Neuroepithelial transforming gene 1 functions as a potential prognostic marker for patients with non-small cell lung cancer

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Abstract. Non-small cell lung cancer (NSCLC) is the most common histological cancer sub-type worldwide. Neuroepithelial transforming gene 1 (Net-1), a Ras homolog family member A-specific guanine nucleotide exchange factor, has been shown to be upregulated in several human cancer types. However, the clinical significance of Net-1 expression in NSCLC has remained elusive. The present study assessed Net-1 mRNA and protein levels by reverse-transcription quantitative polymerase chain reaction and western blot analysis of 64 cases of NSCLC as well as their adjacent normal tissues. Furthermore, Net-1 protein expression in tumor tissues derived from clinically annotated NSCLC cases at stages I-III was detected by immunohistochemical staining. The results showed that Net-1 mRNA and protein levels in NSCLC tissues were significantly elevated compared with those in their corresponding non-tumor tissues. In addition, Net-1 expression was strongly associated with the patients' pathological characteristics, including clinical stage, lymph node metastasis, distant metastasis and differentiation degree ($P<0.05$). In conclusion, the results of the present study suggested that Net-1 expression has a significant role in the tumorigenesis of distinct histotypes and sub-types of NSCLC, and may therefore be utilized as a biomarker as well as an important therapeutic target in NSCLC.

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Abbreviations: Net-1, neuroepithelial transforming gene 1; NSCLC, non-small cell lung cancer

Key words: neuroepithelial transforming gene 1, non-small cell lung cancer, reverse transcription quantitative polymerase chain reaction, western blot, immunohistochemical staining

Introduction

Lung cancer is the major cause of cancer-associated mortality worldwide (1). Non-small cell lung cancer (NSCLC), which accounts for ~85-90% of patients with lung cancer, comprises three sub-types according to their histological characteristics: Adenocarcinoma, squamous cell carcinoma and large cell carcinoma (2,3). Despite recent advances in treatments of NSCLC, they have only yielded modest improvements in NSCLC patient outcomes, with the overall five-year survival rate remaining at 15% (4). Therefore, it is required to discover novel prognostic biomarkers as well as therapeutic targets for NSCLC. Neuroepithelial transforming gene 1 (Net-1) is a 54-kDa oncoprotein (5), which has also been recognized as a Ras homolog family member A (RhoA) guanine nucleotide exchange factor (GEF) (6), and which was initially identified in a neuroepithelioma cell line (5). Net-1 is a member of seven transmembrane four superfamily with two distinct isoforms (Net-1 and Net-1A) and has a crucial role in cell signal transduction, proliferation, migration and invasion; it is also indicative of a poor prognosis of cancer patients (7-10). Overexpression of Net-1 has been documented in a variety of human cancer types, including hepatocellular carcinoma, breast cancer, oesophageal adenocarcinoma, skin squamous cell carcinoma and gastric adenocarcinoma (8,11-14). Silencing of Net-1 by small interfering RNAs (siRNAs) was shown to inhibit cancer cell motility, proliferation and extracellular matrix invasion (8-10). A growing number of studies have suggested that there may be cross-talk between the Net-1 and transforming growth factor β signaling pathways in actin cytoskeletal re-organization (15-17). However, to the best of our knowledge, a comprehensive profiling of Net-1 expression and function in advanced NSCLC has not been performed.

The present study assessed the expression of Net-1 in NSCLC as well as normal adjacent lung tissues and performed correlation analyses with various clinicopathological characteristics in order to explore the p utilization of Net-1 as a potential target for the development of therapeutic agents as well as a novel tissue biomarker for human lung cancer.

Materials and methods

Clinical tissue samples. A total of 64 patients (16 women, 48 men) with NSCLC, who underwent radical surgical resection

at the Department of Thoracic and Cardiovascular Surgery (The Third Xiangya Hospital, Central South University, Changsha, China) from April 2009 to October 2009, were enrolled in the present study. According to the criteria of the World Health Organization (2004) (18), the 64 cases were divided into 29 squamous cell carcinomas, 32 adenocarcinomas and 3 large-cell carcinoma. All tumors were staged on the basis of the tumor-nodes-metastasis (TNM) pathologic classification of the International Association for the Study of Lung Cancer (19). Primary tumor tissues and their corresponding adjacent normal tissues (5 cm from the margin of the tumor) obtained from each surgical specimen were used for reverse-transcription quantitative polymerase chain reaction (RT-qPCR), western blot and immunohistochemical analyses. The protocol of the present study was approved by the Local Ethics committee of the Third Xiangya Hospital, Central South University. All patients provided written, informed consent, with separate written, informed consent obtained for the optional provision of tumour material for biomarker analyses, and from one patient for use of lung tissues.

RNA extraction and RT-qPCR. RT-qPCR was performed as described previously (20). Total RNA was extracted from tissue specimens using TRIzol reagent (Invitrogen Life Technologies, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. RNA yield and purity were assayed using a SmartSpec Plus Spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA), comparing the A230/260 and A260/A280 ratios. First-strand cDNA synthesis (cDNA synthesis kit; Fermentas, Burlington, ON, Canada) was performed using random hexamers on 1 μg of total RNA. The amplification was performed in a total volume of 20 μl containing SYBR Premix Ex Taq (Tli RNaseH Plus; cat no. DRR820S; Clontech Laboratories, Inc., Mountainview, CA, USA). The qPCR protocol was as follows: Initial denaturation, 95˚C for 1 min; primer-extension was performed in 40 cycles, each cycle consisted of 95˚C for 30 sec, 60˚C for 20 sec, 72˚C for 20 sec and final extension at 72 ˚C for 10 min. The qPCR results were quantified using a double standard curve. qPCR was performed using the following primer sets: Net-1 forward, 5'-CTCTCCAGCCCAGTCTCACA-3' and reverse, 5'-CCC TCACACTCTTCGTGCAG-3') and β-actin forward, 5'-GTC CACCTTCCAGCAGATGT-3' and reverse, 5'-CTGTCACCT TCACCGTTCCA-3' (Sangon Biotech Co,. Ltd., Shanghai, China). Amplifications was performed in 40 cycles. Each cycle consisted of 30 sec at 95˚C, 5 sec at 95˚C and 20 sec at 60˚C. mRNA levels were determined using the comparative CT method. The expression levels of each gene were normalized to those of β-actin, which served as an endogenous internal control.

Protein isolation and western blot analysis. Tumor tissues were extracted using radioimmunoprecipitation assay (RIPA) lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA). The protein concentration in all samples was determined using a Bicinchoninic Acid Protein Assay kit (cat no. 23227; Thermo Fisher Scientific). Protein lysates were subjected to 10% SDS-PAGE followed by transfer onto a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). After blocking with 5% non-fat dry milk in Tris-buffered saline containing Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) for 60 min, the membrane was incubated with the primary antibody overnight at 4˚C. The following primary antibodies were used: Rabbit anti-human NET-1 (cat no. 2500682; 1:1,000; Sigma-Aldrich) and β-actin (cat no. sc47778; 1:2,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Following three subsequent washing steps, the membranes were incubated with peroxidase-conjugated polyclonal rabbit anti-human secondary antibody (cat no. P0212; 1:2,000; DakoCytomation, Glotstrup, Denmark) at room temperature for 2 h. The blots were detected using an enhanced chemiluminescence detection kit (Bio-Rad Laboratories, Inc.) according to the manufacturer's instructions. The immunoreactive bands were scanned using an LAS-4000 imaging system (Fuji, Tokyo, Japan) to detect protein expression levels. The results were analyzed using Image Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA) to determine the relative band density ratio.

Immunohistochemical staining. Immunohistochemistry (IHC) was performed on formalin-fixed paraffin-embedded (FFPE) histological sections. FFPE specimens were sectioned at 4 μm onto charged slides and heated for 30 min at 60˚C. Slides were de-paraffinized in three sequential baths of xylene for 3 min each, re-hydrated using a graded series of ethanol for 5 min and then washed for 5 min in distilled water. Following de-paraffinization, antigen retrieval was performed using citrate buffer (0.1 mol/l, pH 6.0) in a pressure cooker for 90 min. Slides were then cooled in water and washed in phosphate-buffered saline (PBS). Intrinsic peroxidase activity was blocked using 3% H_2O_2 (Beyotime Institute of Biotechnology, Haimen, China) in methanol at room temperature for 15 min. The slides were then rinsed three times each with PBS. To block non-specific antibody binding, the sections were incubated with 3% fetal bovine serum for 60 min at 37˚C in a humidified chamber. The slides were washed with PBS and then incubated with rabbit anti-human NET-1 polyclonal antibody (cat no. SAB2500682; 1:1,000; Sigma-Aldrich) overnight at 4˚C. Following incubation, sections were washed with PBS and subsequently incubated with goat anti-rabbit monoclonal secondary antibody (cat. no. M080825; 1:100; Zhong Shan Golden Bridge Biotechnology Co., Ltd) at 37˚C for 30 min. Finally, specimens were stained with 3,3'-diaminobenzidine (Shanghai Mingrui Biological Technology Co., Ltd., Shanghai, China) and counterstained with hematoxylin (Beyotime Institute of Biotechnology) at 37˚C for 30 min. All incubations were performed at room temperature. An experienced pathologist evaluated and scored the immunohistochemical expression using a light microscope (magnification, x200). Net-1 expression was quantified using customary scoring for intensity and the percentage reactivity. Intensity of staining was graded as follows: 0, without stain; 1, straw yellow; 2, brown; 3, dark brown. The extent of staining was evaluated as follows: 0, ≤5% positive cells; 1, 6-25% positive cells; 2, 26-50% positive cells; 3, 51-75% positive cells; and 4, >75% positive cells. The final immunoreactivity score was obtained by multiplying the intensity and reactivity scores: IRS ≤2 was defined as negative, 2-5 point was defined as weak positive (+); 5-9 points was defined as moderate positive (++) and >9 points was defined as strong positive (+++) (21).

Table I. Association between Net-1 protein expression in tumors and various clinicopathological factors of 64 patients with non-small cell lung cancer.

Characteristic	Cases (n)	Net-1 protein expression	P-value
Gender			0.184
Male	15	2.86±1.54	
Female	5	1.87±0.55	
Age, years			0.322
>60	8	3.01±1.90	
≤60	12	2.35±0.98	
Smoking history			0.184
Smoker	15	2.86±1.54	
Non-smoker	5	1.87±0.55	
Tumor size			0.790
>3 cm	15	2.56±1.42	
≤3 cm	5	2.76±1.56	
Histological type			0.351
Scc	15	2.44±1.16	
Ac	5	3.14±2.09	
Tumor differentiation			0.031
Moderate	15	2.44±1.11	
Poor	5	3.29±2.40	
TNM stage			0.042
I	5	2.10±1.31	
II	13	2.62±1.52	
III	2	2.80±1.41	
Lymph node metastasis			0.011
Present	8	2.73±1.11	
Absent	12	2.44±1.86	

Scc, squamous carcinoma; Ac, adenocarcinoma; Net-1, neuroepithelial transforming gene 1; TNM, tumor-nodes-metastasis.

Statistical analysis. Values are expressed as the mean ± standard error of the mean. Differences among the groups were determined by two-way analysis of variance followed by Tukey's post-hoc test. Comparisons between the two independent groups were performed using an unpaired Student's *t*-test. All analyses were performed using SPSS 17.0 for Windows (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Net-1 is overexpressed in NSCLC. To investigate Net-1 expression in NSCLC, the present study quantitatively examined the mRNA expression levels of Net-1 in primary NSCLC tissues and their corresponding adjacent non-cancerous tissues. As shown in Fig. 1, RT-qPCR analysis revealed that NSCLC tissues exhibited higher levels of Net-1 expression compared to those in the corresponding adjacent non-cancerous tissues ($P<0.05$). In order to assess whether the differences in Net-1 expression between tumor and non-neoplastic samples are also present at the protein level, the present study subsequently examined the expression of Net-1 protein in lung cancers as well as in their

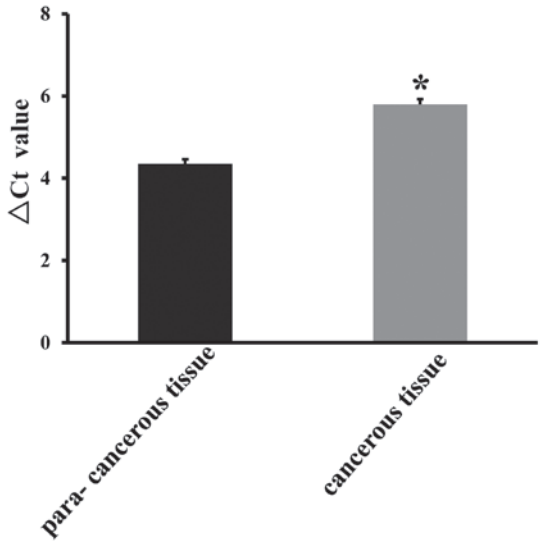

Figure 1. Comparison of Net-1 mRNA expression levels in cancerous and para-carcinoma tissues. Net-1 expression in non-small cell lung cancer and normal lung tissues of 64 patients were determined using reverse-transcription quantitative polymerase chain reaction analysis. Values are expressed as the mean ± standard error of the mean. *P<0.05, vs. para-carcinoma tissues. Ct, cycle threshold; Net-1, neuroepithelial transforming gene 1.

Table II. Correlation between Net-1 and clinicopathological characteristics of 64 patients with non-small cell lung cancer.

Characteristic	Cases (n)	Patients with Net-1 expression score, n (%)				P-value
		-	+	++	+++	
Gender						0.141
Male	15	0 (0.0%)	7 (46.7%)	3 (20.0%)	5 (33.3%)	
Female	5	2 (40.0%)	1 (20.0%)	1 (20.0%)	1 (20.0%)	
Age, years						0.920
>60	8	1 (12.5%)	3 (37.5%)	1 (12.5%)	3 (37.5%)	
≤60	12	1 (8.3%)	5 (41.7%)	3 (25.0%)	3 (25.0%)	
Smoking history						0.141
Smoker	15	0 (0.0%)	7 (46.7%)	3 (20.0%)	5 (33.3%)	
Non-smoker	5	2 (40.0%)	1 (20.0%)	1 (20.0%)	1 (20.0%)	
Tumor size						0.892
>3 cm	15	2 (13.3%)	5 (33.3%)	3 (20.0%)	5 (33.3%)	
≤3 cm	5	0 (0.0%)	3 (60.0%)	1 (20.0%)	1 (20.0%)	
Histological type						0.237
Scc	15	0 (0.0%)	8 (53.3%)	2 (13.3%)	5 (33.3%)	
Ac	5	2 (40.0%)	0 (0.0%)	2 (40.0%)	1 (20.0%)	
Tumor differentiation						0.021
Moderate	15	2 (12.5%)	8 (50.0%)	3 (18.8%)	3 (18.8%)	
Poor	5	0 (0.0%)	0 (0.0%)	1 (25.0%)	3 (75.0%)	
TNM stage						0.023
I	5	0 (0.0%)	4 (80.0%)	1 (20.0%)	0 (0.0%)	
II	13	2 (15.4%)	4 (30.8%)	2 (15.4%)	5 (38.5%)	
III	2	0 (0.0%)	0 (0.0%)	1 (50.0%)	1 (50.0%)	
Lymph node metastasis						0.005
Present	8	1 (12.5%)	0 (0.0%)	2 (25.0%)	5 (62.5%)	
Absent	12	1 (8.3%)	8 (66.7%)	2 (16.7%)	1 (8.3%)	

Scc, squamous carcinoma; Ac, adenocarcinoma; Net-1, neuroepithelial transforming gene 1; TNM, tumor-nodes-metastasis.

matched normal adjacent tissues using western blot analysis. The expression levels of Net-1 protein in NSCLC tissues were significantly higher than those in non-cancerous tissues, which was in accordance with the mRNA expression levels of Net-1 detected by real-time RT-PCR. Representative blots are shown in Fig. 2A and B. The finding that Net-1 is overexpressed in NSCLC specimens compared with non-cancerous tissues suggests the significance of Net-1 as a potential prognostic biomarker.

Net-1 overexpression is associated with poor clinical outcome of NSCLC. To further elucidate the biological and clinicopathological significance of Net-1 in NSCLC, 64 paraffin-embedded NSCLC tissue specimens were examined by IHC staining. The association of Net-1 expression and clinicopathological parameters is illustrated in Table I. As shown in Fig. 3A-D, positive immunostaining for Net-1 was mostly observed in the tumor cells of NSCLC tissues. In addition, Net-1 expression was significantly upregulated in primary NSCLC tissues compared to that in their corresponding adjacent non-cancerous tissues. Semi-quantitative IHC analysis indicated that Net-1 staining in primary NSCLC was higher than that in adjacent non-cancerous tissues. The Net-1 staining intensity gradually increased in accordance with the clinical stages from stages I-III ($P<0.005$) (Fig. 3D). Furthermore, statistical analysis showed that high Net-1 expression was strongly associated with the differentiation degree ($P=0.021$), clinical stage ($P=0.023$), lymph node metastasis ($P=0.005$) and distant metastasis ($P<0.005$) of patients with NSCLC, and this association is further summarized in Table II. However, no statistically significant association between high Net-1 expression and other clinicopathological variables, including gender, age, smoking history, tumor size or histological type, was identified. These results supported the hypothesis that Net-1 is involved in the regulation of the invasive ability of NSCLC.

Discussion

The present study reported for the first time, to the best of our knowledge, that Net-1 mRNA expression was upregulated in NSCLC tissues compared to that in their corresponding adja-

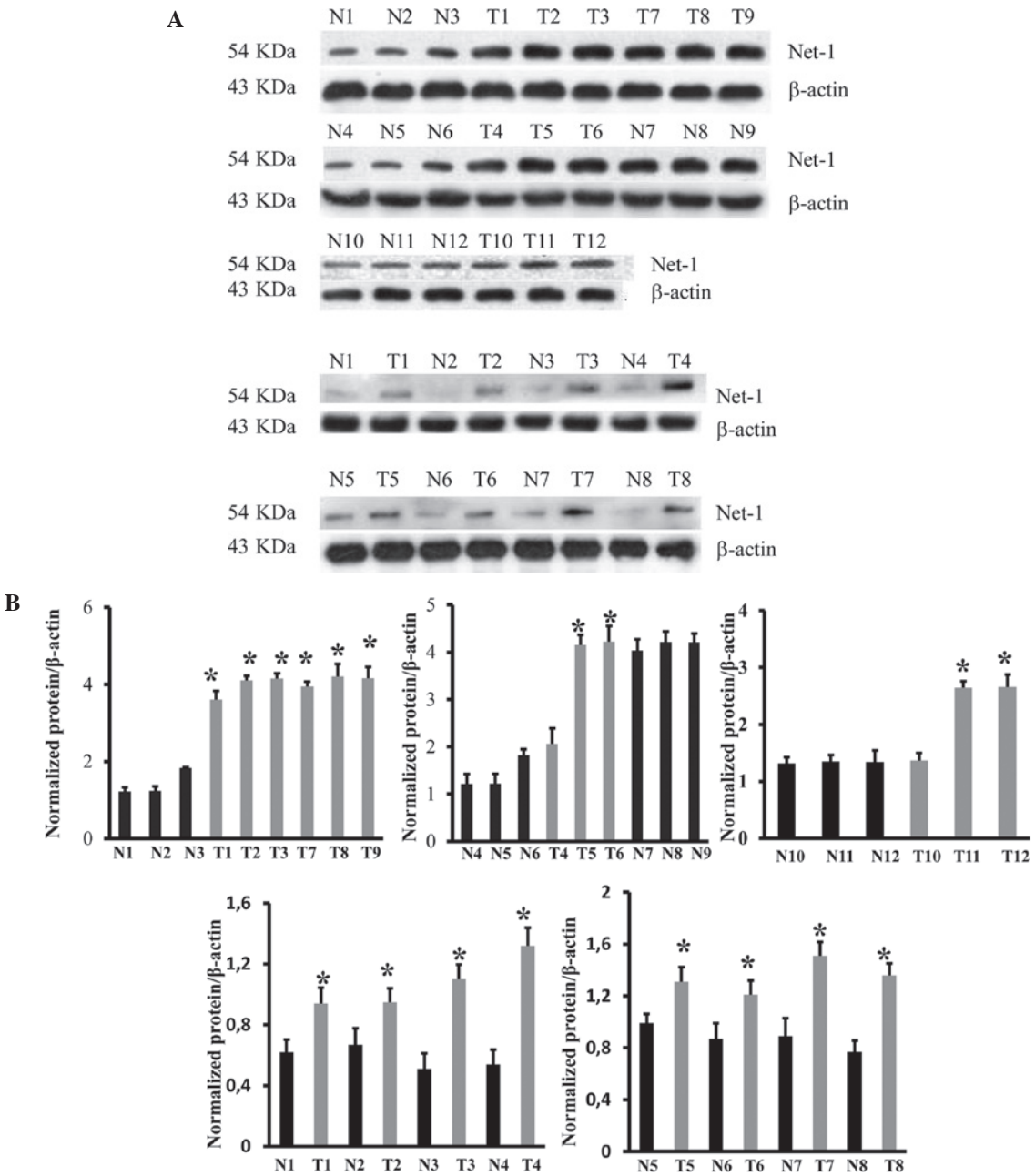

Figure 2. (A) Western blot analysis indicated that Net-1 is overexpressed in NSCLC tissues compared to that in the corresponding adjacent non-cancerous tissues from the same patient. (B) Quantitative results of western blot analysis. *P<0.05, vs. N group. N, normal lung tissue, T, NSCLC specimens; NSCLC, non-small cell lung cancer; Net-1, neuroepithelial transforming gene 1.

cent non-cancerous tissues. High-level Net-1 protein expression was also observed in 12 NSCLC patients, suggesting that elevated expression of Net-1 may contribute to the development of NSCLC. Clinical staging of cancer is based on the TNM classification, which is used to determine the clinical outcome and prognosis (22). By analyzing the correlation between clinicopathological variables of patients and Net-1 protein expression, the present study found that high Net-1 expression in NSCLC was significantly correlated with the differentiation degree, clinical stage, lymph node metastasis and distant metastasis. Multivariate analysis showed that Net-1 may be used as an independent prognostic biomarker for patients with NSCLC.

Previous studies supports the notion that Net-1 is frequently overexpressed in various cancer types, including hepatocellular carcinoma, gastric cancer, gliomas, cervical carcinomas and breast cancer (10,23-26). Using IHC staining, Lahiff *et al* (14) found that Net-1 expression is markedly increased in invasive and metastatic adenocarcinoma of the oesophagogastric junction. Murray *et al* (9) demonstrated that the migration and invasion of AGS gastric cancer cells are inhibited by Net-1-specific small interfering RNA. In addition, the study found that knockdown of Net-1 resulted in the formation of round cells and a loss of definition in the actin cytoskeleton (9). Zhang *et al* (13) further elucidated the role of NET-1 as a vital regulator of invasion and metastasis in the tumor, and reported that overexpression of Net-1 increases the likelihood of aggressive features in patients with squamous cell carcinoma of the skin As a GEF specific for RhoA, Net-1 is able to mediate

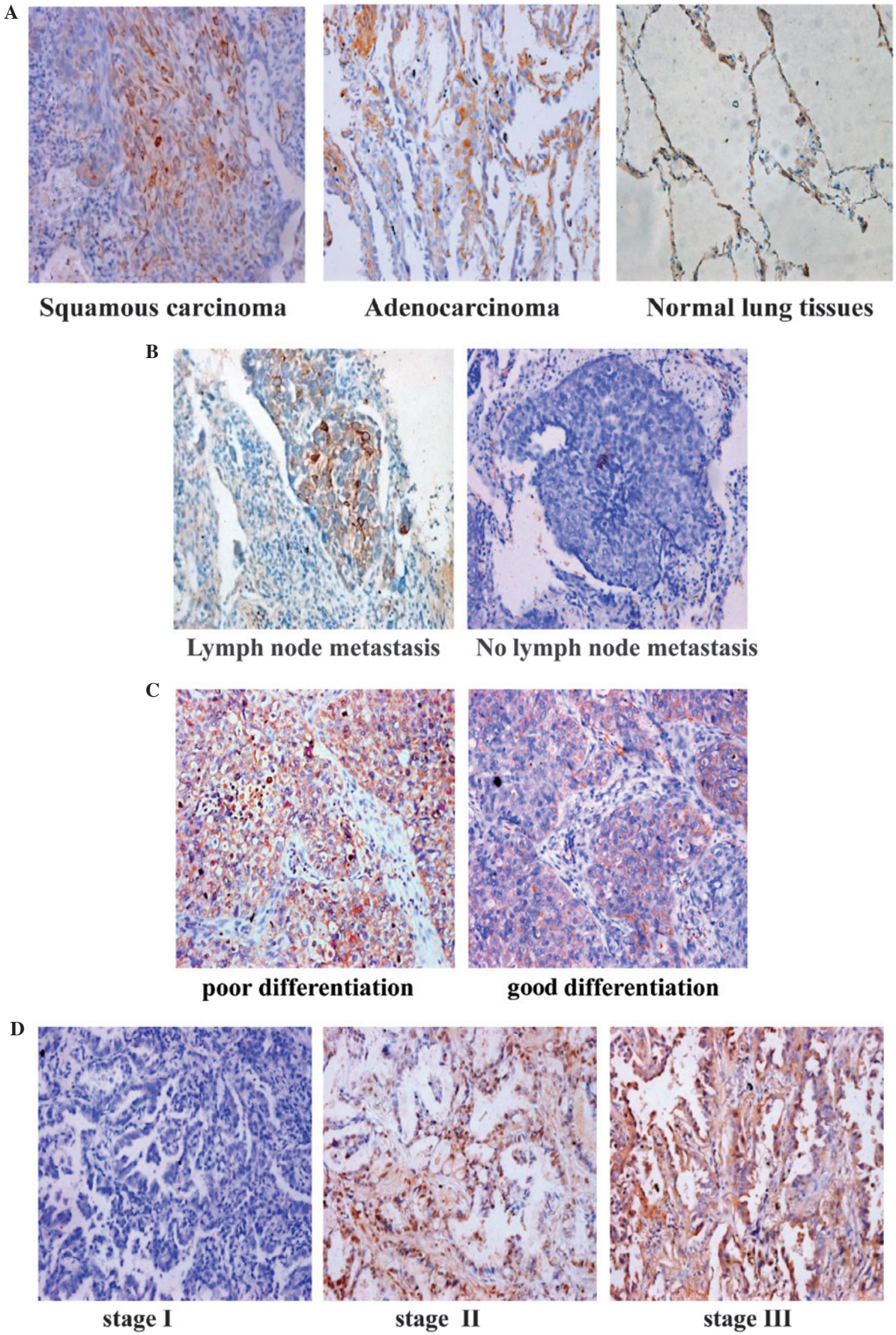

Figure 3. Expression of Net-1 in NSCLC tissues. (A) Representative immunohistochemical images showing high Net-1 expression in NSCLC samples. (B) Expression of Net-1 is strongly associated with lymph node metastasis in lung cancer tissue. (C) Expression of Net-1 is associated with the degree of differentiation of NSCLC. (D) Expression of Net-1 in various clinical stages of NSCLC. Proteins expression of Net-1 exhibited positive staining in tumour tissues and negative staining in normal lung tissues (magnification, x200). NSCLC, non-small cell lung cancer; Net-1, neuroepithelial transforming gene 1.

RhoA activation. Activated Rho proteins are able to stimulate signaling in multiple pathways by binding to target proteins, modulating their activities and thereby regulating a range of biological processes, including cell proliferation, apoptosis, differentiation and cytoskeletal reorganization, signal transduction (6). Recently, Zhang *et al* (13) further elucidated the role of NET-1 as a vital regulator of invasion and metastasis in the tumor and reported that overexpression of Net-1 greatly increases the likelihood of the aggressive feature in human skin squamous cell carcinoma patients. These studies suggested that Net-1 has vital roles in the metastasis of malignant tumors. However, the association between Net-1 and NSCLC has remained elusive. In order to investigate the involvement of Net-1 in NSCLC, the present study detected its mRNA and protein expression in 64 paired NSCLC tissues by RT-qPCR and western blot analysis. Furthermore, Net-1 protein expression in NSCLC tumor specimens was assessed by IHC and subjected to a correlation analysis with regard to clinicopathological features. The results showed that Net-1 expression was significantly elevated in tumor tissues compared to that in their corresponding non-tumor tissues. In addition, the results of the present study indicated that upregulation of Net-1 may be an important event in the development and progression of NSCLC. The levels of Net-1 expression were strongly correlated with the differentiation degree, clinical stage and lymph node metastasis in the NSCLC patients assessed in the present study. Based on these results, Net-1 is likely to be a tumor-promoting molecule and may contribute to the pathogenesis and progression of NSCLC. The results of the present study indicated that Net-1 expression is an independent prognostic factor for the outcome of patients with advanced NSCLC. The findings of the present study not only suggested that Net-1 may be a promising prognostic biomarker, but also implied a possible link between the biological function of Net-1 and the pathogenesis of NSCLC. Net-1 may therefore be an important therapeutic target for NSCLC treatment. However, further study is required in order to elucidate the molecular mechanism of the involvement of Net-1 in the progression of NSCLC.

The present study had certain limitations. The comprehensive mechanisms of Net-1 in NSCLC cells still remains to be investigated. Furthermore, patient data regarding cancer recurrence and mortality were not available for the cohort of the present study. Furthermore, the present study was performed in a retrospective manner on a relatively small number of cases. Thus, the findings of the present study require confirmation by future studies using a larger patient cohort and appropriate design.

In conclusion, to the best of our knowledge, the present study was the first to demonstrate that Net-1 is overexpressed in NSCLC and correlated with the differentiation degree, clinical stage and lymph node metastasis as well as unfavorable prognosis of NSCLC patients. The results of the present study provided novel insight into the function of Net-1 in the development and progression of NSCLC and reported a potential therapeutic target for this malignancy.

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