Expression of NOB1 in endometrial carcinoma and its Clinical Significance

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Abstract: Objective: To investigate NOB1 expression in endometrial carcinoma cell lines and tissues, and to explore its clincal significance. Methods: Quantitative RT-PCR, Western blot and immunofluorensce assay were done to detect the NOB1 expression in endometrial carcinoma cell lines (Ishikawa and RL-952). Immunohistochemistry was performed to determine whether there was difference in NOB1 expression between endometrial carcinoma and normal endometrial tissue. Results: Qualitative RT-PCR and Western blot showed that NOB1 expressed in both Ishikawa and RL-952 cells. Immunofluorescence assay indicated that NOB1 was mainly expressed in the cytoplasm and cell membrane. Immunohistochemistry showed that NOB1 protein was mainly distributed in the glandular epithelium and to a lesser extent in endometrial matrix but almost undetectable in normal endometrial tissue. Conclusion: NOB1 is highly expressed in both endometrial carcinoma cell lines and tissues and may participate in the occurrence and progression of endometrial carcinoma.
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1. Introduction

Human NOB1 gene was firstly cloned by Zhang and colleagues in 2005 (Zhang et al., 2005) and encodes a protein of 412 amino acids. NOB1 is currently known to be involved in the biosynthesis of small subunit ribosome and proteasome. Researches on effects of NOB1 in tumor cells have just started, however, it is strongly suggested that NOB1 may play a role in cancer onset and progression by affecting ribosome assembly, inducing abnormal nuclear division and proliferation, and/or preventing degradation of proto-oncogene products. The expression profiles of NOB1 in ovarian, rectal and esophageal squamous carcinoma have already been demonstrated, but the expression and significance of NOB1 in human endometrial carcinoma remains unclear. The present study investigated the expression of NOB1 in human endometrial carcinoma Ishikawa cells and RL-952 cells and endometrial carcinoma tissues.

2. Material and Methods

Materials

Samples were obtained from a total of 40 patients, including 20 patients with endometrial adenocarcinoma undergoing hysterectomy at the Department of Gynecology, Xiangya Third Hospital between June 2010 and December 2011. Paraffin-embedded normal endometrial tissues in the proliferative phase were collected as a control group from 20 cases of hysteromyoma or uterine prolapse of premenopausal women (with regular menstrual cycle and normal histological appearance). There was no difference (P>0.05) between the age of endometrial adenocarcinoma group (age range: 40-79; mean: 58.45±5.65) and control group (age range: 41-81; mean: 60.22±6.89). The surgically excised tissue of endometrial carcinoma was fixed in 4% formaldehyde and consequently embedded with paraffin for immunostaining. Informed consent was obtained from all patients and the study protocol was reviewed and approved by the ethics committee. The endometrial carcinoma Ishikawa cells and RL-952 cells were provided by Professor Xiaomao Li (Department of Obstetrics and Gynecology, First Affiliated Hospital of Sun Yat-Sen University). The Ishikawa cells and RL-952 cells were maintained and subcultured in DMEM/F12 medium supplemented with 10% fetal bovine serum at 37°C in 5% CO2-containing humidified air.

Reagents

Trizol Reagent (Invitrogen, USA), AMV (Progema,USA), RNasin (Progema, USA), SYBR GREEN I Master Mix (Applied Biosystems, UK), Protease Inhibitor Cocktail (Pierce, USA), goat anti-human NOB1 monoclonal antibody (Upstate, USA), mouse anti-human β-actin antibody (Santa Cruz, USA), mouse anti-human GAPDH antibody (ProMab, USA), rabbit anti-human β-actin antibody (Santa Cruz, USA), PVDF membranes (Amersham, Sweden), HRP-labeled goat anti-mouse IgG (Santa Cruz, USA), protein standard (Amersham, Sweden).

Equipments

PCR Amplifier (Ependorf, Germany), Inverted Fluorescence Microscope (Zeiss, Germany), Type 325 Microtome (Shandon, England), MIAS Biomedical Image Analysis System (Imaging CO., LTD, Beijing

University of Aeronautics & Astronautics), Motic B2 Microscope Camera System (Motic China Group CO., LTD), Mini Format 1-D Electrophoresis Systems (Bio-Rad, USA), Gel Doc 1000 Imaging System (Bio-Rad, USA), Du-70 Ultraviolet Spectrophotometer (Beekman, USA).

Quantitative RT-PCR

All primers were designed using Primer Express 3.0 Software (PE Biosystems, USA) and synthesized by Sangon Biotech Company. The primers for NOB1 are 5′-GTCTGACATGAGCCGAGTGT-3′ (forward) and 5′-GCGTTGGGATTTAAGCGTCT-3′ (reverse). The primers for β-actin are 5'-TAAAGACCTCTATGCCAACACAGT-3'(forward) and 5'-CACGATGGAGGGGCCGGACTCATC-3' (reverse). The PCR products of NOB1 and β-actin gene are the expected size of 378 bp and 241 bp, respectively. After the reaction, the PCR products were subject to electrophoresis on a 1% agarose gel. Each sample was repeated for three times.

Western blot

The Ishikawa and RL-952 cells were harvested and lysed in RIPA buffer. After centrifugation, the supernatant containing total protein extract was collected. The protein concentration was determined using a BCA assay kit. A total of 30 μg protein was loaded per lane for standard SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked with 3% BSA for 2 h at room temperature and then incubated with goat anti-human NOB1 antibody (1:300 dilution) at 4°C overnight. The mouse anti-human GAPDH antibody (1:1000 dilution) was used as a control. After 3 × 10 min washes in TBST, the membrane was incubated with HRP-labeled rabbit anti-goat secondary antibody (1:3000) for 1 h at room temperature. The blots were washed three times in TBST and were visualized using enhanced chemiluminescence (ECL) detection. These experiments were repeated at least three times.

Immunofluorescence

The Ishikawa/RL-952 cells were cultured at a density of 1 × 105 cells per mL on coverslips in a 24-well plate. When the cells reached 30%-50% confluence (24 h after plating), the medium was removed and a mixture of methanol and acetone (1:1 by volume) was added to fix the cells. Subsequently, the fixed cells were blocked with 1% BSA and incubated with NOB1 antibody (1:50 dilution in PBS) for 24 h. After rinsing in PBS, the cells were incubated with FITC-conjugated goat anti-mouse antibody (1:100 in PBS) for 1 h at 37°C in the dark. The nuclei were stained by DAPI (blue fluorescence). PBS (containing no primary antibody) was used as a control. The cells were mounted on glass slides and immediately examined using an invert immunofluorescence microscope.

Immunohistochemistry

The expression and localization of NOB1 in endometrial tissue was detected using the streptavidin peroxidase (SP) method. Tissue sections were incubated with goat anti-human NOB1 antibody (1:500) at 37°C for 4 h. PBS was used as a negative control to replace the primary antibody. The sections were washed in PBS, incubated with HRP-conjugated goat anti-rabbit antibody, and stained with diaminobenzidine (DAB). A double-blind method was adopted by 2 pathologists to evaluate the results of immunohistochemistry. The relative expression level of NOB1 was determined by the optical density (OD) of positive staining, which was calculated by MIAS imaging analysis system over 5 randomly selected high magnification fields in each section.

Statistical analysis

All data were analyzed with SPSS 13.0 software. Results were expressed as means ± standard deviation ($\bar{x}$ ± s). Statistical significance was determined by one-way ANOVA (two-tailed, α= 0.05). A P value < 0.05 was considered significant.

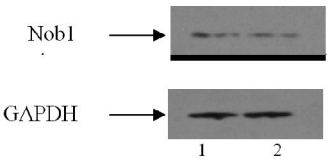
3. Results

Expression of NOB1 mRNA by quantitative RT-PCR

We used quantitative RT-PCR to measure the mRNA levels of NOB1 in Ishikawa and RL-952 cells. The fluorescence absorption values of NOB1 normalized to β-actin showed that there was significant difference (P<0.05) between Ishikawa cells (1.031±0.32) and RL-952 cells (1.287±0.30) in NOB1 mRNA expression.

Expression of NOB1 protein by Western blot

The expression of NOB1 protein in Ishikawa and RL-952 cells was assayed by Western blot using goat anti-human NOB1 monoclonal antibody. GAPDH was used as an internal control. The relative absorbance of NOB1 was determined by gel imaging analysis system. The results showed that the relative absorbance of NOB1 in RL-952 was 0.175±0.028, while it was 0.145±0.031 in Ishikawa cells. The difference between Ishikawa and RL-952 cells is statistically significant (P<0.05) (Figure 1)

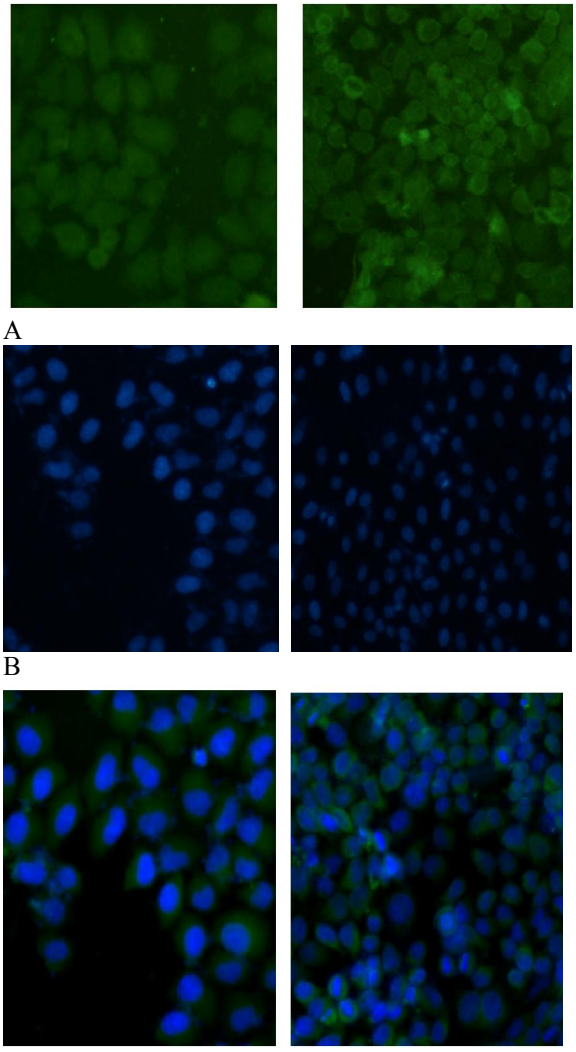


Note: the number “1” and “2” refer to RL-952 cells and Ishikawa cells, respectively.

Fig. 1 The protein level of NOB1 detected by Western blot in Ishikawa and RL-952 cells

Results of immunofluorescence

As shown in Figure 2, NOB1 protein was indicated by green fluorescence and mainly located on cytoplasm and membrane in both Ishikawa and RL-952 cells. Cell nuclei were stained blue with DAPI. (Figure 2)



A

B

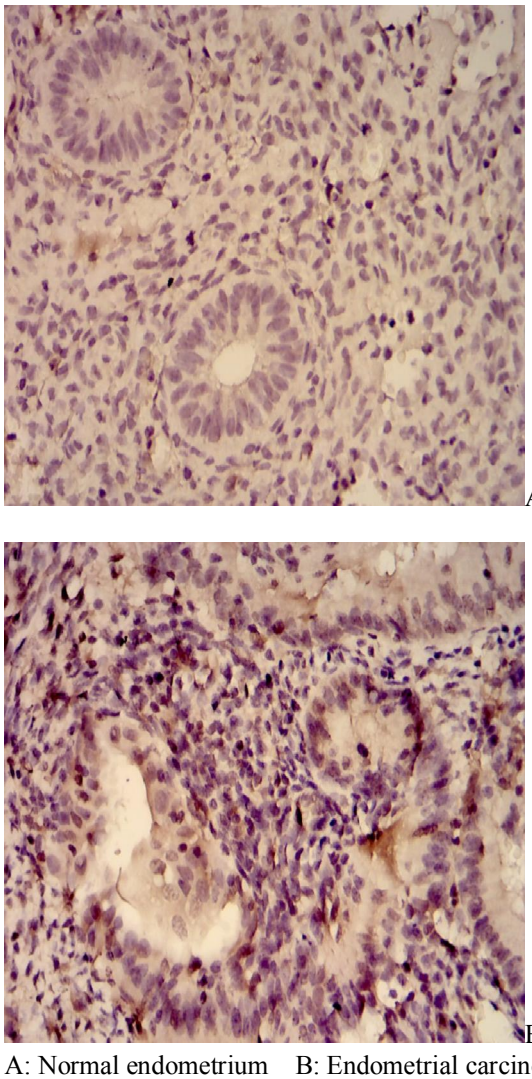
C

Ishikawa RL-952

Figure 2. NOB1 intracellular localization and expression in Ishikawa and RL-952 cells. Green fluorescence indicating NOB1 mostly appeared in the cytoplasm and membrane (A). Cell nuclei appeared as blue fluorescence after staining with DAPI (B). Merged images of stainings are shown (C). Original magnification, x600. Bar =10μm.

Results of immunohistochemistry.

The expression of NOB1 in endometrial carcinoma or normal endometrial tissue was assayed by standard immunohistochemistry method using goat anti-human monoclonal antibody and biotin-labeled secondary antibody. The results showed that in endometrial carcinoma tissue, NOB1 was distributed mainly in glandular epithelium and to a lesser extent in endometrial matrix. In normal endometrial tissue, however, the expression of NOB1 was detected at low levels in matrix and almost undetectable in glandular epithelium. The mean optical density of NOB1-positive staining was measured by MIAS biomedical imaging analysis system. As shown in Figure 3, the expression of NOB1 in endometrial carcinoma was significantly higher than that in normal endometrial tissue (endometrial carcinoma: 2.44±0.21; normal endometrial tissue: 1.37±0.19; $P<0.05$). (Figure 3)



A

B

A: Normal endometrium B: Endometrial carcinoma

Figure 3 Localization of NOB1 in normal endometrium and endometrial carcinoma by immunohistochemical staining using SP method, DAB and hematoxylin staining (×400).

4. Discussions

NOB1 gene, orthologue of yeast NOB1p, is localized on human chromosome 16q22.1 and composed of nine exons and eight introns. The assembled cDNA of NOB1 is 1749 bp in length. NOB1 gene encoded an RNA-binding protein of 412 amino acids with a predicted molecular weight of 46675 Da. NOB1 is involved in the assembly of 40S (small subunit) ribosome, the biosynthesis of 26S proteasome and the regulation of cell cycle. The predicted NOB1 protein comprises a PIN domain which contains RNase activity in the amino terminus, and a zinc ribbon domain which is required for RNA binding in the carboxy terminus. Fatica et al reported that a point mutation in the PIN domain was lethal to cells, resulting in the accumulation of 20S rRNA and loss of synthesis of 18S rRNA (Smith et al., 2004). The functional role of yeast NOB1p in the biosynthesis of ribosome has been extensively studied. The assembly of 40S ribosome small subunit requires cleavage at site D to form mature 18S rRNA. Using RNA binding assay and dimethyl sulfate (DMS) probe, Lamanna and co-workers found that recombinant human NOB1 not only binds to site D via PIN domain, but also interacts with helix44 (H44) in 18S rRNA (Lamanna et al., 2009). Moreover, NOB1 encircled site D and interacted with the domain adjacent to cleavage site A2. Therefore, NOB1 endonuclease activity is essential for cleavage at site D and plays an important role in maturation of 18S rRNA, which is directly related to synthesis of ribosome small subunit. It is known that normal biosynthesis of rRNA is a prerequisite for cell cycle progression (Raychaudhuri et al., 2009; Meraner et al., 2006). The abnormality of NOB1 expression is likely to cause loss of synthesis of ribosome and excessive degradation of ribosomal precursor particle, thus disturbing the regulation of cell growth cycle (Granneman et al., 2005; Ramos et al., 1998). Another aspect of NOB1 functions is that it participates in the synthesis of 26S proteasome through the ubiquitin-proteasome pathway, which is an important mechanism for intracellular protein degradation. The ubiquitin-proteasome pathway is composed of ubiquitin (Ub), ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), ubiquitin-protein ligase (E3), 26S proteasome and deubiquitinating enzyme (DUB). Ubiquitinated proteins are targeted for degradation by 26S proteasome in a cascade fashion. The 26S proteasome consists of a 20S proteolytic core particle (CP) and a 19S regulatory particle (RP). As a component of 26S proteasome, NOB1 is involved in the biosynthesis of 26S proteasome and plays a key role in its protease activity(Ferrell et al,2000). Impaired degradation of target proteins by ubiquitin-proteasome system can lead to accumulation of oncogene products, degradation of anti-oncogene products, decreased apoptosis and accelerated proliferation, all of which contributes to tumorigenesis (Ni et al,2007). It is found that many nuclear oncogene proteins such as c-myc, c-fos, p53 and E1A are degraded through ubiquitin-dependent pathway (Ciechanover,1984; Ciechanover A, et al,1991).

The role of NOB1 gene in tumor occurrence and development has attracted scholars' attention, however, the mechanism of NOB1 effects in tumors have not been well characterized. Li and colleagues discovered that NOB1 expressed at high level in ovarian carcinoma and that gene silencing of NOB1 by small interfering RNA (siRNA) inhibited cell proliferation and caused arrest of cell cycle in G1 phase in ovarian cancer HEY and SKOV3 cells (Lin,2012; Wu,2012). Wu and co-workers compared the expression of NOB1 in colorectal adenocarcinoma and normal colorectal tissue by immunohistochemistry methods and found that NOB1 expression was significantly increased in colorectal adenocarcinoma, compared with normal colorectal tissue (Huo et al,2011). Moreover, NOB1 knockdown by siRNA led to decreased proliferation in RKO cells. Xiaolei He and Lili Ma reported a high expression of NOB1 in esophageal squamous cell carcinoma and that the expression level of NOB1 was correlated with tumor differentiation degree and lymph node metastasis but unrelated to gender, age or depth of tumor invasion (Ma et al,2011). NOB1 expression and effects in endometrial carcinoma have not been reported. In the present study, we found that NOB1 expressed in both the endometrial carcinoma cell lines and tissues and that the expression of NOB1 was significantly higher in endometrial carcinoma than in normal endometrial tissue. The correlation of NOB1 expression with clinical stage and pathological grade of endometrial carcinoma was not discussed owing to limited sample size and remains to be addressed in future studies. Abnormal nuclear division and proliferation requires participation of ribosomes, and both abnormal nuclear division and inhibited degradation of oncogene products can cause malignant tumorigenesis. NOB1 plays an important role in the biosynthesis of both ribosome small subunit and 26S proteasome, thus providing a possible common pathway of tumor occurrence and progression. Therefore, NOB1 gene is likely to be a proto-oncogene which promotes malignant tumor proliferation such as endometrial and ovarian carcinoma. To further explore the mechanism of NOB1 effects on tumor growth may offer new insights regarding the control of cancer and indicate a novel tumor marker and a potentially important therapeutic target.

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