

EXPRESSION OF NEDD9 IN TRANSBRONCHIAL BIOPSIES OF LUNG ADENOCARCINOMA

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SUMMARY – Lung cancer as the major cause of cancer mortality worldwide includes several histologic subtypes evolving from numerous genetic and epigenetic changes emerging in alveolar, bronchiolar and bronchial epithelium. The majority of cases are diagnosed in advanced stage (i.e. stages IIIB and IV), often with scanty amount of tissue in transbronchial biopsies or cytology specimens. The aim of the present study was to investigate the expression of the scaffolding protein neural precursor cell expressed, developmentally downregulated 9 (NEDD9) in small biopsies of lung adenocarcinoma. The expression of NEDD9 was analyzed immunohistochemically in 71 formalin-fixed and paraffin-embedded transbronchial biopsy specimens of primary lung adenocarcinoma. Nuclear and cytoplasmic NEDD9 expression was detected indicating activation of the epithelial-mesenchymal transition process. Direct relationship between the expression of NEDD9 and survival was not demonstrated.

Key words: *Lung Neoplasms; Adenocarcinoma; Biopsy, Needle; Adaptor Proteins, Signal Transducing; Croatia*

Introduction

The declining incidence rates of lung cancer in women in the mid-2000s followed the lung cancer decreasing incidence rate in male population in the 1980s as a direct result of the anti-smoking campaigns in previous decades. However, lung cancer is still the leading cause of cancer mortality worldwide¹. In 2016, lung cancer preceded 14% and 13% of the most common newly diagnosed malignancies in men and women, respectively, and was the second most common malignancy following prostate cancer in men and breast cancer in women². Since cancer cells have to un-

dergo multiple genetic and epigenetic changes promoting multiplied proliferation potential in parallel with some degree of resistance to programmed cell death during cancer growth, identification of these changes has become an ultimate clinical goal in the past decade. Due to the continuous increase in the incidence of lung adenocarcinoma in the past decades, now it is the most common histologic type of lung cancer³.

Neural precursor cell expressed, developmentally downregulated 9 (NEDD9) triggers mitotic checkpoint, regulates cellular attachment and migration, and ciliary resorption, but also apoptosis and anoikis, i.e. programmed cell death due to the loss of attachments. NEDD9 is involved in the control of cellular differentiation, migration and chemotaxis, acting as a scaffolding protein in the complex process of epithelial-mes-

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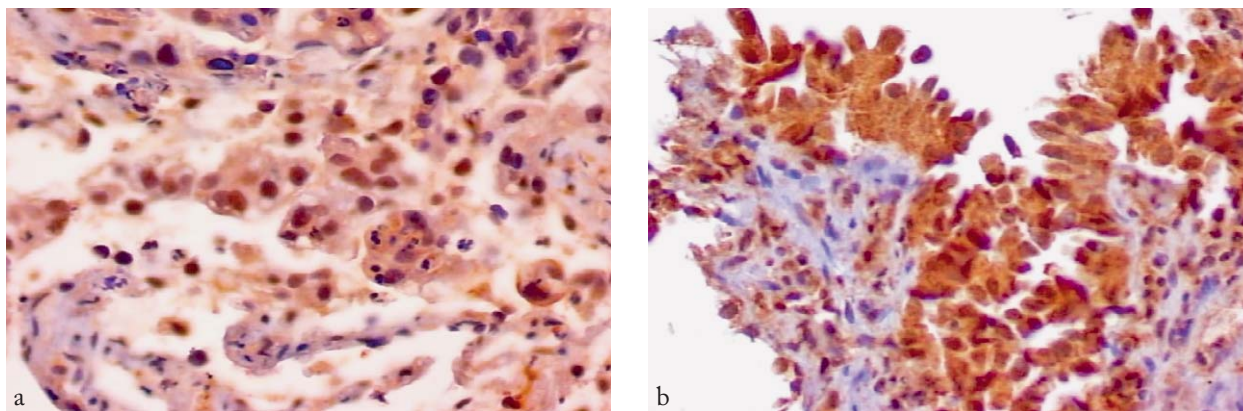


Fig. 1. (a) Strong nuclear expression of NEDD9 in lung adenocarcinoma (x400); (b) strong cytoplasmic expression of NEDD9 in lung adenocarcinoma (x400).

enchymal transition (EMT)⁴. NEDD9 has been identified as an integral part of the complex process of metastatic potential acquisition in mammary adenocarcinoma, glioblastoma and metastatic melanoma⁵⁻⁷. Moreover, some recent data suggested correlation between NEDD9 expression in resected specimens of lung adenocarcinoma and survival⁸.

However, lung carcinomas are often diagnosed in scanty amounts of tissue obtained by transbronchial biopsies or in cytology samples in up to 70% of all cases, even in the high-resource centers. Therefore, we aimed to investigate the expression of NEDD9 and its correlation with survival in small biopsy samples of lung adenocarcinoma.

Materials and methods

Materials

Out of 90 formalin-fixed paraffin-embedded routine diagnostic transbronchial biopsies from the archives of the Institute of Pathology, School of Medicine, University of Zagreb, 71 were eligible for the study conducted between January 2002 and December 2004. Identity protection was in strict accordance with the Declaration of Helsinki, 7th revision. Patient survival was defined as the time elapsed from the date of tissue collection to the end of the follow-up period or the date of death according to the National Cancer Registry. The samples were stained with hematoxylin and eosin and the diagnosis was confirmed by immunohistochemistry using TTF-1, CK7 and napsin A panel of antibodies for adenocarcinoma confirmation and

p63 and CK5/6 antibodies to exclude squamous cell carcinoma.

Methods

Immunohistochemistry was performed on 4 µm thick sections by standard protocols with primary antibodies including TTF-1 (Novocastra, Great Britain), CK7 (Dako, Denmark), napsin A (Novocastra, Great Britain), CK5/6 (Dako, Denmark) and p63 (Novocastra, Great Britain).

The NEDD9 monoclonal antibody (Abcam, San Francisco, CA, USA) was used in 1:400 dilution with EnVision kit for visualization. Normal bronchial epithelium present in tumor surroundings was used as negative control. The 'hot spot' was determined by inspection on whole specimen at x40 magnification by two investigators blinded for the clinicopathologic data. The immunohistochemical reaction was examined at x400 magnification.

The percentage of NEDD9 positive tumor cells was assessed semiquantitatively, as follows: 0-25% of positive cells scored 0 or negative; 26%-50% as 1 (weakly positive); 51%-75% as 2 (mildly positive); and more than 75% of positive tumor cells as 3 (strongly positive). Positivity was evaluated separately for nuclei and cytoplasm. The intensity of staining was also scored in the range of 0-3 (negative, weakly stained, mildly or strongly stained) (Fig. 1).

Statistical analysis

All data analyses were performed using IBM SPSS Statistics, version 24.0. Quantitative data distribution

was analyzed by the Kolmogorov-Smirnov test, correlations between categorical and nominal data were analyzed using the χ^2 -test, and correlations between immunoreactivity and survival by Spearman's coefficient of correlation. Survival curves were generated using the Kaplan-Meier method and the log-rank test was performed for survival data analysis. The level of statistical significance was set at $p < 0.05$.

Results

Seventy-one out of 90 selected transbronchial biopsy samples were eligible for analysis; 8 samples contained insufficient tumor tissue for further analysis, 7 were pulmonary metastases, immunohistochemically confirmed, and 4 were diagnosed as squamous cell carcinomas.

Fifty-five out of 71 lung adenocarcinoma cases were male (77.5%). Mean age of all patients was 74.3 years. The patients diagnosed with pulmonary adenocarcinoma were part of a larger cohort study and the diagnosis was confirmed by immunohistochemical staining for TTF-1, CK7, p63, CK5/6 and napsin A. Table 1 shows results of testing for different antibodies, and Table 2 shows different combinations of antibodies found positive in study subjects.

Assessment methods (percentage scale of staining intensity and semiquantitative scale) correlated significantly ($p < 0.001$) in both nuclei and cytoplasm, with Spearman correlation coefficient of 0.905 for nuclei and 0.786 for cytoplasm. Because of such high correlation coefficients, suggesting both scales to measure the same attribute, only the latter (semiquantitative) method will be discussed hereafter. Table 3 shows the number and percentage of specimens by the intensity of NEDD9 staining of both nucleus and cytoplasm.

Analysis of staining results for the nuclei and cytoplasm of our samples revealed an inverse pattern in staining intensity between them, i.e. only a small fraction of nuclei stained with intensity of 3 while a similar small fraction of cytoplasm stained with intensity of 0. To overcome this, further grouping of staining categories was performed, so that groups 0 and 1 were merged, as well as groups 2 and 3. Survival analysis was performed on such transformed groups of NEDD9 staining intensity and results are presented in Table 4 and Figure 2. Although the level of statistical significance was not reached, the results suggested stronger

Table 1. Number of specimens positive for each of antibodies tested

Antibody	Number (%) of positive specimens
TTF-1	38 (55.5)
CK7	63 (91.3)
p63	1 (1.4)
CK5/6	0 (0)
Napsin A	31 (43.7)

Table 2. Number of specimens positive for different antibody combinations

Antibody	Positive (+/-)								
TTF-1	-	+	+	-	-	+	-	+	+
CK7	+	+	+	+	-	-	-	-	+
p63	-	-	-	-	-	-	-	-	+
CK5/6	-	-	-	-	-	-	-	-	-
Napsin A	-	+	-	+	-	+	+	-	-
Total	25	24	10	3	3	2	2	1	1

Table 3. Staining intensity for NEDD9 in nuclei and cytoplasm of adenocarcinoma specimens

Staining intensity	Number (%) of specimens	
	Nuclei	Cytoplasm
0	30 (42.3)	7 (9.9)
1	10 (14.1)	23 (32.4)
2	27 (38.0)	23 (32.4)
3	4 (5.6)	18 (25.4)

NEDD9 staining intensity to correlate with longer survival. This was true for both nuclei and cytoplasm staining intensity, with the effect being significantly more pronounced in cytoplasm.

Discussion

The last decade was characterized by a growing scientific interest for the members of the Crk-associated substrate (CAS) adaptor protein family, namely p130Cas/BCAR1 and NEDD9/HEF1/Cas-L. Although the catalytic function has not yet been demonstrated for any member of the CAS family, it is clear that these proteins serve as hubs for many vital cellular processes owing to multiple protein interaction motifs on their surfaces. NEDD9 has numerous roles in

Table 4. Mean survival time in months (with 95% confidence intervals) based on NEDD9 staining intensity

Staining intensity	Survival (months)	
	Nuclei	Cytoplasm
0 and 1	26.7 (14.3-39.1)	21.0 (8.6-33.4)
2 and 3	35.5 (17.4-53.6)	38.1 (22.2-54.1)
p	0.687	0.199

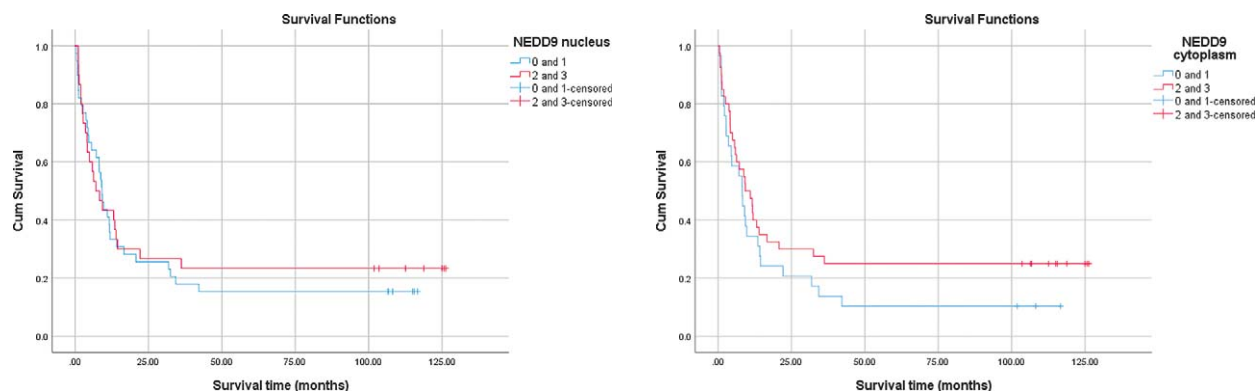


Fig. 2. Survival time in months based on intensity of NEDD9 staining of nuclei (left) and cytoplasm (right) of adenocarcinoma specimens.

modulating and controlling normal cell cycle, cellular attachment, migration and chemotaxis, and therefore has been studied mostly in the context of epithelial-mesenchymal transition, i.e. cancer growth, invasion and metastasis formation. To date, overexpression of NEDD9 has been demonstrated in various malignancies. Recently, its positive correlation with lymph node metastasis in non-small cell lung carcinoma (NSCLC) has been described in a series of 105 resected tumors⁸. However, since the vast majority of lung cancers are diagnosed by use of small biopsy and cytology specimens, we aimed to analyze NEDD9 expression and its correlation with survival in small biopsy samples of lung adenocarcinoma diagnosed according to the latest World Health Organization classification of lung tumors⁹. To our knowledge, our study is the first one analyzing NEDD9 expression in transbronchial biopsies of lung adenocarcinoma.

In our series of transbronchial biopsies, the diagnosis of adenocarcinoma was confirmed by TTF-1, CK7 and/or napsin A positive reaction. Of note, napsin A stained positive in 8 (11.3%) cases of TTF-1-negative tumors, two of which were double-negative for TTF-1/CK7. This finding has been reported in recent litera-

ture with napsin A described as a particularly useful tool in identifying double-negative (TTF-1/CK7) tumors as adenocarcinoma, since it is persistently negative in squamous cell carcinoma^{10,11}.

The NEDD9 has been shown to concentrate at focal adhesions in response to binding of various intrinsic and extrinsic partner proteins to the SH3-domain and highly conserved carboxy-terminal domain that

allows homo- and heterodimerization of the NEDD9 molecule and subsequent interaction with downstream molecules mediating cellular migration. Also, NEDD9 concentrates at the mitotic spindle and interacts with the centrosome and proteasomal machinery with both nuclear and cytoplasmic pools, where its expression and activities are closely regulated by rapid turnover¹². In our study, significant levels of expression were found in both nuclear (41/71, 57.7%) and cytoplasmic departments (64/71, 90.1%) of adenocarcinoma cells. This finding is in concordance with the results of an earlier study in which Chang *et al.* investigated NEDD9 localization and expression in 60 cases of surgically treated lung adenocarcinoma¹³. It has been postulated that NEDD9 serves as a sort of switch driving cell migration in the early stages of neoplastic transformation and contributes to the invasive properties¹⁴. McLaughlin *et al.* found that increased expression of NEDD9 correlated with the transformation from breast carcinoma *in situ* to invasive tumor¹⁵. On the other hand, NEDD9 expression correlated inversely with recurrence-free survival, but also in patients treated with curative surgery, i.e. earlier stages of the disease¹⁶. Furthermore, NEDD9 expression was

correlated with smoking history, TNM stage and lymph node metastasis (but with no apparent correlations with other clinical features), indicating the role of NEDD9 in the disease progression¹⁷. Recently, Miao *et al.* demonstrated inverse correlation between NEDD9 expression and overall survival in patients with resectable NSCLC (but interestingly, not with the TNM stage)⁸. In our study, the investigated relationship between NEDD9 expression and survival did not reach statistically significant difference. Quite surprisingly, there was a trend towards correlation of stronger NEDD9 staining intensity to longer survival. This was true for both nuclear and cytoplasmic expression, with the effect being significantly more pronounced in cytoplasm. Considering numerous functions of NEDD9 in complex processes of cellular differentiation and migration, it is expected that the overexpression in lung carcinomas is a marker of a higher malignant potential. On the other hand, it has been demonstrated that expression of NEDD9 over certain level inevitably leads to apoptosis¹⁸. Furthermore, Minn *et al.* independently demonstrated down-regulation of NEDD9 as part of metastatic signature in mammary tumors¹⁹. It is plausible to conclude that the levels of NEDD9 must be precisely and timely regulated, and the overexpression can be tolerated only in tumor cells with extensive prior lesions that enable escaping cellular death. It is also possible that elevated expression of NEDD9 may indicate better responses to therapy¹⁶. Pallarès *et al.* have described unexpected discovery of NEDD9 (but not BCAR1) expression as an independent prognostic factor of favorable prognosis in intermediate-risk acute myeloid leukemia, which is in contrast with the previously reported higher aggressiveness in BCR-ABL-dependent acute lymphoblastic leukemia²⁰.

We demonstrated both nuclear and cytoplasmic expression of NEDD9 in transbronchial biopsies of lung adenocarcinoma indicating activation of the epithelial-mesenchymal transition process. However, we were unable to demonstrate relationship between the expression of NEDD9 and survival.

Our study had several limitations; data were obtained on a rather small population, with the possibility of selection bias, and it was performed on small biopsies. It would be of interest to extend our study to the resected tumors and correlate these results with other clinical parameters (such as TNM stage, tumor

size, and response to therapy) in a larger group. Taking into account the complex functions of NEDD9 and precise regulation of numerous different interconnected signaling networks, more future studies are warranted before targeting NEDD9 as one of the possible routes in precision oncology.

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Sažetak

EKSPRESIJA NEDD9 U TRANSBRONHALNIM BIOPTATIMA ADENOKARCINOMA PLUĆA

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Karcinom pluća objedinjuje zloćudne tumore nastale brojnim genetskim i epigenetskim promjenama epitelnih stanica bronha, bronhiola i alveola, i predstavlja najčešći uzrok smrti među malignim bolestima. U većini slučajeva dijagnoza se postavlja u uznapredovalim stadijima bolesti (III B. i IV.) iz uzoraka transbronhalnih biopsija ili citoloških materijala. U ovom istraživanju ispitana je ekspresija regulatorne bjelančevine nazvane *neural precursor cell expressed, developmentally downregulated 9* (NEDD9) u 71 uzorku malih biopsija adenokarcinoma pluća. Utvrđena je izražena ekspresija NEDD9 u jezgri i citoplazmi stanica adenokarcinoma, koja ukazuje na aktivaciju epitelnno-mezenhimne tranzicije. Pritom nije dokazana nedvojbeno povezanost ekspresije NEDD9 s preživljenjem.

Ključne riječi: *Plućni tumori; Adenokarcinom; Biopsija, punkcijsko-aspiracijska; Adaptorni proteini, prijenosnici signala; Hrvatska*