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ORIGINAL ARTICLE

Downregulation of lncRNA TUBA4B is Associated with Poor Prognosis for Epithelial Ovarian Cancer

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Abstract A host of studies have revealed that long non-coding RNAs (lncRNAs) are critically involved in the development and progression of epithelial ovarian cancer. LncRNA TUBA4B is recently identified to be a critical mediator in non-small cell lung cancer. However, the clinical roles and biological functions of lncRNA TUBA4B in epithelial ovarian cancer have yet to be fully clarified. The present study was conducted to explore the expression of lncRNA TUBA4B in human epithelial ovarian cancer tissues and potential roles of lncRNA TUBA4B in ovarian cancer cells. The matched epithelial ovarian cancer specimens and adjacent normal tissues were employed to detect the expression of lncRNA TUBA4B. The prognostic value of lncRNA TUBA4B for tumor progression and survival rate was investigated. The effects of lncRNA TUBA4B on ovarian cancer cell proliferation and migration were also explored. The expression of lncRNA TUBA4B was significantly decreased in epithelial ovarian cancer tissue specimens. The low lncRNA TUBA4B level was closely related with pathological grade, FIGO stage and lymph node metastases, and serum CA125 level. Enforced expression of lncRNA TUBA4B obviously reduced the proliferation of SKOV3 cells, and attenuated the activation of ERK and Akt signaling pathways. Our data demonstrate for the first time that lower lncRNA TUBA4B may be a novel independent prognostic biomarker for overall survival of epithelial ovarian cancer. Overexpression of lncRNA TUBA4B inhibits the proliferation of ovarian cancer cells. LncRNA TUBA4B may be an important target for therapeutic intervention in ovarian cancer.

Keywords Ovarian cancer · lncRNA · Overall survival · Proliferation

Introduction

Epithelial ovarian cancer is considered as one of the worst gynecologic malignancies among women, and 85–90% of the ovarian cancers are demonstrated to be epithelial ovarian cancer [1, 2]. Accumulating evidence indicates that prognosis of patients with epithelial ovarian cancer may be better in early diagnosis. Therefore, identification of novel biomarkers and therapeutic targets may be useful for predication of early diagnosis and prognosis of ovarian cancer.

Long non-coding RNAs (lncRNAs) are identified to be more than 200 nucleotides, and play key roles in various biological processes including chromatin remodeling, gene regulation and RNA maturation [3]. A wide coverage of studies have established that abnormal expressions of lncRNAs may be essential for proliferation, migration, invasion and metastasis of cancer cells [4]. A recent study has disclosed that lncRNA TUBA4B levels are significantly decreased in non-small cell lung cancer tissues and cells. The downregulated lncRNA TUBA4B expressions are employed as a poor predictor for prognosis of non-small cell lung cancer [5]. However, the roles of lncRNA TUBA4B in tumorigenesis, and the prognostic significance of ovarian cancer remain incompletely unknown. Therefore, this study investigated whether lncRNA TUBA4B may be served as a prognostic

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factor for ovarian cancer, and the possible mechanism of lncRNA TUBA4B in proliferation of ovarian cancer cells were also identified.

Methods

Patients and Specimens

The study was reviewed and approved by the Research Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University. All experiments were conformed to the Code of Ethics of the World Medical Association (Declaration of Helsinki). Signed written informed consent was provided from all subjects. A total of 116 ovarian cancer patients who underwent a curative resection were included from Department of Gynaecology, the First Affiliated Hospital of Wenzhou Medical University ranged from February 2002 to October 2008. The diagnosis of ovarian cancer cases was based on the clinical presentations, immunohistochemical staining and morphological criteria. The patients who had history of radiation, chemoradiation, or hormonal therapy were excluded from this study. The clinicopathological and demographic data pre- and post-operation were preserved in medical records. The characteristics of patients including age, tumor grade, Federation of Gynecology and Obstetrics (FIGO) stage, histological subtype, tumor size, serum CA-125 level and lymphatic invasion were summarized in Table 1. The survival information of each patient was collected by letter, e-mail or phone calls. The overall survival time was considered as the time from the date of surgery to the date of death or the date of last follow-up. The paired tumor tissues and adjacent normal ovarian tissues were obtained from patients who underwent a curative resection at our hospital. All species were immediately frozen in liquid nitrogen and kept at −80 °C prior to use.

RNA Isolation and Quantitative Real-Time PCR

Total RNA in each sample was isolated from frozen specimen by the TRIzol (Invitrogen, Carlsbad, CA, USA) protocol according to the manufacturer's instructions. The reverse transcription of RNA was then performed using PrimeScript RT-PCR kit (Takara, Otsu, Shiga, Japan). The quantitative real-time polymerase chain reaction (RT-PCR) was performed using the SYBR Premix Ex Taq TM (Takara, Otsu, Shiga, Japan) on ABI 7500 Real Time PCR system (Applied Biosystems, Foster City, CA, USA). The relative proportion of target gene expression was quantified by normalizing the targeted gene level to that of internal control by the $\Delta\Delta Ct$ method. Primer sequences used are as follows: lncRNA TUBA4B: forward: 5′-ATCAATCACCAGCCTCCC-3′; reverse: 5′-CCACCTCCTT-GTAATCCTTCT-3′;

glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward: 5′-TGACTTCAACAGCGACACCCA-3′; reverse: 5′-CACCCTGTTGCTGTAGCCAAA -3′ [5].

Cell Culture

The ovarian cancer cell lines including A2780, IGROV1, SKOV3, and OVCAR3 and a control human ovarian surface epithelial cell line (HOSE 6.3) were purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China). OVCAR-8 and SKOV-3 were cultured in DMEM medium, but A2780 and IGROV-1 were cultured in RPMI 1640 medium, supplemented with 10% FBS (FBS; Gibco, Grand Island, NY, USA) coupled with 100 U/ml penicillin sodium, and 100 mg/ml streptomycin sulfate at 37 °C in a humidified incubator with 5% CO2. HOSE 6.3 cells were cultured in MCDB Medium supplemented with 10% FBS.

Cell Proliferation Assay

SKOV3 cell proliferation was assessed with the cell counting kit-8 (CCK-8) assay kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) in accordance with the manufacturer's suggestions. Cells were seeded at an initial density of 2×10^4 cells/mL in 96-well plates. The OD450 absorbance was determined to measure cell viability. The DNA synthesis of SKOV3 cells was determined using a Cell-Light™ EdU Apollo®488 following the manufacturer's instructions. The EdU positive cells were normalized by the total number of Hoechst 33,342 stained cells [6]. SKOV3 cells were transfected of lentivirus (LV)-mediated overexpression vector targeting lncRNA TUBA4B or negative control at a density of 2×10^5 cells for indicated time in six-well plates according to the manufacturer's suggestions.

Western Blot

The cell lysates were collected by using radioimmunoprecipitation assay (RIPA) buffer (Beyotime Biotechnology, China). The total protein concentration in the supernatant was quantified with the Bradford assay (BCA; Pierce, Santa Cruz, CA, USA). Total cellular proteins were loaded to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Bio-Rad Laboratories Inc., Hercules, CA, USA) and transferred to immobilon polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% nonfat milk at room temperature and incubated with designed primary antibodies overnight at 4 °C. The positive signals from HRP-coupled secondary antibodies (Santa Cruz, CA, USA) were visualized. The densitometric analysis of the band intensities was measured and

Table 1 Correlation of lncRNA TUBA4B expression with epithelial ovarian cancer patients' clinicopathologic features

Variables	All Cases (%)	LncRNA TUBA4B expression Low (%)	High (%)	χ^2	P
Age (years)					
≤ 50	68 (58.6%)	32(47.1%)	36(52.9%)	0.124	0.725
> 50	48 (41.4%)	21(43.8%)	27(56.2%)		
Pathological grade					
G1 + G2	72 (62.1%)	32(44.4%)	40(55.6%)	5.047	0.025
G3	44 (37.9%)	29(65.9%)	15(34.1%)		
FIGO stage					
I + II	51 (44.0%)	23(45.1%)	28(54.9%)	5.166	0.023
III + IV	65 (56.0%)	43(66.2%)	22(33.8%)		
Tumor size (cm)					
≤ 2	78 (67.2%)	36(46.2%)	42(53.8%)	0.848	0.357
> 2	38 (32.8%)	21(55.3%)	17(44.7%)		
Histological type					
Serous	49 (42.2%)	22(44.9%)	27(55.1%)	0.917	0.821
Endometrioid	27 (23.3%)	13(48.1%)	14(51.9%)		
Mucinous	18 (15.5%)	10(55.6%)	8(44.4%)		
Clear cell	22 (19.0%)	12(54.5%)	10(45.6%)		
Lymph node involvement					
Negative	41(35.3%)	20(48.8%)	21(51.2%)	9.811	0.002
Positive	75(64.7%)	58(77.3%)	17(22.7%)		
Serum CA125 level (U/L)					
≤ 900	50 (43.1%)	22(44.0%)	28(56.0%)	6.818	0.009
> 900	66 (51.7%)	45(68.2%)	21(31.8%)		

FIGO Federation of gynecology and obstetrics

normalized to the band intensities of GAPDH using the Image J softwere (NIH, USA) [7].

Chemicals

The primary antibodies against proliferating cell nuclear antigen (PCNA), phosphorylated histone H3 (P-H3) and GAPDH were obtained from Santa Cruz (Santa Cruz, CA, USA). The antibodies of phosphorylated Akt, phosphorylated ERK, Akt and ERK were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). The lentivirus (LV)-mediated vector containing TUBA4B as well as a negative control was synthesized from Genechem (Shanghai, China).

Statistical Analysis

All data about continuous variables were presented in the form of means ± standard deviation (SD), and the categorical data were expressed as the percentage. SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The chisquare or Fisher's exact tests, or t-test were used to evaluate the relationship between expression of lncRNA TUBA4B and other clinical parameters. One-way or two-way ANOVA followed by post hoc Bonferroni test was used when multiple comparisons were made. Survival analysis was estimated with the Kaplan-Meier method. Differences between survival curves were analyzed using the log-rank test. The Cox proportional hazards model was applied for the multivariate survival analysis. The diagnostic efficacy of lncRNA TUBA4B was evaluated by receiver operating characteristic (ROC) curve. A two-sided P value of <0.05 was considered statistically significant.

Results

Downregulation of lncRNA TUBA4B Expression in Epithelial Ovarian Cancer Patients

RT-PCR results showed that lncRNA TUBA4B mRNA levels were significantly lower in epithelial ovarian cancer tissues than paired adjacent tissue specimens (Fig. 1a). In addition,
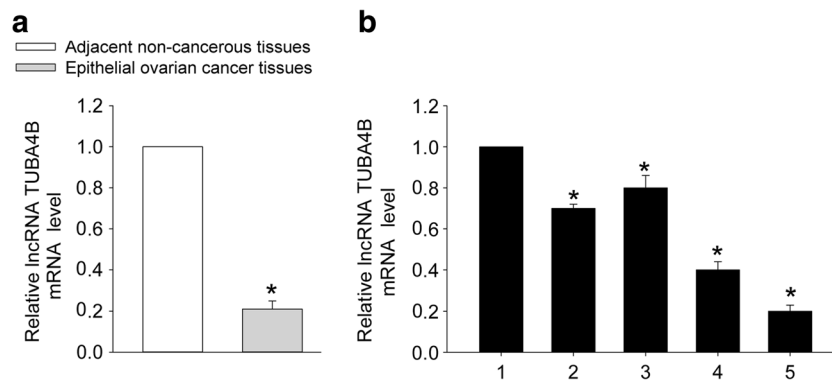
Fig. 1 Expressions of lncRNA TUBA4B in paired adjacent non-cancerous tissues and epithelial ovarian cancer tissues detected by real-time quantitative RT-PCR assay (**a**); (**b**) Lowler expression levels of lncRNA TUBA4B were detected in 4 ovarian cancer cell lines (2,A2780), (3,OVCAR3), (4,IGROV1), (5,SKOV3), compared with values obtained for the normal human ovarian surface epithelial cell line (1, HOSE 6.3) determined with RT-PCR. The results showed that lncRNA TUBA4B expression level was significantly decreased in epithelial ovarian cancer tissues and cell lines. * $P < 0.05$ vs. Adjacent non-cancerous tissues or 1, HOSE 6.3. Values are mean ± SD. $n = 6$ for each group

the levels of lncRNA TUBA4B mRNA were also dramatically decreased in ovarian cancer cell lines including SKOV3, IGROV1, A2780, and OVCAR3 in comparison with normal human ovarian surface epithelial HOSE 6.3 cells (Fig. 1b). It is interesting that SKOV3 cell line exhibited the lowest lncRNA TUBA4B mRNA level, thus SKOV3 cell line was selected for the in vitro experiments.

Diagnostic Efficacy of lncRNA TUBA4B in Epithelial Ovarian Cancer Patients

The receiver operating characteristic curve (ROC) curve analysis revealed that AUC was 0.185 ($P = 0.002$). When the cutoff value = 1.1, the diagnostic sensitivity (60.2%) and specificity (89.8%) reached their peak values ($P < 0.05$). Thus the lncRNA TUBA4B expression was further classified into the low expression group (n = lncRNA TUBA4B expression < 1.1, $n = 79$) and high expression group (lncRNA TUBA4B expression ≥1.1, $n = 37$) as the threshold ROC curve value of 1.1 (Fig. 2).

Correlation of lncRNA TUBA4B Expression with Clinical Parameters of Epithelial Ovarian Cancer Patients

As shown in Table 1, the decreased lncRNA TUBA4B expression is closely associated with the pathological grade ($P = 0.025$), FIGO stage ($P = 0.023$), and lymph node involvement ($P = 0.002$) and serum CA125 level ($P = 0.009$) in epithelial ovarian cancer patients. However, we found no significant correlations between lncRNA TUBA4B expression and age ($P = 0.725$), tumor size ($P = 0.357$), histological type ($P = 0.821$) in patients with ovarian cancer.

Relationship between lncRNA TUBA4B Expression and Overall Survival

The Kaplan-Meier curves between high or low lncRNA TUBA4B expression and overall survival demonstrated that the ovarian cancer patients with lower lncRNA TUBA4B expression had an obviously shorter overall survival ($P = 0.000$, Fig. 3). Multivariate Cox proportional hazards regression analysis revealed that pathological grade, FIGO stage, lymph node involvement, serum CA125 level and lncRNA TUBA4B expression were closely related with overall survival rate in epithelial ovarian cancer patients, and these parameters may be employed to be independent prognostic indicators for overall survival of ovarian cancer patients ($P < 0.05$, Table 2).

Overexpression of lncRNA TUBA4B Retarded the Proliferation of SKOV3 Cells

Compared with day 1, the absorbance of SKOV3 cells in both control group and NC group was significantly increased after day 3, 5 and 7 (Fig. 4a). However, the SKOV3 cells transfected with LV-mediated lncRNA TUBA4B vector obviously diminished the SKOV3 cell proliferation at day 5 and 7 in comparison with control group and NC group (Fig. 4a). The increased EdU-positive SKOV3 cells in both control group and NC group were obviously inhibited by enforced introduction of lncRNA TUBA4B (Fig. 4b and c). Furthermore, the proliferating markers including PCNA and phosphorylated histone H3 (P-H3) were also remarkably depressed by lncRNA TUBA4B overexpression (Fig. 4d).
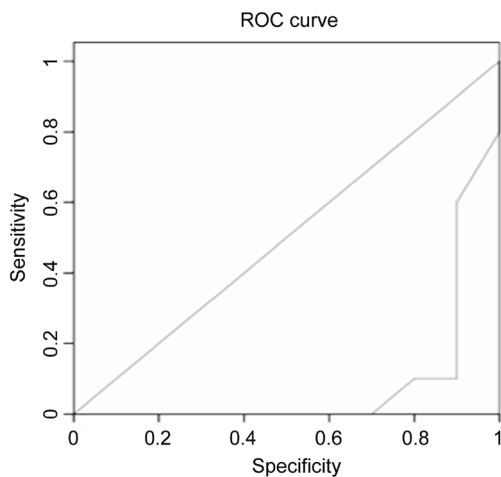
Fig. 2 ROC curve analysis of lncRNA TUBA4B in epithelial ovarian cancer patients. Assessment of the diagnostic efficacy of lncRNA TUBA4B in epithelial ovarian cancer tissues patients by calculating the area under the receiver operating characteristic curve. (AUC = 0.185, $P = 0.002$, Sensitivity = 0.602; Specificity = 0.898)

Effects of lncRNA TUBA4B on PI3K/Akt and ERK Signaling Pathways

The increased phosphorylation levels of PI3K/Akt and ERK signaling pathways were suppressed by overexpression of lncRNA TUBA4B (Fig. 5).

Discussion

The absence of useful biomarkers has disappointed the clinical outcome of ovarian cancer therapy. The utility of novel biomarkers may provide a better understanding of cancer biological behavior, thus the useful biomarkers for cancer are continuously characterized in recent years [8]. In this study, we established an intimate relationship of lncRNA TUBA4B with aggressive clinicopathological parameters including
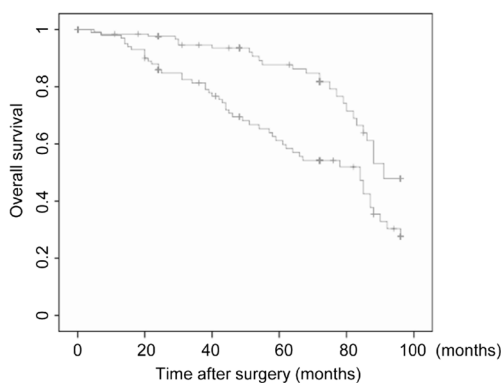

Fig. 3 Kaplan-Meier survival analysis of lncRNA TUBA4B in epithelial ovarian cancer patients. Kaplan-Meier survival analysis stratified according to lncRNA TUBA4B expression in epithelial ovarian cancer patients. The patients with lower lncRNA TUBA4B expression had significantly shorter overall ($P < 0.001$)

pathological grade, FIGO stage and lymph node metastases, and serum CA125 level in ovarian cancer patients. LncRNA TUBA4B may be a crucial component in the pathogenesis of ovarian cancer associated with the deteriorated proliferation of ovarian cancer cells via ERK and Akt signaling pathways.

Deficiency of diagnostic and prognostic factors may be largely responsible for the poor outcome of ovarian cancer. It has been recommended that early and accurate diagnosis of ovarian cancer is beneficial for the appropriate therapy [8]. LncRNAs are emerged to play important roles in human tumor biology [9]. LncRNAs have attracted considerable attention in cancer research due to their fundamental actions in cancer initiation, progression, proliferation, differentiation, apoptosis, invasion and metastasis [9]. It is recently reported that lncRNA urothelial cancer associated 1 is significantly increased in ovarian cancer tissues, and may be taken as a valuable factor in predicting the prognosis in patients with ovarian cancer [10]. LncRNA HOST2 is highly expressed in human ovarian cancer, and stimulates the proliferation, migration and invasion of epithelial ovarian cancer cells [11]. It is also verified that lncRNA HOTAIR expression is significantly elevated in epithelial ovarian cancer tissues, and high HOTAIR level may be closely associated with FIGO stage, the histological grade of the tumor, lymph node metastasis [12]. TUBA4B (Tubulin, Alpha 4b) is identified to be a pseudogene. High-throughput microarray assay reveals that lncRNA TUBA4B is obviously downregulated in non-small cell lung cancer tissues [13]. The same group further characterizes that low lncRNA TUBA4B expression is remarkably related with advanced TNM stage and lymph node metastasis [5]. However, the potential clinical significance of lncRNA TUBA4B in ovarian cancer remains largely obscure. In this study, we showed that the expression of lncRNA TUBA4B was significantly decreased in epithelial ovarian cancer tissue specimens and ovarian cancer cells. The low lncRNA TUBA4B level was closely related with pathological grade, FIGO stage and lymph node metastases, and serum CA125 level. The ovarian cancer patients with lower lncRNA TUBA4B expression had an obviously shorter overall survival. Multivariate Cox proportional hazards regression analysis revealed that pathological grade, FIGO stage, lymph node involvement, serum CA125 level and lncRNA TUBA4B expression were closely related with overall survival rate in epithelial ovarian cancer patients. To our knowledge, our results firstly identified the prognostic significance of lncRNA TUBA4B in ovarian cancer. These results hinted that lncRNA TUBA4B may be a new prognostic biomarker for ovarian cancer.

The abnormal growth of cancer cells was one of the most important events in the tumorigenesis and tumor progression [14, 15]. In the present study, we showed that lncRNA TUBA4B overexpression eliminated the SKOV3 cell proliferation. The increased EdU-positive SKOV3 cells were obviously inhibited by enforced introduction of lncRNA TUBA4B. Furthermore, the proliferating markers including

Table 2 Cox multivariate analysis of the clinicopathological parameters for overall survival

Variables	Hazard ration	95% CI	P value
Ages (≤50 vs. >50)	1.143	0.544–2.402	0.352
Pathological grade (G1 + G2 vs. G3)	0.414	0.190–0.901	0.020
FIGO stage (I + II vs. III + IV)	0.420	0.198–0.893	0.018
Tumor size (≤2 cm vs. >2 cm)	0.694	0.318–1.512	0.235
Lymph node involvement (Negative vs. Positive)	0.279	0.123–0.632	0.003
Serum CA125 (≤900 U/L vs. >900 U/L)	0.367	0.171–0.785	0.013
LncRNA TUBA4B expression (High vs. Low)	1.675	0.347–3.382	0.004

CI Confidence Interval

PCNA and phosphorylated histone H3 (P-H3) were also obviously suppressed in SKOV3 cells in response to overexpression of lncRNA TUBA4B. These results suggested that lncRNA TUBA4B may function as a negative modulator in the proliferation of ovarian cancer cells.
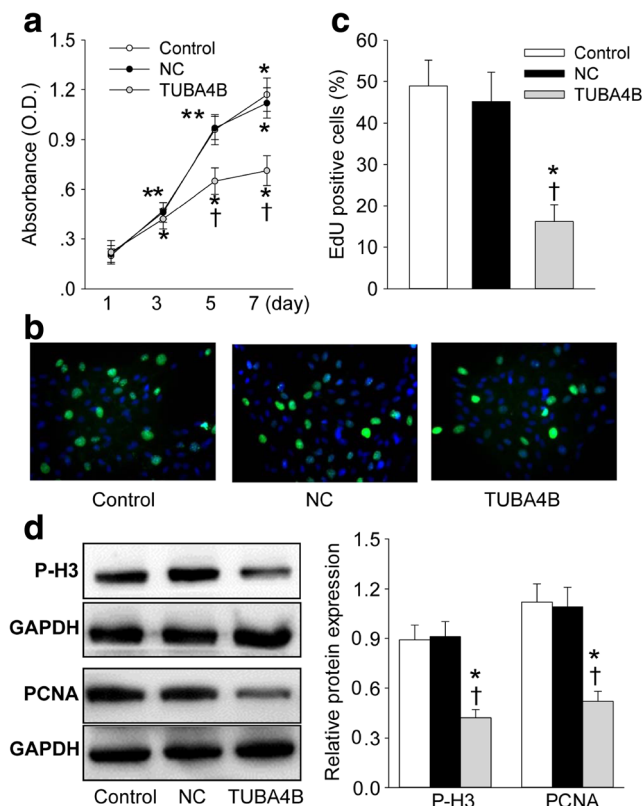

Fig. 4 Effect of lncRNA TUBA4B on the proliferation of SKOV3 cell lines. (**a**) SKOV3 cells were transfected with Control, LV-mediated empty vector or LV-mediated lncRNA TUBA4B vector and measured at day 1, 3, 5 and 7 posttransfection determined with CCK-8 kits; (**b**) Effect of lncRNA TUBA4B pretreatment on DNA synthesis in SKOV3 cells treated for 7 days evaluated with EdU incorporation assay; (**c**) The data were expressed as a ratio of EdU-positive cells to total cells. Blue fluorescence (Hoechst 33,342) shows cell nuclei and green fluorescence (Edu) stands for cells with DNA synthesis; (**d**) Effect of lncRNA TUBA4B on the proliferating cell nuclear antigen (PCNA) and phosphorylated histone H3 (P-H3) levels (markers of proliferation) in SKOV3 cells. Values are mean ± SD. *$P < 0.05$ vs. Control or 1 day. †$P < 0.05$ vs. NC. $n = 6$ for each group

Both MAPK/ERK and Akt pathways are requisite for proliferation of cancer cells during the processes of cancers, including ovarian cancers [16, 17]. Herein, we showed that lncRNA TUBA4B overexpression markedly prevented the phosphorylation levels of Akt and ERK in ovarian cancers cells. These results indicated both Akt and ERK signaling pathways involved in lncRNA TUBA4B-mediated growth of human ovarian cancers cells. However, the potential roles of lncRNA TUBA4B in the migration and invasion of ovarian cancers cells and its underlying molecular mechanisms are needed to be further defined.

In conclusion, our results showed that decreased lncRNA TUBA4B may participate in the progression of ovarian cancer. We also highlighted the potential usefulness of lncRNA TUBA4B for prognosis in patients with ovarian cancer. LncRNA TUBA4B may be considered as a novel molecular target for the diagnosis and treatment of ovarian cancer.
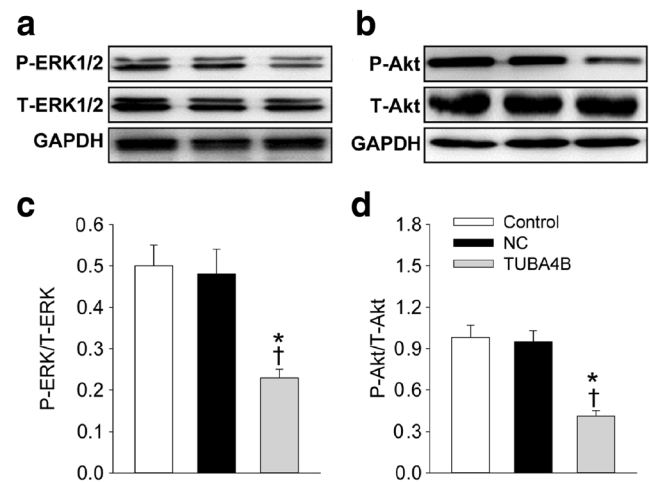

Fig. 5 Involvement of PI3K/Akt and ERK signaling pathways in the proliferation of SKOV3 cell lines. (**a**) Representative photographs showing the effect of lncRNA TUBA4B overexpression on the phosphorylation of ERK; (**b**) Measurement of the relative gray scale of phosphorylation of ERK; (**c**) Representative photographs showing the effect of lncRNA TUBA4B overexpression on the phosphorylation of Akt; (**d**) Measurement of the relative gray scale of phosphorylation of Akt. Values are mean ± SD. *$P < 0.05$ vs. Control. †$P < 0.05$ vs. NC. $n = 6$ for each group

Compliance with Ethical Standards

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