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RESEARCH

NOB1 in Non-small-cell Lung Cancer: Expression Profile and Clinical Significance

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Abstract Nin one binding (NOB1) gene has been reported up-regulated in several types of cancer. The aim of this study was to investigate the expression profile of NOB1 in non-small-cell lung cancer (NSCLC) and assess the clinical significance. qRT-PCR was used in the detection of NOB1 mRNA expression both in NSCLC tissue and in adjacent normal lung tissue. Western blot analysis and immunohistochemistry were used in the detection of NOB1 protein expression. The clinicopathological implications of NOB1 were analyzed statistically. It was confirmed by RT-qPCR that expression of NOB1 mRNA in NSCLC cells was higher than in human lung cells ($P<0.05$), and NOB1 mRNA was also over-expressed in NSCLC tissue when compared with adjacent tissue and normal lung tissue ($P<0.05$). Western blot analysis showed that NOB1 protein was significant increased in NSCLC cell lines compared with human lung cell line. Western blot analysis and immunohistochemistry showed that NOB1 protein was significant increased in NSCLC tissue compared with adjacent tissue and normal lung tissue ($P<0.05$). There were significant associations between NOB1 expression and TNM stage, lymph node metastasis, and histopathological grade ($P<0.05$), but not gender, age, smoke, or tumor diameter ($P>0.05$). Our results suggest that enhanced expression of NOB1 gene plays an important role in the occurrence and development of NSCLC. NOB1 may be a potential therapeutic target in NSCLC.

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Introduction

Lung cancer was the most commonly diagnosed cancer as well as the leading cause of cancer death in males in 2008. Among females, it was the fourth most commonly diagnosed cancer and the second leading cause of cancer death. Lung cancer accounts for 13 % (1.6 million) of the total cases and 18 % (1.4 million) of the deaths in 2008 globally [1]. In the United States, lung cancer was the second most commonly diagnosed cancer, accounting for nearly 228,190 estimated new cases, and the first leading cause of cancer death, accounting for nearly 159,480 estimated deaths annually [2]. It is important to understand the molecular mechanisms responsible for the development, progression and metastasis of Lung cancer, and to develop novel strategies for its early detection, prevention and treatment.

Nin one binding (NOB1) protein is a subunit of the 26S proteasome and plays a crucial role in protease function and RNA metabolism [3]. Recent studies have been increasingly recognized that abnormal expression of NOB1 plays a significant role in tumorigene. Lin S et al. reported NOB1 was higher expressed in papillary thyroid carcinomas, compared with normal thyroid and benign thyroid tumour tissue [4]. In human ovarian cancer cells research, Lin Y et al. reported the RNA interference-mediated the downregulation of NOB1 expression markedly reduced the proliferative and colony-formation ability of ovarian cancer cells, and NOB1 shRNA-expressing lentivirus-treated ovarian cancer cells tended to arrest in the G0/G1 phase [5]. In human hepatocellular carcinoma cells research, Lu Z et al. reported, the growth and proliferation of SMMC-7721/pGCSIL-GFP-shNob1 cells

were significantly reduced compared with the SMMC-7721/pGCSIL-GFP-shNC, and the colony formation was impaired after the suppression of Nob1 in SMMC-7721 cells [6]. Huang WY et al. also reported NOB1 deletion caused significant decline in cell proliferation in breast cancer cell lines [8].

NOB1 protein has been found expressed mainly in liver, lung and spleen [9]. However, no study was found accessing the relationship between NOB1 abnormal expression and lung cancer. Non-small-cell lung cancer (NSCLC) is the most common type of lung cancer, about 85 % to 90 % of lung cancers are NSCLC [10, 11]. The aim of this study was to investigate the expression of NOB1 in NSCLC tissues and to assess its clinicopathological significance.

Materials and Methods

NSCLC Cell Lines and Cell Culture

NSCLC cell lines (A549, H1299, H1975 and H1650) and human lung cell line BEAS-2B were obtained from the cell bank of the Central South University in Changsha, China. They were cultured in DMEM medium containing 10 % fetal calf serum (FCS) at 37 °C, 5 % CO_2.

Patients and Tissue Samples

Paired NSCLC and adjacent normal lung tissue were obtained from 15 patients who underwent primary surgical resection of NSCLC in the department of thoracic surgery of our hospital. Following surgical removal, the tissue samples were immediately cut into small pieces and snap frozen in liquid nitrogen until RNA extraction and protein quantization. We also collected 70 paraffin-embedded NSCLC tissue samples from Department of Pathology in our hospital for histopathologic and immunohistochemical examination. The clinicopathologic characteristics of 85 patients were collected prospectively and retrospectively. No patient had received preoperative chemotherapy or radiotherapy. The study protocol was approved by the Human Research Ethics Committee of our hospital.

Quantitative Reverse-Transcriptase Polymerase Chain Reaction (qRT-PCR)

Total RNA from the five cell lines mentioned above and frozen tissue extracted using TRIzol Reagent (Applied Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from total RNA with random primers. The NOB1 and β-actin PCR primer sequences were designed as follows: NOB1, forward 5′- ATCTGCCCTACAAGCCTAAAC -3′; reverse 5′- TCCT CCTCCTCCTCCTCAC- 3′; β-actin, forward 5′- GTGGGG CGCCCCAGGCACCA -3 ′; reverse 5′- CTCCTTAATGTC ACGCACGATTTC -3′. Reverse transcription was performed for 30 min at 42 °C, and 5 min at 85 °C. The PCR conditions included an initial denaturation step of 95 °C for 10 min, followed by 45 cycles of 95 °C for 20 s, 55 °C for 30 s, 72 °C for 30 s, 95 °C for 1 min, and 55 °C for 30 s. NOB1 mRNA was quantified by qRT-PCR using SYBR Premix Ex Taq (Applied Takara Bio) and normalized to β-actin.

Western Blot

Total protein was isolated from the five cell lines mentioned above and frozen tissue using ice-cold protein lysis buffer which contained 1 % Triton X-100, 50 mM Tris–HCl, 150 mM NaCl, 0.1 % SDS, 1 mM PMSF, and 1 mM EDTA. Suspensions were shaken at 4 °C for 1 h, and insoluble molecules were removed by centrifugation at 40,000 9×*g* at 4 °C for 1 h. Protein extracts were separated on a SDS-polyacrylamide gel, blotted onto a nitro-cellulose membrane and incubated with anti-NOB1 antibody (Abcam, Cambridge, UK) or anti β-actin antibody (Santa Cruz, CA, USA). Membranes were washed three times (10 min each) with TBS and incubated for 1 h with horseradish peroxidase (HRP) -conjugated goat anti-rabbit IgG. Specific proteins were detected using an ECL kit and AlphaImager (FluorChem5500; Alpha Innotech).

Immunohistochemistry

For immunohistochemistry, 4 μm sections were dewaxed and rehydrated through descending grades of alcohol to distilled water, followed by blocking of the endogenous peroxidase by using 3 % (v/v) hydrogen peroxidase in PBS. The sections were then subjected to microwave antigen retrieval in 0.02 M EDTA. They were washed in PBS and blocked with goat serum for 2 h, then incubated overnight at 4 °C with NOB1 antibody. After three washes in PBS, the sections were incubated with HRP-conjugated secondary antibody for 1 h at room temperature. Immunoreactivity was demonstrated using diaminobenzadine for increased sensitivity. The negative controls were incubated with a solution that was devoid of any primary antibody.

Percentages of NOB1 positive cells were scored into four categories according to staining: 0 for 0 %, 1 for 1–33 %, 2 for 34–66 %, and 3 for 67–100 %. The staining intensities were also scored into four grades: 0 for no stain, 1 for light yellow, 2 for yellowish brown, and 3 for brown. The sum of the percentages and intensity scores was used as the final staining score: 0 for -, 1–3 for +, 4–6 for ++, and 7–9 for +++.

Statistical Analysis

Statistical significance was determined by Stata 12.0 (Stata Corp, College Station, Texas). Analysis of variance and Chi-square test was performed for multiple comparisons among

individual samples. Two-sided $P<0.05$ was considered statistically significant.

Results

The Expression Profile of NOB1 mRNA in NSCLC Cell Lines

It was confirmed by RT-qPCR that the NOB1 gene expression was present in all cell line at different levels. The NOB1 mRNA level in NSCLC cell lines (A549, H1299, H1975 and H1650), especially in H1975, was higher than that in BEAS-2B ($P<0.05$). The NOB1 mRNA level in all cell lines was shown in Fig. 1.

The Expression Profile of NOB1 mRNA in NSCLC Tissue

The qRT-PCR results in 15 pairs of NSCLC tissue, adjacent tissue, and normal lung tissue showed that the NOB1 was over-expressed in NSCLC tissue when compared with adjacent tissue and normal lung tissue ($P<0.05$).

The NOB1 Protein Expression in NSCLC Cell Lines

The Western blot results showed that NOB1 protein was significant increased in NSCLC cell lines (A549, H1299, H1975 and H1650), compared with human lung cell line (BEAS-2B) ($P<0.05$). The NOB1 protein in all cell lines was shown in Fig. 2.

The Expression Profile of NOB1 Protein in NSCLC Tissue

The Western blot results showed that NOB1 protein was significant increased in NSCLC tissue, compared with adjacent tissue, and normal lung tissue ($P<0.05$). The NOB1 protein in two typical pairs of NSCLC tissue, adjacent tissue, and normal lung tissue was shown in Fig. 3. In Fig. 4, we also found NOB1 protein expression was higher in lung tissue than that in normal lung tissue by immunohistochemistry stain.

Correlation of NOB1 Expression with Clinicopathological Characteristics in NSCLC

There were significant associations between NOB1 protein expression and TNM stage, lymph node metastasis, and histopathological grade ($P<0.05$). However, NOB1 protein expression was not significantly associated with gender, age, smoke, histological type, or tumor diameter ($P>0.05$). The correlation was listed in Table 1.

Discussion

During the past decade, an array of molecular genetic tumor markers (MGTMs) has been identified based on the biological characterization of tumors, such as tumor development, growth, invasion and metastasis in NSCLC. Several markers (ERCC1, RRM1, BRCA1, P53, KRAS, β-tubulin, and EGFR) are suggested to be important prognostic markers regardless of treatment, and other markers (ERCC1, RRM1, BRCA1, P53, KRAS, and EGFR) are suggested to predictive markers after treatment [11–13]. NOB1p (Nin one binding protein) was first isolated by the yeast two-hybrid screening method [14]. The human NOB1 gene is composed of nine exons and eight introns and is localized on human chromosome 16q22.1. The predicted NOB1 protein comprises a PIN (PilT amino terminus) domain and a zinc ribbon domain [8]. NOB1 has a critical function of the ubiquitin proteasome

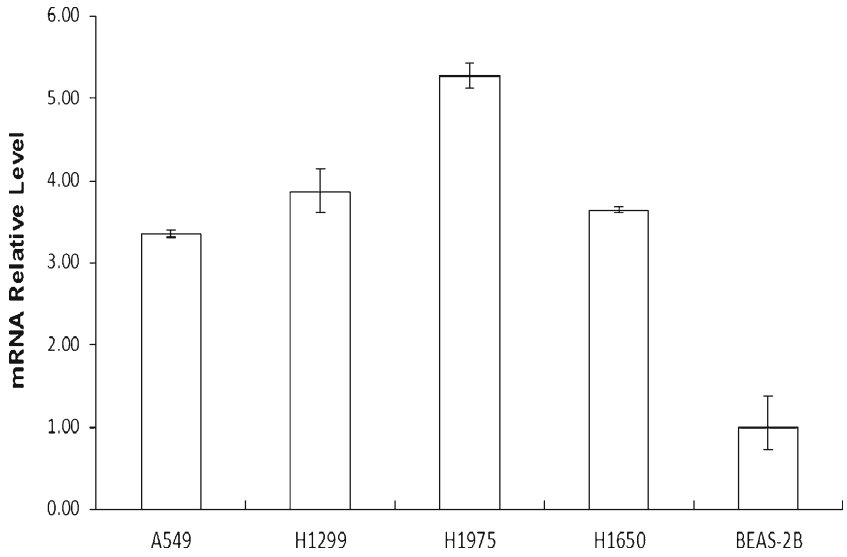

Fig. 1 NOB1 mRNA level for the qRT-PCR result. The NOB1 mRNA level in NSCLC cell lines (A549, H1299, H1975 and H1650), especially in H1975, was higher than that in human lung cell line (BEAS-2B) ($P<0.05$)

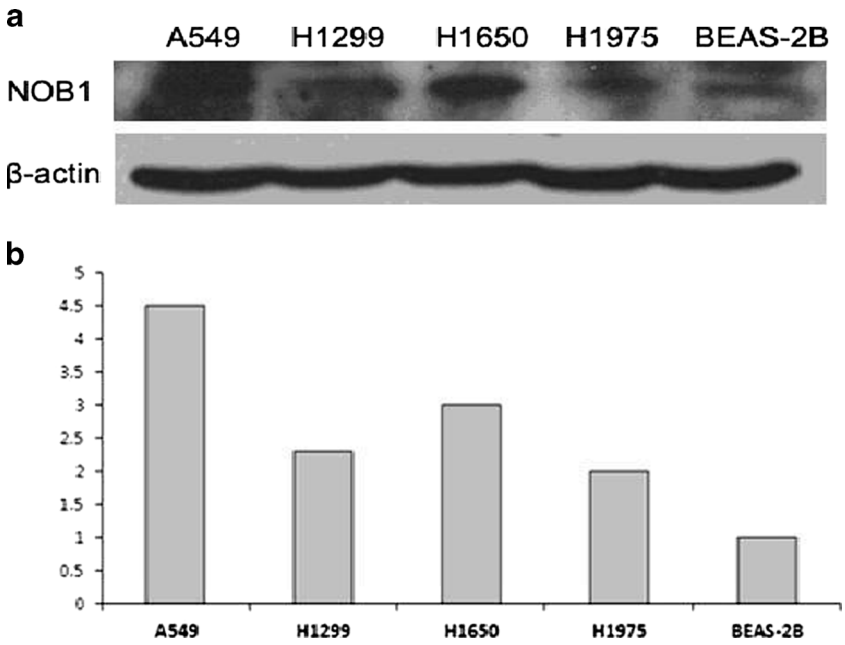

Fig. 2 Evaluation of NOB1 protein expressions in cell lines by western blot. The NOB1 mRNA level in NSCLC cell lines (A549, H1299, H1975 and H1650), was higher than that in human lung cell line (BEAS-2B) ($P<0.05$)

pathway (UPP) on cell cycle regulation. NOB1p serves as a chaperone to join the 20S proteasome with the 19S regulatory particle in the nucleus and facilitates the maturation of the 20S proteasome and degradation of Ump1p, and NOB1p is then internalized into the 26S proteasome and degraded to complete 26S proteasome biogenesis [15]. The 26S proteasome catalyzes protein degradation via the UPP, which is required for the degradation of cyclic proteins, CDKs, and regulates multiple aspects of the cell cycle progression [16]. Some studies have proved abnormal expression of NOB1 play a significant role in tumorigene. In this study, we investigate the clinicopathological significance of NOB1 in NSCLC.

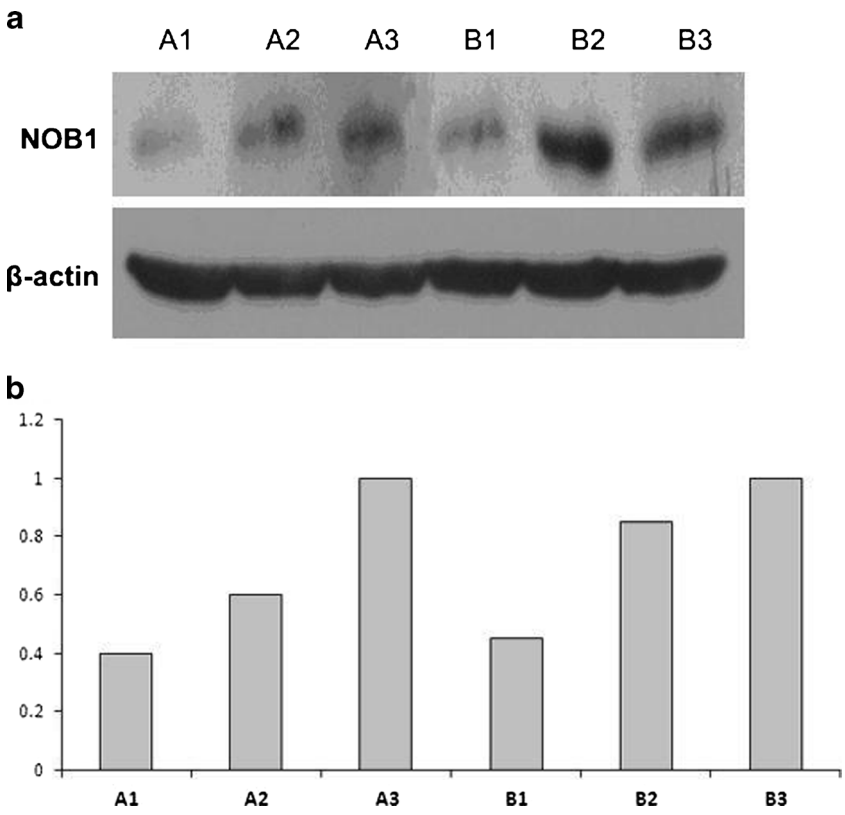

Fig. 3 Evaluation of NOB1 protein expressions in NSCLC tissue, adjacent tissue, and normal lung tissue by western blot. A and B were two typical NSCLC patients; 1 normal lung tissue, 2 adjacent tissue, 3 NSCLC tissue. The NOB1 protein level in NSCLC tissue was higher than that in adjacent tissue and normal lung tissue ($P<0.05$)

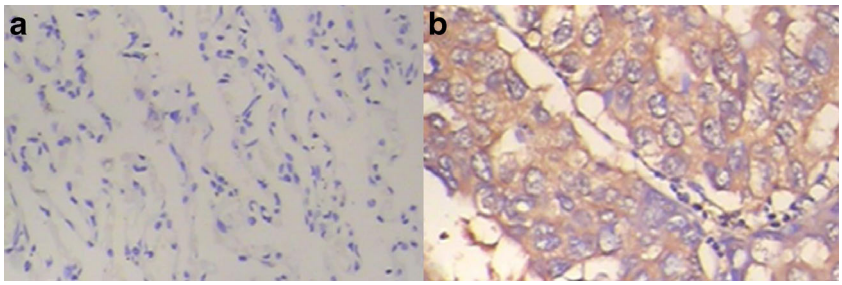

Fig. 4 Evaluation of NOB1 protein expressions in NSCLC tissue, and normal lung tissue by immunohistochemistry. **a** and **b** were two typical immunohistochemistry stain. **a** showed in normal lung tissue, NOB1 expression was 0; **b** showed in NSCLC tissue, NOB1 expression was +++

In the present study, our current data of RT-qPCR showed that the NOB1 mRNA expression in NSCLC cells was higher than NOB1 expression in lung cells. In 15 pairs of tissue, the NOB1 was over-expressed in NSCLC tissue when compared with adjacent tissue and normal lung tissue. Further more, expression of NOB1 protein in the four NSCLC cell lines and a lung cell line were measured by Western blot and Immunohistochemistry. The Western blot results showed that NOB1 protein was significant increased in NSCLC cell lines and NSCLC tissue. The Immunohistochemistry stain showed the expression pattern of NOB1 protein, mainly in the nucleus of NSCLC cells, was in accordance with previous reports [8]. The results also showed NOB1 protein expression was higher in lung tissue than that in normal lung tissue. These data were similar to previous reports of NOB1 and cancer [4–7]. We also found high NOB1 protein expression was significant associations between and TNM stage, lymph node metastasis, and histopathological grade. The results showed NOB1 play an important role in the occurrence and development of NSCLC. However, the reason and mechanism of NOB1 overexpression is still unclear. Further researches are needed.

Table 1 Correlation of NOB1 expression with clinicopathological characteristics in NSCLC

Clinico-pathological characteristics	N	NOB1 expression					χ^2	P
		–	+	++	+++	High expression rate		
Gender								
Male	39	10	13	10	6	15.38 %	0.007	0.932
Female	31	4	11	11	5	16.13 %		
Age (years)								
<50	9	4	1	3	1	11.11 %	0.165	0.684
>50	61	10	23	18	10	16.39 %		
Smoke								
No	32	5	10	12	5	15.63 %	0.000	0.985
Yes	38	9	14	9	6	15.79 %		
Histological type								
Squamous cell carcinoma	43	10	16	11	6	14.0 %	0.261	0.609
Adenocarcinoma	27	4	8	10	5	18.5 %		
Tumor diameter (cm)								
<3	42	8	14	14	6	14.28 %	0.162	0.688
≥3	28	6	10	7	5	17.88 %		
pTNM stage								
I, II	47	12	19	12	4	8.51 %	5.604	0.018
III	23	2	5	9	7	30.43 %		
Lymph node metastasis								
No	29	10	14	4	1	3.45 %	5.624	0.018
Yes	41	4	10	17	10	24.39 %		
Histopathological grade								
Well	1	0	1	0	0	0.00 %	8.593	0.014
Medium	45	11	18	13	3	6.67 %		
Low	24	3	5	8	8	15.71 %		

Conclusion

Our results suggest that enhanced expression of NOB1 gene plays an important role in the occurrence and development of NSCLC. NOB1 may be a potential therapeutic target in NSCLC.

Contributorship Kun LIU: research design, and acquisition, analysis, interpretation of data, approval of the submitted and final versions

Ming-Ming GU: research design, and acquisition, analysis, interpretation of data, approval of the submitted and final versions

Hong-Lin CHEN: acquisition, analysis, interpretation of data and drafting the paper, approval of the submitted and final versions

Qing-Sheng YOU: acquisition, analysis, interpretation of data, approval of the submitted and final versions

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Competing interests None

The study protocol was approved by the Human Research Ethics Committee of Affiliated Hospital of Nantong University

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