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# Expression of TBC1D16 Is Associated with Favorable Prognosis of Epithelial Ovarian Cancer

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Epithelial ovarian cancer (EOC) is the most lethal gynecologic malignancy with high recurrence and poor prognosis duo to the lack of effective biomarkers. TBC1 domain family member 16 (TBC1D16), a GTPase-activating protein, is involved in regulating intracellular trafficking in tumorigenesis and metastasis. However, the clinical significance of TBC1D16 in EOC remains unknown. In the present study, we investigated the expression and prognostic significance of TBC1D16 in EOC and its relationship with the expression of vascular endothelial growth factor (VEGF). The tissue specimens included 156 histologically confirmed EOC and 30 normal ovarian tissues. The expression of TBC1D16 and VEGF was detected by immunohistochemistry (IHC), and the immunoreactive score was calculated with signal intensity and percentage of positive cells. IHC results showed that TBC1D16 and VEGF were both mainly localized in cytoplasm of epithelial cells in normal ovarian tissues and were expressed in cancer cells. Based on the immunoreactive score, TBC1D16 expression in EOC was categorized as "high expression," compared with normal ovarian tissues ($P < 0.05$). The Chi-square test showed that high TBC1D16 expression was related to advanced pT stages ($P = 0.029$), but not correlated with other clinical features. Moreover, the TBC1D16 expression was significantly higher in EOC specimens with low VEGF expression ($P < 0.001$). Importantly, in both univariate and multivariate survival analyses, high expression of TBC1D16 was significantly correlated with good overall survival (OS). In conclusion, TBC1D16 is a predictive marker for favorable prognosis of EOC.

**Keywords:** biomarker; epithelial ovarian cancer; prognosis; TBC1 domain family member 16; vascular endothelial growth factor
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## Introduction

Ovarian cancer is the seventh most common cancer in women, with 239,000 new cases and 152,000 deaths estimated in 2012 around the world (Ferlay et al. 2015). Due to difficulties in early detection and diagnosis, the overall survival (OS) of patients with ovarian cancer is poor (Chiang et al. 2013; Kurosaki et al. 2016). Epithelial ovarian cancer (EOC) is the majority of ovarian cancer, and its OS could be affected by many factors, including cancer stage, histological type, early recognition, patient management, and age (Chiang et al. 2013; Kajiyama et al. 2014; Bristow et al. 2015; Wright et al. 2015; Anuradha et al. 2016). Approximately 75% of patients are diagnosed with advanced ovarian cancer (stage III or IV), in which the 5-year survival rate is 15-20% (Dunberger et al. 2013). Therefore, it is urgent to ascertain an effective diagnostic method related to prognosis in the early stage of EOC.

The Tre2-Bub2-Cdc16 (TBC) domain-containing Rab-specific GTPase-activating proteins are vital in regulating intracellular vesicular-membrane trafficking routes (Fukuda 2011; Frasa et al. 2012). As a member of the TBC domain-containing family of proteins, TBC1D16 is involved in various kinds of diseases, such as systolic blood pressure (Franceschini et al. 2014), obesity (Pietiläinen et al. 2016) and cancer (Akavia et al. 2010; Hoek et al. 2010). In cancer, it has been demonstrated that TBC1D16 overexpression enhanced melanoma progression by targeting epidermal

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growth factor receptor (EGFR) (Vizoso et al. 2015). Further research shows that TBC1D16 can regulate transferrin receptor recycling and EGFR signaling by activating Rab4A in melanoma (Goueli et al. 2012). However, it is unclear whether TBC1D16 influences EOC progression.

The cancer progression depends on the formation of an adequate vascular support and amoeboid mode of cancer cell invasion (Patterson et al. 2011). Vascular endothelial growth factor (VEGF) is a potent mediator of angiogenesis that induces the formation of novel blood vessels, and growth and metastasis of cancers. Several researches have revealed that VEGF plays an important role in promoting endothelial cell proliferation and migration, and induces a direct effect on cancer proliferation and invasiveness (Awazu et al. 2013; Hu et al. 2013; Sun et al. 2014). It has been reported that TBC domain containing proteins also participate in cancer proliferation and angiogenesis via VEGF (Kim et al. 2003; Ji et al. 2004). However, the function of TBC1D16 in angiogenesis has not been reported.

In this study, we investigated the role of TBC1D16 in the progression of EOC and its relevance to VEGF by detecting TBC1D16 and VEGF in 156 EOC samples with immunohistochemistry (IHC). Subsequently, we analyzed the potential correlation between expression of TBC1D16 and VEGF with their clinicopathological features and evaluated their prognostic values in EOC.

## Patients and Methods

*Human tissues*

A total of 214 tissue samples were obtained from patients diagnosed with ovarian tumor according to the criteria of the World Health Organization in the First Affiliated Hospital of Sun Yat-Sen University from 1996 to 2008. The selected tumors contained 156 histologically confirmed EOC, 28 borderline tumors and 30 benign cystadenomas (Table 1). All patients selected had not received radiotherapy, chemotherapy or hormonal therapy before surgical resection. The study was reviewed and permitted by the Institute Research Medical Ethics Committee of First Affiliated Hospital of Sun Yat-Sen University.

*Tissue microarray (TMA) construction*

The tissue specimens were fixed in formalin and then embedded in paraffin after surgical resection. The TMA was constructed with the paraffin-embedded samples according to previously described methods (Yang et al. 2010). Briefly, two representative tissue cylinders were cut from every donor block and then were re-embedded in a new blank paraffin block in predetermined position, with a tissue arraying instrument (Beecher Instruments, Silver Spring, MD).

*Immunohistochemistry*

The TMA was cut into 5 $\mu$m by rotary microtome and adhered onto slides. The slides were deparaffinized and rehydrated through graded ethanol in standard procedures. Subsequently, the slides were blocked with 3% $H_2O_2$ reagent for 10 minutes, incubated with anti-TBC1D16 antibody (Abcam; 1:200 dilution) or anti-VEGF antibody (Proteintech; 1:500 dilution) overnight at 4°C. Then, the slides were incubated with the secondary antibody and stained with 3,3-diaminobenzidine (DAB). Negative controls were generated by omitting the primary antibody.

*Immunoreactive score*

The immunoreactive scores were obtained based on a semi-quantitative staining index with two experienced pathologists blinded to the study. The immunoreactive score (with scores from 0 to 9) was calculated by multiplying the signal intensity (negative, 0; weakly positive, 1; moderate positive, 2; strong positive, 3) and the percentage of positive tumor cells (< 10%, 1; 10-50%, 2; > 50%, 3). Determination of the immunoreactive score of 4 as the cut-off point to evaluate TBC1D16 and VEGF expression was based on the median of TBC1D16 and VEGF staining results in EOC. Protein expression with the immunoreactive score of ≥ 4 was categorized as "high expression" or "overexpression."

*Statistical analysis*

All statistical analyses were analyzed by SPSS 20.0 software (SPSS Inc., Chicago, IL). The Chi-square test was applied to analyze the relationship between the expression of TBC1D16 and VEGF and the factors of clinical pathology in EOC. Univariate survival analysis was performed by the Kaplan-Meier method, and the significant differences in survival were calculated by log-rank analysis. Multivariate survival analysis was performed by the Cox proportional hazards regression model. The P value of $< 0.05$ was considered statistically significant.

## Results

*Expression of TBC1D16 and VEGF in ovarian tissues*

We investigated the expression of TBC1D16 and VEGF in different ovarian tissues with IHC. TBC1D16 and VEGF were both mainly localized in cytoplasm of epithelial cells in normal ovarian tissues and were expressed in cancer cells (Fig. 1). According to the immunoreactive score described in Methods, the protein level was divided into low expression and high expression. The Chi-Square test showed that increased frequencies of TBC1D16 and VEGF high-expression were detected in EOC, compared

Table 1. The expression of TBC1D16 and VEGF in different types of ovarian tissues.

| | | TBC1D16[a] | | VEGF[a] | |
|---|---|---|---|---|---|
| | Cases | Low | High | Low | High |
| **Normal ovary** | 30 | 30 (100.0%) | 0 (0.0%) | 30 (100.0%) | 0 (0.0%) |
| **Cystadenoma** | 30 | 27 (90.0%) | 3 (10.0%) | 27 (90.0%) | 3 (10.0%) |
| **Borderline tumor** | 28 | 23 (82.1%) | 5 (17.9%) | 19 (67.9%) | 9 (32.1%) |
| **Epithelial ovarian cancer** | 156 | 96 (61.5%) | 60 (38.5%) | 69 (44.2%) | 87 (55.8%) |

[a]A significant increasing frequency of overexpression of TBC1D16 or VEGF was observed in cystadenomas, borderline tumors and EOC ($P < 0.05$, Chi-Square Test for Trend).

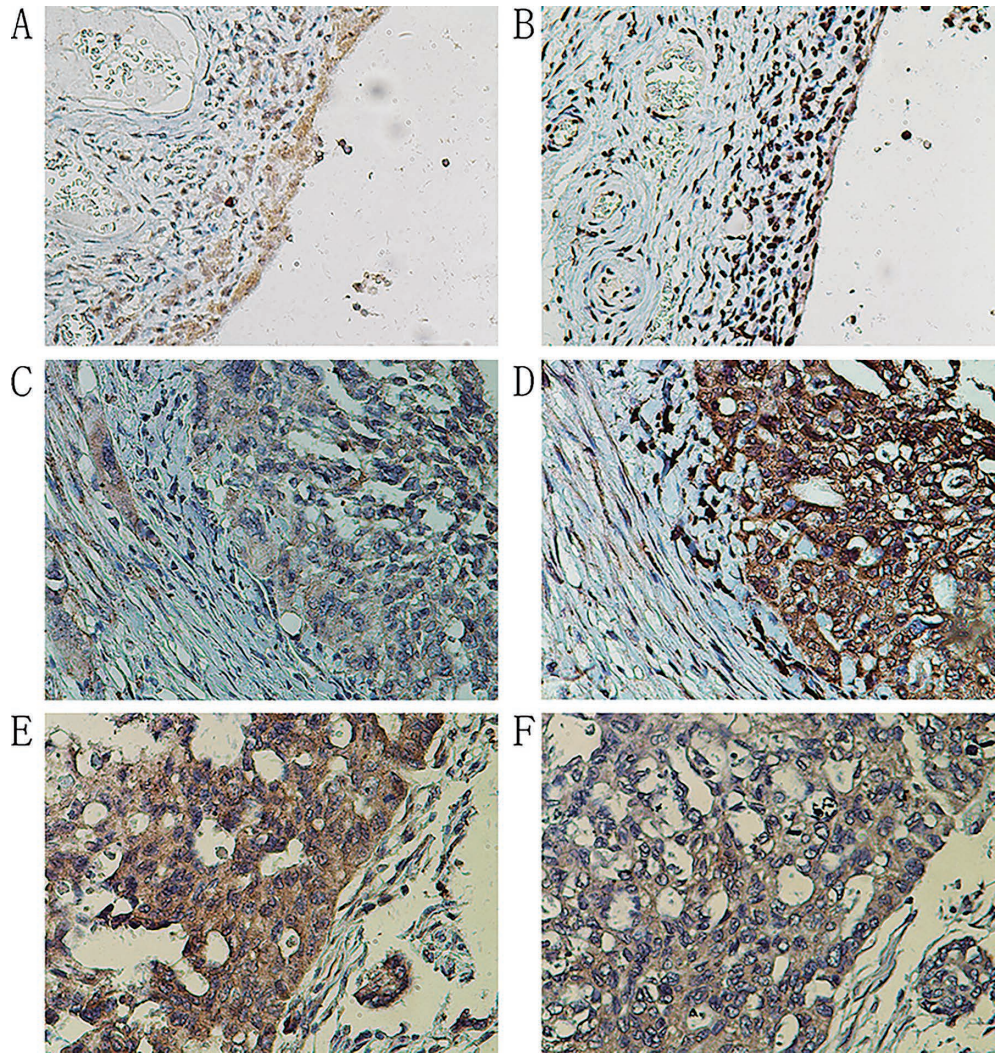


Fig. 1. TBC1D16 and VEGF expression in human ovarian tissues.
(A, B) Normal ovarian tissues from patient 1. (A) Low expression of TBC1D16: both the staining intensity and positive cell percentage scores were 1, total score is 1. (B) Low expression of VEGF: both the staining intensity and positive cell percentage scores were 1; thus, total score is 1.
(C, D) Serous EOC tissues from patient 2. (C) Low expression of TBC1D16: the score of staining intensity is 1 and the score of positive cell percentage is 3; total score is 3. (D) High expression of VEGF: the score of staining intensity is 3 and the score of positive cell percentage is 3; total score is 9.
(E, F) Serous EOC tissues from patient 3. (E) High expression of TBC1D16: the score of staining intensity is 3 and the score of positive cell percentage is 3; total score is 9. (F) Low expression of VEGF: the score of staining intensity is 1 and the score of positive cell percentage is 3; total score is 3. Original magnification: × 400.

with borderline ovarian tumors and cystadenomas and normal ovarian tissues, respectively ($P < 0.05$, Table 1).

*Correlation of TBC1D16 and VEGF expression with the clinicopathological characteristics of patients with EOC*

The association between expression of TBC1D16 and VEGF in EOC and clinicopathological parameters was further analyzed (Table 2). TBC1D16 overexpression was closely related to advanced pT stages ($P = 0.029$) of EOC, while VEGF overexpression was correlated to histological types and advanced pN stages of EOC ($P = 0.038$ and $P = 0.036$, respectively). The other clinicopathological features were not related to the expression TBC1D16 and VEGF, such as age, histological grade (Silveberg), FIGO stages (International Federation of Gynecology and Obstetrics) and pM stages ($P > 0.05$, Table 2). The Chi-square test revealed that TBC1D16 overexpression had a significant correlation with low VEGF expression ($P < 0.01$, Table 3, Fig. 1C-F).

*OS analysis of EOC patients*

Univariate analysis was performed using the Kaplan-Meier method. The results showed that OS of EOC patients was correlated to several clinical pathological prognostic

Table 2. Correlation of TBC1D16 expression with clinicopathological characteristics of EOC.

| | | TBC1D16 expression | | P | VEGF expression | | P |
|---|---|---|---|---|---|---|---|
| | Cases | Low (n, %) | High (n, %) | value[c] | Low (n, %) | High (n, %) | value[c] |
| **Age at surgery（years）** | | | | 0.100 | | | 0.872 |
| ＜ 50[a] | 78 | 43（55.1%） | 35（44.9%） | | 35（44.9%） | 43（55.1%） | |
| ＞ 50 | 78 | 53（67.9%） | 25（32.1%） | | 34（43.6%） | 44（56.4%） | |
| **Histological type** | | | | 0.258 | | | 0.038[d] |
| Serous | 107 | 62（57.9%） | 45（42.1%） | | 44（41.1%） | 63（58.9%） | |
| Mucinous | 18 | 14（77.8%） | 4（22.2%） | | 13（72.2%） | 5（27.8%） | |
| Others[b] | 31 | 20（64.5%） | 11（38.6%） | | 12（38.7%） | 19（61.3%） | |
| **Histological grade (Silveberg)** | | | | 0.669 | | | 0.057 |
| G1 | 26 | 18（69.2%） | 8（30.8%） | | 17（65.4%） | 9（34.6%） | |
| G2 | 91 | 55（60.4%） | 36（39.6%） | | 37（40.7%） | 54（59.3%） | |
| G3 | 39 | 23（59.0%） | 16（41.0%） | | 15（38.5%） | 24（61.5%） | |
| **pT status** | | | | 0.029[d] | | | 0.327 |
| $pT_1$ | 45 | 35（77.8%） | 10（22.2%） | | 24（53.3%） | 21（46.7%） | |
| $pT_2$ | 29 | 16（55.2%） | 12（44.8%） | | 11（37.9%） | 18（62.1%） | |
| $pT_3$ | 82 | 45（54.9%） | 37（45.1%） | | 34（41.5%） | 48（58.5%） | |
| **pN status** | | | | 0.188 | | | 0.036[d] |
| $pN_0$ | 78 | 52（66.7%） | 26（33.3%） | | 41（52.6%） | 37（47.4%） | |
| $pN_1$ | 78 | 44（56.4%） | 34（43.6%） | | 28（35.9%） | 50（64.1%） | |
| **pM status** | | | | 0.391 | | | 0.323 |
| $pM_0$ | 133 | 80（60.2%） | 53（39.8%） | | 61（45.9%） | 72（54.1%） | |
| pM1 | 23 | 16（69.6%） | 7（30.4%） | | 8（34.8%） | 15（65.2%） | |
| **FIGO stage** | | | | 0.054 | | | 0.123 |
| I | 30 | 24（80.0%） | 6（20.0%） | | 19（63.3%） | 11（36.7%） | |
| II | 17 | 8（47.1%） | 9（52.9%） | | 7（41.2%） | 10（58.8%） | |
| III | 86 | 48（55.8%） | 38（44.2%） | | 35（40.7%） | 51（59.3%） | |
| IV | 23 | 16（61.5%） | 7（31.5%） | | 8（34.8%） | 15（65.2%） | |

[a]Mean age.
[b]Endometrioid, clear cell and undifferentiated types.
[c]The Chi-square test.
[d]Statistically significant.

Table 3. Association between the expression of TBC1D16 and VEGF in EOC tissues.

| | TBC1D16 expression | | |
|---|---|---|---|
| **VEGF expression** | **Low** | **High** | **P value[a]** |
| **Low** | 56 | 13 | < 0.001[b] |
| **High** | 40 | 47 | |

[a]The Chi-square test.
[b]Statistically significant.

parameters, include histological grade (P = 0.012), pT stages (P = 0.002), pN stages (P < 0.001), pM stages (P < 0.001), and FIGO stages (P < 0.001) (Table 4). Furthermore, high TBC1D16 expression in EOC predicted good survival (P = 0.007, Table 4, Fig. 2). The results of Cox proportional hazards model indicated TBC1D16 may be an independent prognostic factor for OS (Relative risk (RR) = 0.326, 95% confidence interval (CI) = 0.183-0.581, P < 0.001, Table 5). In addition, pN status (P = 0.024) and FIGO stage (P < 0.001) were also confirmed as independent prognostic factors of EOC. Receiver operating characteristic (ROC) curve analysis suggested the potential predictive value of TBC1D16 in OS for EOC patients (area under curve (AUC) = 0.613, P = 0.016, Fig. 3).

## Discussion

EOC is the most lethal gynecologic malignancy and more than 70% of EOC patients at advanced stage will develop recurrence (Jelovac and Armstrong 2011; Petrillo et al. 2013). Due to the high rate of relapse, the mortality of EOC patients at advanced stage is not optimistic, even after standard front-line therapy (Chatterjee et al. 2016). The poor survival of EOC patients is mainly caused by the lack of effective diagnostic methods. Thus, it is paramount to explore an effective diagnostic method of early stage of EOC patients (Tung et al. 2008).

Table 4. Univariate survival analysis (log-rank test) of the expression of TBC1D16 and VEGF with clinicopathological parameters in EOC patients.

| Variable | Cases | Mean survival (months) | Median survival (months) | P value |
|---|---|---|---|---|
| **Age at surgery（years）** | | | | 0.674 |
| ＜ 50[a] | 78 | 102.3 | 136 | |
| ＞ 50 | 78 | 109.1 | 147 | |
| **Histological type** | | | | 0.977 |
| Serous | 107 | 98.8 | 136 | |
| Mucinous | 18 | 107.7 | NR[c] | |
| Others[b] | 31 | 112.0 | 147 | |
| **Histological (Silveberg)** | | | | 0.012[d] |
| G1 | 26 | 136.7 | 147 | |
| G2 | 91 | 110.9 | NR[c] | |
| G3 | 39 | 63.9 | 36 | |
| **pT status** | | | | 0.002[d] |
| $pT_1$ | 45 | 150.8 | NR[c] | |
| $pT_2$ | 29 | 97.8 | 147 | |
| $pT_3$ | 82 | 85.9 | 49 | |
| **pN status** | | | | ＜ 0.001[d] |
| $pN_0$ | 78 | 133.2 | NR[c] | |
| $pN_1$ | 78 | 66.9 | 45 | |
| **pM status** | | | | ＜ 0.001[d] |
| $pM_0$ | 133 | 122.7 | NR[c] | |
| $pM_1$ | 23 | 36.1 | 14 | |
| **FIGO stage** | | | | ＜ 0.001[d] |
| Ⅰ | 30 | 173.5 | NR[c] | |
| Ⅱ | 17 | 133.8 | 147 | |
| Ⅲ | 86 | 92.7 | 136 | |
| Ⅳ | 23 | 36.1 | 14 | |
| **TBC1D16** | | | | 0.007[d] |
| Low | 96 | 97.8 | 64 | |
| High | 60 | 128.7 | NR[c] | |
| **VEGF** | | | | 0.878 |
| Low | 69 | 111.7 | 147 | |
| High | 87 | 94.1 | NR[c] | |

[a]Mean age.
[b]Endometrioid, clear cell and undifferentiated types.
[c]Not reached.
[d]Statistically significant.

TBC1D16 is a GTPase-activating protein for RAB family proteins, which contains a conserved Tre2/Bub2/Cdc16 (TBC) domain. It is mainly located in cytoplasm and participates in cellular events by regulating vesicular trafficking. In recent studies, it has been found that TBC1D16 could regulate transferrin receptor recycling and EGFR signaling by activating Rab4A in melanoma and epigenetic reactivation of TBC1D16 is associated with poor clinical outcome in melanoma (Goueli et al. 2012; Vizoso et al. 2015). However, there were no studies about the role of TBC1D 16 in ovarian cancer. In this study, we detected the expression of TBC1D16 in 156 EOC samples by IHC and found that TBC1D16 was highly expressed in EOC. Compared with normal ovarian tissues, cystadenomas and borderline specimens, TBC1D16 was significantly overexpressed in EOC ($P < 0.05$). Further analysis indicated that high expression of TBC1D16 was correlated with advanced pT stages ($P = 0.029$). However, this result appears to be contradictory to the survival data of EOC patients; namely, high expression of TBC1D16 was correlated with good survival for EOC patients by univariate and multivariate analysis ($P < 0.05$). This might be caused by the limitation of sample size and single detective method of the present study. Although further trials are needed to study the relationship between TBC1D16 and pT stages, overexpression of TBC1D16 protein has positive impact on survival for EOC patients and may serve as a predictor for favorable prognosis.

As a novel tumor-related gene, TBC1D16 was found to promote EGFR degradation and decrease EGFR signaling in melanoma (Goueli et al. 2012), and it is unclear whether TBC1D16 could influence VEGF to mediate angiogenesis in cancer, which is another powerful growth factor in angiogenesis (Reinthaller 2016). In our study, the

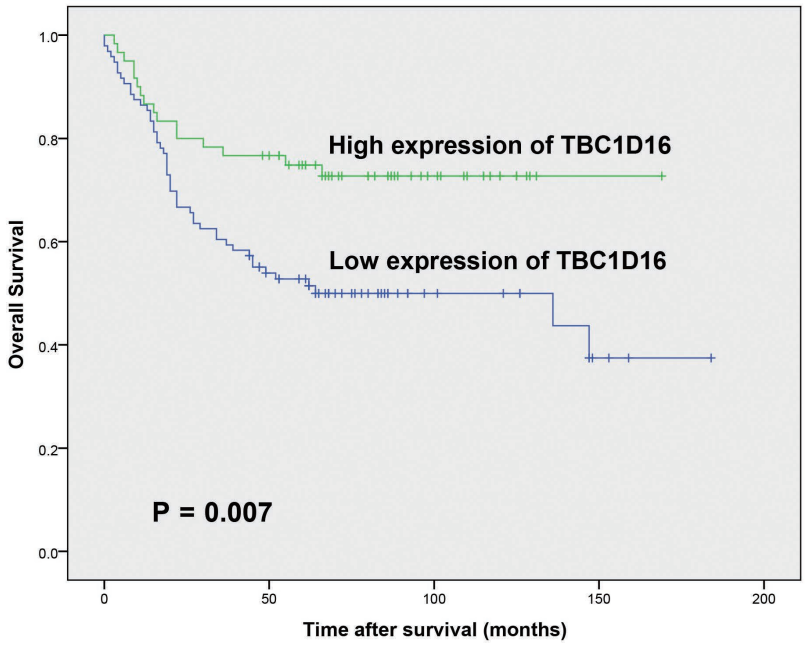


Fig. 2. TBC1D16 expression in 156 EOC patients with Kaplan-Meier survival analysis.
Compared with Low expression of TBC1D16 (n = 96), High expression of TBC1D16 (n = 60) showed better prognostic value for EOC patients (P = 0.007).

Table 5. Cox proportional hazards analysis of overall survival for EOC patients.

| Variable | Relative risk | 95% confidence interval | P value |
|---|---|---|---|
| **FIGO stage**[a] | 3.392 | 2.152-5.347 | 0.000[e] |
| **pN status**[b] | 1.827 | 1.082-3.086 | 0.024 |
| **pT status**[c] | 1.377 | 0.984-1.928 | 0.062[e] |
| **TBC1D16**[d] | 0.326 | 0.183-0.581 | 0.000[e] |

[a]Stage I vs. II vs. III vs. IV.
[b]pN0 vs. 1.
[c]pT1 vs. 2 vs. 3.
[d]High expression vs. Low expression.
[e]Statistically significant.

expression of TBC1D16 was significantly higher in EOC specimens with VEGF low expression (the Chi-square test, $P < 0.001$). This suggested that TBC1D16 may be a potential impact on angiogenesis through VEGF signaling in EOC. In the next step, we need more fundamental experiments in vitro and vivo to prove the relationship and reveal the mechanisms of TBC1D16 functions in progression of EOC.

In conclusion, our study shows that the expression of TBC1D16 is related to VEGF expression and prognosis of EOC patients. High expression of TBC1D16 is involved in the good outcome of patients with EOC, and TBC1D16 may be an independent prognostic factor. This prognostic maker could play an important role in stratifying patients in clinical trials.

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## Conflict of Interest

The authors declare no conflict of interest.

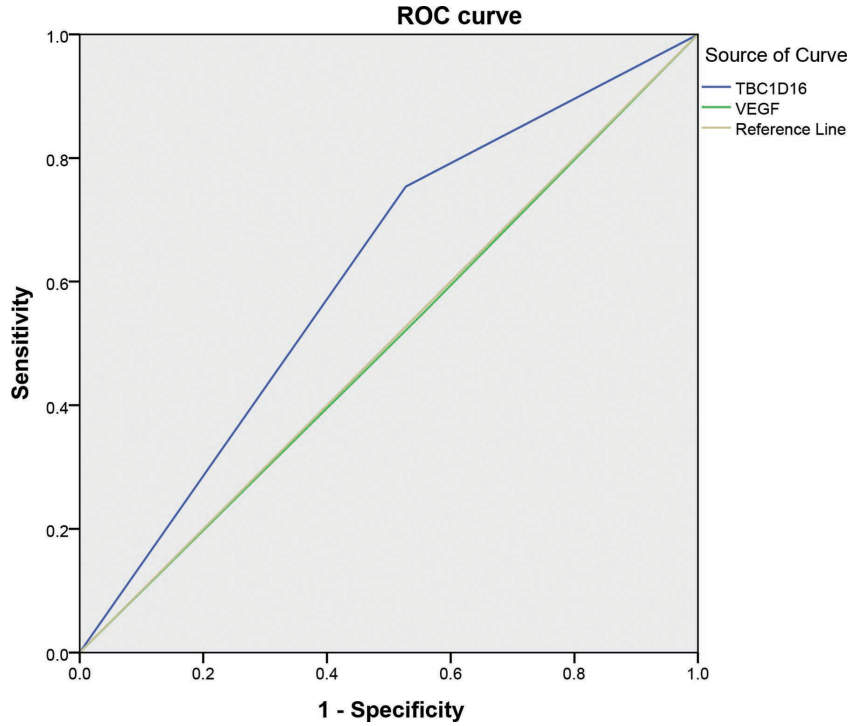


Fig. 3. ROC curve analysis for TBC1D16 and VEGF to evaluate survival status in 156 EOC patients. TBC1D16 expression (area under the curve (AUC) = 0.613, P = 0.016) is significantly associated with survival, whereas VEGF expression (AUC = 0.497, P = 0.944) is not correlated with survival. Note that the ROC curve for VEGF is close to the reference line.

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