UBE2T is upregulated, predicts poor prognosis, and promotes cell proliferation and invasion via inhibiting autophagy in ovarian cancer

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Research

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Abstract

Background: Aberrant upregulation and oncogenic roles of UBE2T have been revealed in several cancers. However, the expression, clinical significance, and functions of UBE2T has not been explored in ovarian cancer (OC).

Methods: In this study, the mRNA and protein expression of UBE2T in OC were detected via analyzing the online databases and immunohistochemical staining. Moreover, relations of UBE2T expression with clinopathological features and prognosis OC patients were further analyzed. Besides, the effects of UBE2T knockdown on growth, proliferation, and invasion of OC cells were investigated by CCK-8, plate clone formation, and Transwell assays. Finally, the underlying mechanism of UBE2T associated functions in OC was analyzed.

Results: The results indicated that UBE2T was significantly upregulated in OC tissues. UBE2T expression was notably correlated with clinical features such as primary T stage, TNM stage in OC patients. UBE2T, acting as an independent prognostic indicator, was inversely associated with prognosis of OC patients. UBE2T knockdown remarkably suppressed the growth, proliferation, and invasion of OC cells indicating by impaired cell viability, less cell clones and invasive cells. Mechanistically, the oncogenic roles of UBE2T was exerted by inhibiting autophagy via maintaining AKT/mTOR activity in OC.

Conclusion: Collectively, our findings confirm UBE2T upregulation predicts poor prognosis and promotes malignant progression via suppressing autophagy in OC, suggesting the promising values of UBE2T both in diagnosis and treatment of OC.

Introduction

Ovarian cancer (OC) is one of aggressive gynecologic cancers with the poor prognosis, whose five-year overall survival rate is less than 50%. Consistent with the circumstances for most carcinomas, the favorable therapeutic and prognostic outcomes are just achievable for OC patients at early stage. However, the actual rate of OC patients diagnosed at early stage is as low as 20%, indicating the most OC patients are diagnosed at advanced stages, which largely accounts for the unsatisfactory prognosis of OC patients [1, 2]. Obviously, the unfavorable outcomes suggest the defects of the current biomarkers and treatments of OC and it is vital to explore novel diagnostic and prognostic biomarkers, and therapeutic targets to improve the efficiency of diagnosis and treatment in OC.

Dysregulations of catalytic enzymes, involving regulation of protein stability and degradation, exert critical roles in malignant transformation and progression of cancers [3, 4]. Severing as the main pathway of protein degradation, ubiquitin-proteasome system (UPS) is closely related with carcinogenesis, reflected by the aberrant expression of proteins accounting for catalyzing the ubiquitination reaction [3, 5]. The substrates ubiquitination are sequentially catalyzed by ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3), with ubiquitin. Given their capacities of determining substrate specificity in ubiquitination, the expressions and roles of E3 ligases have been
widely explored in cancers and their roles in malignances are comprehensively revealed [5, 6]. Interestingly, apart from E3 ligases, the aberrant expressions and critical functions of ubiquitin-conjugating enzymes, being considered as a family of constitutive enzymes before, in tumorigenesis have been indicated by the recent work [7].

Ubiquitin-conjugating enzyme E2T (UBE2T) is firstly identified in the study of Fanconi anaemia syndrome (FA) and plays vital roles in the development of FA via catalyzing the mono-ubiquitination of FANCD2 and FANCI. Accordingly, high mutation rate of UBE2T is observed in patients with FA [8]. Recently, the roles of UBE2T have been revealed in cancers. Upregulation of UBE2T, severing as an unfavorable prognostic indicator, has been confirmed in both solid tumors, such as gastric cancer [9, 10], hepatocellular carcinoma [11, 12], breast cancer [13], lung cancer [13, 14], nasopharyngeal carcinoma [15], osteosarcoma [16], prostate cancer [17] and renal cell carcinoma [18], and non-solid tumor like multiple myeloma (MM) [19]. Consistently, UBE2T depletion notably suppresses malignant progression via modulation the activity AKT [18] and p53 [12]. Nevertheless, the expression, clinical significance, functions and the corresponding mechanism of UBE2T has not been revealed in OC.

Therefore, in this study, we examined the mRNA and protein expression, prognostic value of UBE2T in OC via mining the online data and immunohistochemistry assay in local OC cohort. Moreover, we also explored the primary functions of UBE2T in OC by detecting the effects of UBE2T knockdown on cell phenotypes, including growth, proliferation, and invasion, and analyzed the role of autophagy in UBE2T related functions. The outcomes reveal that UBE2T mRNA and protein is upregulated and severs as an indicator for poor prognosis of OC patients. Furthermore, UBE2T knockdown significantly activated autophagy via suppressing AKT/mTOR, and subsequently inhibits growth, proliferation, and invasion of OC cells.

side) <0.05 was considered statistically significant.

**Results**

**UBE2T is upregulated in OC.**

Firstly, we analyzed the expression of UBE2T via several web portals. As shown in Fig. 1A, compared with the normal counterparts, the mRNA expression of UBE2T was significantly upregulated in OC tissues reflected by two data sets in Oncomine [23]. Accordingly, through analyzing the data in GEPIA [24], which integrated the data from TCGA and GTEx databases, upregulation of UBE2T mRNA in OC was further validated (Fig. 1B). Furthermore, Mass-spectrometry-based proteomic data from UALCAN [25] indicated upregulation of UBE2T protein in OC (Fig. 1E). Finally, we analyzed the protein level of UBE2T in a cohort of OC via IHC staining. The results showed that the staining intensity of UBE2T was significantly stronger in OC specimens than in normal samples (Fig. 1F). Thus, these results revealed UBE2T was upregulated in OC both at mRNA and protein level.

**UBE2T correlates to the clinical features of patients with OC.**
Subsequently, we explored the relation between UBE2T expression and clinopathological variables. According to Oncomine data, the UBE2T mRNA level was comparative among different histological types including mucinous, serous and endometrioid type of OC (Fig. 1C), which was confirmed by the results of IHC (Fig. 1F, Table 1). Interestingly, based on the data from GEPIA, UBE2T mRNA level was inversely associated with the stage of OC patients (Fig. 1D). However, the IHC data revealed that although no significant difference of UBE2T protein among stage I, II, III and IV, notable upregulation of UBE2T was observed in advance stage (III+IV) OC tissues than that in early stage (I+II) (Fig. 1F, Table 1). Moreover, UBE2T level positively correlated with primary T stages in OC (Table 1). However, possibly due to the limited sample volumes, UBE2T showed no significant relation with distant metastasis and lymph node metastasis (Table 1). These results revealed that UBE2T was associated with stages of OC patients indicating UBE2T may involve in the progression of OC.

UBE2T inversely associated with the prognostic outcomes and served as an independent indicator for OC patients.

Next, we investigated the relation between UBE2T and prognosis in OC via online and experimental data. The data from Kaplan-Meier Plotter portal [26] indicated UBE2T upregulation was negatively correlated with overall survival (OS), progression-free survival (PFS), and post-progression survival (PPS) of OC patients (Fig. 2A). Besides, GEPIA data confirmed that higher UBE2T mRNA was significantly correlated with poor OS of OC patients (Fig. 2B). Consistently, upregulation of UBE2T protein served as a poor indicator for OC patients as well (Fig. 2C). The univariate Cox proportional hazards regression analysis revealed that primary T stage, TNM stage, distant metastasis, and the level of UBE2T were significantly related to the OS of OC patients (Table 2). The multivariate Cox proportional hazards regression analysis indicated that UBE2T upregulation severed as an independent predictor for poor OS for OC patients (Table 2). Thus, these outcomes confirmed UBE2T was inversely related to the prognosis and served as an independent indicator for poor prognosis for OC patients.

UBE2T depletion inhibits growth, proliferation, and invasion of OC cells.

Next, we further explored the functions of UBE2T in OC. Therefore, we checked the expression of UBE2T, depleted UBE2T expressions with si-UBE2T transfection, and subsequently detected the influences on OC cells. As the qPCR and western blot results shown (Fig. 3A), both mRNA and protein expression of UBE2T were remarkably higher in OC cells, OVCAR3 and SKOV3 than that in IOSE80 cell, an immortalized normal ovarian epithelial cell line. As indicated by Fig. 3B, UBE2T expression was successfully knocked down in OVCAR3 and SKOV3 cells. The CCK-8, plate clone formation and Transwell invasion assays manifested UBE2T depletion significantly suppressed the growth, proliferation, and invasion of OC cells, demonstrated by impaired viability (Fig. 3C), less cell clones (Fig. 3D) and invasive cells (Fig. 3E). Herein, these results unveiled UBE2T depletion could inhibit growth, proliferation and invasion of OC cells.

UBE2T knockdown suppresses malignant progression of OC cells via activating autophagy by inhibiting AKT/mTOR.
The roles of autophagy in OC have been widely explored [27, 28]. Considering the regulatory effects of UBE2T on AKT [18] and p53 [12], which are vital regulators of autophagy [28], we explored the function of UBE2T in autophagy of OC cells. As the results shown, UBE2T knockdown activated the autophagy of OC cells indicating by upregulated BECN1, accumulated LC3B, and decreased p62 (Fig. 4A). Moreover, the accumulative effects of LC3B were augmented under CQ (50 μM) treatment (Fig. 4B), further confirming autophagy activation induced by UBE2T knockdown. Simultaneously, UBE2T depletion inhibited AKT/mTOR, reflected by decreased p-AKT (S473) and p-mTOR (S2448) (Fig. 4A), but exerted no influence on p53 (Fig. 4A), suggesting AKT/mTOR axis mediating the regulation of UBE2T on autophagy. Therefore, further experiments were performed to validate this notion. Accordingly, ectopic UBE2T expression could re-inhibit autophagy in OC cells with endogenous UBE2T knockdown, and the rescue effect was deprived when AKT inhibitor MK2206 (2.5 μM) was added, demonstrating UBE2T could inhibit autophagy via sustaining AKT/mTOR activity (Fig. 4C). Meanwhile, consistent phenotypes were observed indicating by CCK-8 (Fig. 4D), plate clone formation (Fig. 4E), and Transwell invasion results (Fig. 4F). Thus, we revealed that UBE2T knockdown suppressed malignant progression of OC cells via activating autophagy by inhibiting AKT/mTOR.

**Discussion**

Here, we demonstrated that the mRNA and protein level of UBE2T was notably upregulated in OC tissues and cells. Upregulation of UBE2T was inversely correlated with stages and prognosis of OC patients. UBE2T inhibition remarkably suppressed the growth, proliferation and invasion of OC cells via activating autophagy by suppressing AKT/mTOR. Collectively, our findings suggest the promising values of UBE2T as a prognostic biomarker and therapeutic target in OC.

The roles of UBE2T have been primarily revealed in FA related ubiquitin signaling [8]. UBE2T is critical for maintenance of genome integrity in FA involving its regulatory roles in mono-ubiquitination of FANCD2 and FANCI. Notably, FA patients bearing related gene mutations are particularly prone to malignant outcomes, suggesting the vital roles of FA related genes in tumorigenesis [8]. Indeed, aberrant upregulation of UBE2T has been observed in a host of cancers. For example, UBE2T is remarkably upregulated in MM cells, especially in the early stage. UBE2T level is significantly associated with IgG serotype of MM and UBE2T upregulation predicts poor prognosis, including OS and event-free survival time, of patients with MM [29]. Furthermore, UBE2T is upregulated in gastric cancer tissues and cells, positively associated with poor differentiation, advanced T stage, and short OS time in patients with gastric cancer [9, 10]. Accordingly, UBE2T upregulation, severing as a poor prognostic biomarker, has been confirmed in nasopharyngeal carcinoma [20], osteosarcoma [16], lung cancer [13, 14], breast cancer [16], prostate and hepatocellular cancer [11, 12, 17, 26]. Consistently, we demonstrated that UBE2T mRNA and protein was upregulated in OC tissues and cells and UBE2T upregulation was associated with stages and poor prognosis OC patients.

The functions of UBE2T have been explored in cancers. UBE2T knockdown exerts significantly inhibitory effects on cell characteristics via different mechanisms. By inhibiting AKT and related pathways, UBE2T
Ubiquitin conjugating enzymes can exert essential regulatory roles in physiopathologic processes, including cancers, via modulating autophagy [8, 33, 34]. UBE2L6 depletion can activate autophagy and regulate the chemosensitivity of esophageal cancer cells [33]. Serving as either oncogenic or anti-tumor regulator, autophagy is widely implicated in regulation of stem maintenance, proliferation, invasion, metastasis, and therapy resistance of OC [27, 28, 35]. Here, we validated that UBE2T inhibition could promote autophagy, subsequently suppress the malignant characteristics of OC cells via constraining AKT/mTOR.

Conclusion

In conclusion, we initially and comprehensively explored the expression, clinical significance, fundamental functions, and underlying mechanisms of UBE2T in OC. The outcomes reveal that UBE2T is notably increased in OC, which severs as an indicator for poor prognosis in OC patients. UBE2T knockdown suppresses AKT/mTOR activity, subsequently activates autophagy, and eventually inhibits growth, proliferation, and invasion of OC cells. These findings contribute a better understanding of development and progression of OC and present UBE2T as a promising biomarker and therapeutic target in diagnosis and treatment of OC, respectively.

Methods

Tissue samples, Ethical Statement and Immunohistochemistry (IHC)

An OC tissue chip, containing 70 OC tissues and 10 normal tissues (#OVC1504) was purchased from the shanghai Superbiotek Pharmaceutical Technology Inc. (Shanghai, China). The study was approved by the Academic Ethics Committee of the Second Xiangya Hospital of Central South University and performed under the instructions of Declaration of Helsinki. IHC and scoring were applied according to our previous description [20] with primary antibody UBE2T (dilution: 1:50, BBI, Shanghai, China).

Cell Culture

Normal ovarian epithelial cell line IOSE80, and OC cell lines, SKOV3 and OVCAR3, were purchased from the American Type Culture Collection (ATCC, VA, USA). SKOV3 was maintained in RPMI-1640 medium plus 10% fetal bovine serum (FBS, Thermo, MA, USA). OVCAR3 and IOSE80 were cultured in DMEM
medium plus 10% FBS (Thermo, MA, USA). The cells were culture in a humidified incubator at 37°C and 5% CO₂. The chloroquine (CQ, Sigma-Aldrich, MO, USA) and MK2206 (Selleck, TX, USA) were added into the culture medium for functional experiments as indicated in figure legends.

Small interfering RNAs (siRNAs) and plasmids transfection

The UBE2T siRNA (si-UBE2T) and scrambled siRNA (si-NC) were purchased from RiboBio Inc. (Guangzhou, China). The UBE2T expression plasmid, pENTER-UBE2T, and the control plasmid, pENTER-vector, were obtained from Vigene Inc. (Jinan, China). The si-UBE2T sequences are 5'-GTCTGGTTCATCTTAGTTAA-3', which targets the 3' untranslated region of UBE2T mRNA. The siNC sequences were not offered by the manufacturer. SKOV3 and OVCAR3 cells were transfected with si-UBE2T or co-transfected with siUBE2T and pENTER-UBE2T using Lipofectamine™ 2000 (Thermo, MA, USA) according to our previous description [21].

RNA isolation and quantitative real-time PCR(qPCR)

The total RNA isolation and qPCR was carried out as previous description [22]. The expressions of UBE2T was determined by qPCR with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control. The primer sequences of UBE2T and GAPDH are as follows. UBE2T, Forward 5'-ATCCCTCAACATCGCAACTGT-3', Reverse 5'-CAGCCTCTGGTAGATTATCAAGC-3'; GAPDH, Forward 5'-ATGGAGAAGGCTGGGGCTC-3', Reverse 5'-AAGTTGTCATGGATGACCTTG-3'.

Cell growth assay

The cell growth assay was performed as previously described [21]. The effects of UBE2T rescue and MK2206 addition on cell growth were reflected by inhibitory growth rate with si-NC group as control. The experiments were independently performed in triplicate.

Plate clone formation assay

Plate clone formation assay was carried out as previously described [22]. The experiments were independently performed for three times.

Transwell invasion assay

Transwell invasion assay was performed as previous description [22]. The experiments were independently performed for three times.

Western blot

The western blot was performed as previous description [22]. Primary antibodies, including UBE2T (BBI, Shanghai, China), p53(Santa Cruz, TX, USA), BECN1(Abcloonal, Wuhan, China), p62(Abcloonal, Wuhan, China), LC3A/B (Abcloonal, Wuhan, China), p-AKT(S473) (CST, MA, USA), AKT(CST, MA, USA), mTOR (CST,
p-mTOR(S2448) (CST, MA, USA), β-actin (Proteintech, Wuhan, China), were used to detect their levels under specific treatments.

**Statistical analysis**

Statistical analyses and statistical charts were analyzed and produced using SPSS20.0 software and GraphPad Prism version 8. For comparisons between two groups, a Student t-test or chi-square test was carried out. Survival curves were obtained via Kaplan-Meier method, and the statistical analysis was evaluated by Log-rank test. The univariate and multivariate Cox regression was performed to analyze the relationship of among UBE2T expression, clinicopathological parameters, and survival in OC patients. For all analyses, \( P \) (two side) <0.05 was considered statistically significant.

**Declarations**

**Ethics approval and consent to participate**

The study was approved by the Academic Ethics Committee of the Second Xiangya Hospital of Central South University (OV_XXM_20191128) and performed under the instructions of Declaration of Helsinki. The informed consent were obtained from the all enrolled patients.

**Consent for publication**

Not applicable.

**Availability of data and materials**

The online databases, including Oncomine(www.oncomine.org), GEPIA(gepia.cancer-pku.cn), and UALCAN(ualcan.path.uab.edu), were used. Other data and materials are available from the corresponding author on reasonable request.

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**Author’s contributions**

X-M. X and WH designed this study, wrote and revised the manuscript. X-M. X and X-L. F supervised this study. H-Y. H and WH performed the most experiments. Y-Z. X and LW performed the online analyses. T-T. Z and LW contributed to the statistical analysis and picture process.

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Not applicable.
Competing interests

There are no conflicts of interest in this work by all authors.

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Tables

Table 1. Correlation between UBE2T expression and clinicopathological characteristics in Ovarian Cancer (n=70, χ2 test)

| Variables                      | n   | Expression level | χ2  | P       |
|--------------------------------|-----|------------------|-----|---------|
|                                |     | Low (1-3)        | High (4-6) |       |
| Age (years)                    |     |                  |       |         |
| ≥50                            | 37  | 9                | 28   | 1.204   | 0.273  |
| <50                            | 33  | 12               | 21   |         |        |
| Primary tumor (T) stage        |     |                  |       |         |
| T1-2                           | 36  | 18               | 18   | 14.118  | 0.000172 |
| T3-4                           | 34  | 3                | 31   |         |        |
| Lymph node (N) metastasis      |     |                  |       |         |
| N0                             | 67  | 21               | 46   | -       | 0.549  |
| N1-3                           | 3   | 0                | 3    |         |        |
| Distant metastasis (M)         |     |                  |       |         |
| M0                             | 65  | 21               | 44   | -       | 0.313  |
| M1                             | 5   | 0                | 5    |         |        |
| Clinical TNM stage             |     |                  |       |         |
| I-II                           | 35  | 18               | 17   | 15.306  | 0.000091 |
| III-IV                         | 35  | 3                | 32   |         |        |
| Pathological Classification    |     |                  |       |         |
| Endometrioid                   | 15  | 6                | 9    | 2.074   | 0.549  |
| Mucinous                       | 7   | 3                | 4    |         |        |
| Serous                         | 48  | 12               | 36   |         |        |
Table 2. Univariate analysis and Multivariate analysis of prognostic factors for overall survival using Cox proportional hazards regression model (N=70)

| Variables                | Univariate analysis | Multivariate analysis |
|--------------------------|---------------------|-----------------------|
|                          | HR(95% CI)          | P                     | HR(95% CI)          | P                     |
| Age (years)              |                     |                       |                      |                       |
| ≥50 vs ≥50               | 1.292(0.642-2.602)  | 0.473                 | 1.062(0.521-2.166)  | 0.868                 |
| Primary tumor (T) stage  |                     |                       |                      |                       |
| T1-2 vs T3-4             | 4.393(2.026-9.524)  | 0.0002                | /                    | /                    |
| Lymph node (N) metastasis|                     |                       |                      |                       |
|                         | 4.547(0.530-39.010) | 0.167                 | 4.729(0.532-42.062) | 0.163                 |
| Distant metastasis (M)   |                     |                       |                      |                       |
|                         | 8.480(2.698-26.655) | 0.0003                | 6.428(1.994-20.720) | 0.002                 |
| TNM stage                |                     |                       |                      |                       |
|                         | 4.353(2.007-9.442)  | 0.0003                | /                    | /                    |
| Pathological Classification|                    |                       |                      |                       |
|                         | 1.423(0.898-2.255)  | 0.133                 | 1.272(0.8-2.022)     | 0.308                 |
| UBE2T                    |                     |                       |                      |                       |
| High vs Low              | 5.992(1.823-19.696) | 0.003                 | 4.986(1.476-16.849)  | 0.01^a                |

HR: hazard ratio; 95% CI: 95% confidence interval;  

**Figures**
Figure 1

The expression of UBE2T in OC and its relation to clinical features of patients with OC. Oncomine data (A) and GEPIA data (B) indicated UBE2T mRNA upregulated in OC. (C) The Oncomine data showed no difference of UBE2T mRNA expression among mucinous, serious and endometriod OC tissues. (D) GEPIA data showed UBE2T mRNA significantly correlated to stage of OC. (F) UALCAN data showed UBE2T protein was upregulated in OC tissues. (F) IHC results of UBE2T protein level in normal tissues and OC tissues with different pathological types and stages. *P < 0.05, ** P < 0.01, ***P < 0.001, ns, no significant statistical difference.
Figure 2

UBE2T upregulation predicts poor prognosis of patients with OC. (A) UBE2T mRNA level was inversely associated with OS (left panel), PFS (middle panel), and PPS (right panel) of OC patients indicated by Kaplan-Meier Plotter data. (B) UBE2T mRNA level was inversely associated with OS of OC patients indicated by GEPIA data. (C) UBE2T protein level was negatively related to OS of OC patients demonstrated by our data.
Figure 3

UBE2T depletion inhibits growth, proliferation and invasion of OC cells. (A) qPCR and western blot indicating the UBE2T expression among IOSE80, SKOV3 and OVCAR3 cell. (B) Western blot indicating the knockdown efficacy of siUBE2T in OC cells. Analyzing the effects of UBE2T knockdown on growth, proliferation, and invasion via CCK-8 (C), plate clone formation (D), and transwell invasion (E). *P <0.05, ** P <0.01, ***P <0.001.
Figure 4

UBE2T depletion inhibited malignant progression of OC cells by inducing autophagy via suppressing AKT/mTOR axis. (A) Western blot indicating the level of p53, p-AKT (S473), p-mTOR (S2448), and autophagy related proteins in OC cells with UBE2T knockdown. (B) Western blot indicating the accumulated LC3B of OC cells with UBE2T knockdown under CQ (50μM) treatment. (C) Western blot indicating the influences of MK2206 (2.5μM) on the rescue effects of UBE2T on autophagy in OC cells with UBE2T knockdown. CCK-8 (D), plate clone formation (E), and transwell invasion (F) assays indicating the influences of MK2206 (2.5μM) on the rescue effects of UBE2T on autophagy in OC cells with UBE2T knockdown.