I and II	66	20	33.3	54.15	6	9.1
Grade III	13	3	23.1	43.30	0	0.0
Adenocarcinoma	36	19	52.8	88.42	3	8.6
Grade I and II	21	11	52.4	68.64	1	5.0
Grade III	15	8	52.3	115.60	2	13.3
Brain metastases	35	30	85.7	94.39	2	5.7

Table 2. Clinicopathological and histopathological characteristics of NSCLC patients and their relation to disease stage.

	Total	Stage I	Stage II	Stage IIIa
Number of patients	114	31	27	56
Male	92	29	20	43
Female	22	2	7	13
Age	60.3	61.0	60.6	59.5
Squamous cell carcinoma	79	21	13	45
Grade I and II	66	18	13	35
Grade III	13	3	0	10
Adenocarcinoma	35	10	14	11
Grade I and II	20	7	9	4
Grade III	15	3	4	8

Table 3. Comparison of nestin expression in adenocarcinomas versus squamous cell carcinomas.

	N	H-index			
		0	>0	0+≤10	>10
Adenocarcinoma	35	20 57.2%	15 42.8%	24 68.6%	11 31.4%
Squamous cell c.	79	54 68.4%	25 31.6%	63 79.8%	16 20.2%
Total	114	74 64.9%	40 35.1%	87 76.3%	27 23.7%
p-value		0.25		0.30	

both the cytoplasmic and the nuclear nestin epitopes but according to the authors, most of the nestin positivity was located in the nuclei. In our study, we also found a significantly higher expression of nestin in brain metastases of squamous cell carcinomas than the metastases of

adenocarcinomas. Unfortunately, we could not compare these results with nestin expression in relevant primary tumours. We described a higher occurrence of nestin positive cells surrounding tumour cell nests which was observed in brain metastases of squamous cell carcinomas but not in metastases of adenocarcinomas.

Table 4. Nestin expression and nestin-positive vascularisation in adenocarcinomas and squamous cell carcinomas as a whole in relation to stage of disease.

Number of cases	Total	Stage I	Stage II	Stage IIIa	<i>P</i>
Nestin-positive vascul.	9	1	1	7	0.02
Nestin-positive cases	31	6	8	17	0.04
Nestin H-index mean	68.98	69.5	97.3	53.2	0.30
Nestin H-index median	90	90	93	60	0.54

Compared stage I and II vs. stage IIIa

Table 5. Comparison of nestin expression in brain metastases of adenocarcinoma and squamous cell carcinoma.

H-index	N	Minimum	Maximum	Median	Mean	Std. deviation
Adenocarcinoma	24	0	110	80	63.33	39.75
Squamous cell carcinoma	11	50	250	100	125.45	68.17

Total number 35 $P \leq 0.003$

We hypothesise that these nestin-positive metastatic tumour cells may represent a pool of stem/progenitor populations of proliferating cells similar to those recently described in the basal/myoepithelial layer of the mammary gland and the basal epithelial breast cancer subtype^{12,38,39}.

Currently, nestin is considered a marker of activated endothelium⁴. Also in fetal tissues, nestin is strongly expressed in endothelial cells during the vasculogenesis. However, nestin expression is down-regulated in the vasculature of postnatal and adult animals where it can be restricted only to newly formed blood vessels⁴⁰. Significantly higher nestin positive vascularization in advanced NSCLC in stage IIIa than in stages I and II found in this study support our previous findings describing an increase in nestin-positive vascularization in less differentiated cancers¹⁵. Microvessel density (MVD) is also considered an independent prognostic marker for different invasive carcinomas^{21-23,25-31}. Thus a higher MVD of nestin positive blood vessels may be responsible for higher risk of recurrence and metastasis of non-small cell lung cancers.

In this study we found that nestin expression seems to be associated with poorer prognosis since it is significantly more expressed in tumours of higher stage as well as with greater nestin-positive microvessel density. These results are in agreement with previous reports concerning NSCLC although, to date, no description of a significant association of nestin-expression with stage of NSCLC has been published. On the other hand, no association with tumour grade was confirmed. This is not in agreement with either Chen or Ryuge findings^{34,37}. The fact that we found no significant association of nestin expression with disease-free and overall survival is in contrast with common assumptions. Perhaps, a higher degree of neoangiogenesis during the initial disease stages can be exploited to deliver a larger dose of chemotherapeutics to the tumour

during a certain period of its growth. Our finding of better disease-free survival during the first 18 months of disease supports this idea. Although the precise function of nestin remains to be elucidated, several studies suggest that it may play a role in the regulation of cell proliferation, survival and differentiation^{35,36}. From the data available, the role of nestin in the regulation of migration and invasion of NSCLC cells also cannot be excluded.

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