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RESEARCH ARTICLE

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# Down-regulation of POTEG predicts poor prognosis in esophageal squamous cell carcinoma patients

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POTE ankyrin domain family, member G (*poteg*) belongs to POTE family. The POTE family is composed of many proteins which are very closely related and expressed in prostate, ovary, testis, and placenta. Some POTE paralogs are related with some cancers. Here we showed that down-regulation of POTEG was detected in about 60% primary esophageal squamous cell carcinoma (ESCC) tumor tissues. Clinical association studies determined that POTEG down-regulation was significantly correlated with tumor differentiation, lymph nodes metastasis and TNM staging. Kaplan-Meier analysis determined that POTEG down-regulation was associated with poorer clinical outcomes of ESCC patients ($P = 0.026$). Functional studies showed that POTEG overexpression could suppress tumor cell growth and metastasis capacity in vitro and in vivo. Molecular analyses revealed that POTEG downregulated CDKs, leading to subsequent inhibition of Rb phosphorylation, and consequently arrested Cell Cycle at G1/S Checkpoint. POTEG overexpression induced apoptosis by activating caspases and PARP, and regulating canonical mitochondrial apoptotic pathways. On the other side, POTEG inhibited epithelial-mesenchymal transition and suppressed tumor cell metastasis. In conclusion, our study reveals a functionally important control mechanism of POTEG in esophageal cancer pathogenesis, suggesting potential use in the ESCC intervention and therapeutic strategies.

**KEYWORDS**
apoptosis, EMT, ESCC, metastasis

## 1 | INTRODUCTION

Esophageal cancer is the 8th most common cancer in the world. It is estimated that the incidence of new cases is 456 000 per year. It ranks the sixth common cause of cancer-related mortality worldwide.[1] Esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma are the two main histological subtypes of esophageal cancer. ESCC is the major histological type and accounts for 80% of cases of esophageal cancer worldwide.[2,3] The incidence of ESCC is high in specific ethnic groups and certain locations, and is affected by environmental factors (alcohol consumption and tobacco use) and genetic factors (mutations in enzymes that metabolize alcohol).[4] In high-incidence areas of northern China,

**Abbreviations:** POTEG, POTE ankyrin domain family, member G; EMT, epithelial-mesenchymal transition; ESCC, esophageal squamous cell carcinoma; HE, hematoxylin-eosin; IHC, immunohistochemistry; Rb, retinoblastoma; RCA-I, Ricinus communis agglutinin I; STS, staurosporine; TMA, tissue microarray; TSG, tumor suppressor gene.

familial aggregation was frequently observed. It suggests that genetic susceptibility may play a key role in ESCC tumorigenesis.[5] As many solid tumors, the ESCC development is believed as a multiple-step process caused by the accumulation of inactivation of tumor suppressor genes (TSG) and activation of oncogenes.[6]

POTE ankyrin domain family member G (*poteg*), also named ANKRD26-like family C member 2 and POTE-14, located at 14q11.2. POTEG belongs to POTE family, which is mostly expressed in prostate, testis, ovary, and placenta.[7] The *poteg* encodes a predicted protein of 508 amino acids in length and amino acid sequence analysis reveals that POTE proteins are made up of three distinctive regions (N-terminal region, central region, and C-terminal region), indicating their possible function as scaffold or adaptor proteins.[8] The amino terminal region is comprised of cysteine-rich repeats (37 amino acids each); the central region is comprised of ankyrin repeat motifs (33 amino acids each); the carboxyl terminal region contains spectrin like a helices.[9] Ankyrin repeat motif is a protein recognition module correlated with many cellular functions. The helical region of the protein family is predicted to contain structures which are similar to the α-helical coiled coil domain of spectrins. These domains mediate oligomerization of proteins, as well as protein-protein interaction.[10] The ankyrin repeat motifs and spectrin-like coiled coil domain existed in a single protein, and POTEG is located in plasma membrane, indicating that POTEG might be functional in signal transmission across the plasma membrane.[11] Recently, there is a study showing that POTE family is involved in apoptosis, as POTE enhanced expression induces apoptosis in HeLa cells and POTE increases when cells are undergoing Fas receptor-dependent apoptosis.[12] However, no study was reported to investigate the role of POTEG in cancer development and/or progression.

In the present study, down-regulation of POTEG was observed in about 60% ESCC tumor tissues. To examine the possible function of POTEG in ESCC, POTEG was overexpressed in two ESCC cell lines, EC109 and KYSE510. The in vitro and in vivo assays indicated that POTEG could inhibit tumor cell growth and motility. The mechanisms of its tumor-suppressing effect were also explored.

## 2 | MATERIALS AND METHODS

### 2.1 | Cells and primary tumor tissues

ESCC cell lines (KYSE30, KYSE140, KYSE180, KYSE410, KYSE510, and KYSE520) were acquired from DSMZ (the German Resource Center for Biological Material).[13,14] Chinese ESCC cell lines HKESC1, EC18, EC109, EC9706, and immortalized esophageal epithelial cell line NE1 were kindly provided by Dr Srivastava and Dr Tsao at the University of Hong Kong.[15]

The primary ESCC tumor and corresponding non-tumor tissues were collected at Linzhou Cancer Hospital (Henan Province, China). No patient has received any preoperative treatment. The clinical research was approved by the Committees for Ethical Review of Research Involving Human Subjects at Zhengzhou University and Sun Yat-Sen University Cancer Center.

### 2.2 | Tissue microarray (TMA) and immunohistochemistry (IHC)

A total of 300 pairs of primary ESCC (tumor and paired non-tumor tissues) cases were collected from Linzhou Cancer Hospital. The ESCC tissue microarray was constructed as described previously.[16] A total of 73 ESCC tumor and paired non-tumor specimens were collected from Sun Yet-sat University Cancer Center. Patients recruited in the study have not received follow-up radiation or chemotherapy. For IHC experiment, the slides were deparaffinized, rehydrated, and blocked by 0.3% hydrogen peroxide at room temperature for 30 min. The antigen was retrieved by bathing slides in 10 mM EDTA buffer (pH 8.0) for 15 min. The slides were incubated with anti-POTEG (Novus Biologicals, Littleton, CO) at a dilution of 1:600 at 4°C overnight and the nucleus was counterstained using Meyer's hematoxylin. A staining index (0-12) was calculated by staining intensity (negative-0; weak-1; moderate-2; or strong-3) multiplying the percentage of POTEG-positive staining (<5%-0; 5% ~ 25%-1; 25% ~ 50%-2; 50% ~ 75%-3; >75%-4). Down-regulation of POTEG was defined as score ≤1.

### 2.3 | Establishment of POTEG overexpressed ESCC cell lines

Lentiviral plasmid pEZ-LV105-POTEG (GeneCopoeia, Guangzhou, China) was transfected into 293FT with Lenti-Pac™ HIV Expression Packaging Kit (GeneCopoeia) to generate lentivirus. ESCC cell lines were transduced and selected with the corresponding antibiotic resistance (puromycin) to establish POTEG overexpressed cell lines.

### 2.4 | Cell growth assay, foci formation assay, and soft agar assay

The effect of POTEG overexpression on cell proliferation was evaluated by using CCK-8 kit (Dojindo, Kumamoto, Japan). Anchorage dependent (foci formation) and independent (colony formation in soft agar) assays were done as described.[17] The assays were repeated three times.

### 2.5 | Cell migration and invasion assays

Cell chamber (BD Biosciences, Flanklin Lakes, NJ) was applied in migration assay according to the manufacturer's instructions. Migrated cells were fixed and stained with Crystal Violet, counted using microscope. The assays were repeated three times. For invasion test, BioCoat™ Matrigel™ Invasion Chamber (BD Biosciences) was used according to the manufacture's protocol. Triplicate independent experiments were performed.

### 2.6 | Cell cycle analysis

Tested cells were fixed in pre-cooled 75% ethanol, stained with PI (propidium iodide, Sigma-Aldrich, Saint Louis, MO) and DNA content of the cells was examined by CytoFLEX (Beckman Coulter, Fullerton,

CA). The profile of cell cycle was calculated with CytoExpert and ModFitLT software. Three independent assays were performed.

## 2.7 | Apoptosis assay

Cells were starved for 12 h, then treated with or without staurosporine (STS, Selleck, Houston, TX) at 0.2 μM for 24 h. At last cells were harvested and stained with Annexin V/PI Staining Kit (KeyGEN BioTECH, Shanghai, China). Apoptosis was detected by CytoFLEX (Beckman Coulter). Triplicate independent experiments were performed.

## 2.8 | Western blotting and antibodies

Western blotting was performed according to the standard protocol. Antibodies used were: POTEG (Novus Biologicals, Littleton, CO), GAPDH, Caspase 3, Caspase 6, Caspase 8, Caspase 9, PARP, Cleaved PARP, Bcl-2, Bcl-xL, Bax, Rb, p-Rb (ser807/811), p-Rb (ser795), Cyclin D1, CDK2, CDK4, CDK6, p18 INK4C, and p15 INK4B (Cell Signaling Technology, Danvers, MA).

## 2.9 | Animal experiments

All animal experiments were approved and performed complying with the guidelines of the Welfare of Experimental Animals in Sun Yat-Sen University Cancer Center. 510-POTEG ($2 \times 10^6$) and vector control cells were subcutaneously injected into the dorsal flanks of nude BALB/c mice ($n = 5$, 4-week-old, male), respectively. The growth of xenografts was monitored every 5 days. Xenograft volume was defined by $V = 0.5 \times L$ (length of tumor) $\times W^2$ (width of tumor). After mice were sacrificed, xenografts were excised, weighed, fixed, and embedded in paraffin block for hematoxylin-eosin (HE) staining and IHC study.

For the metastasis assay, $8 \times 10^5$ EC109 derivative cells were injected intravenously through the tail vein into 4-week-old male nude

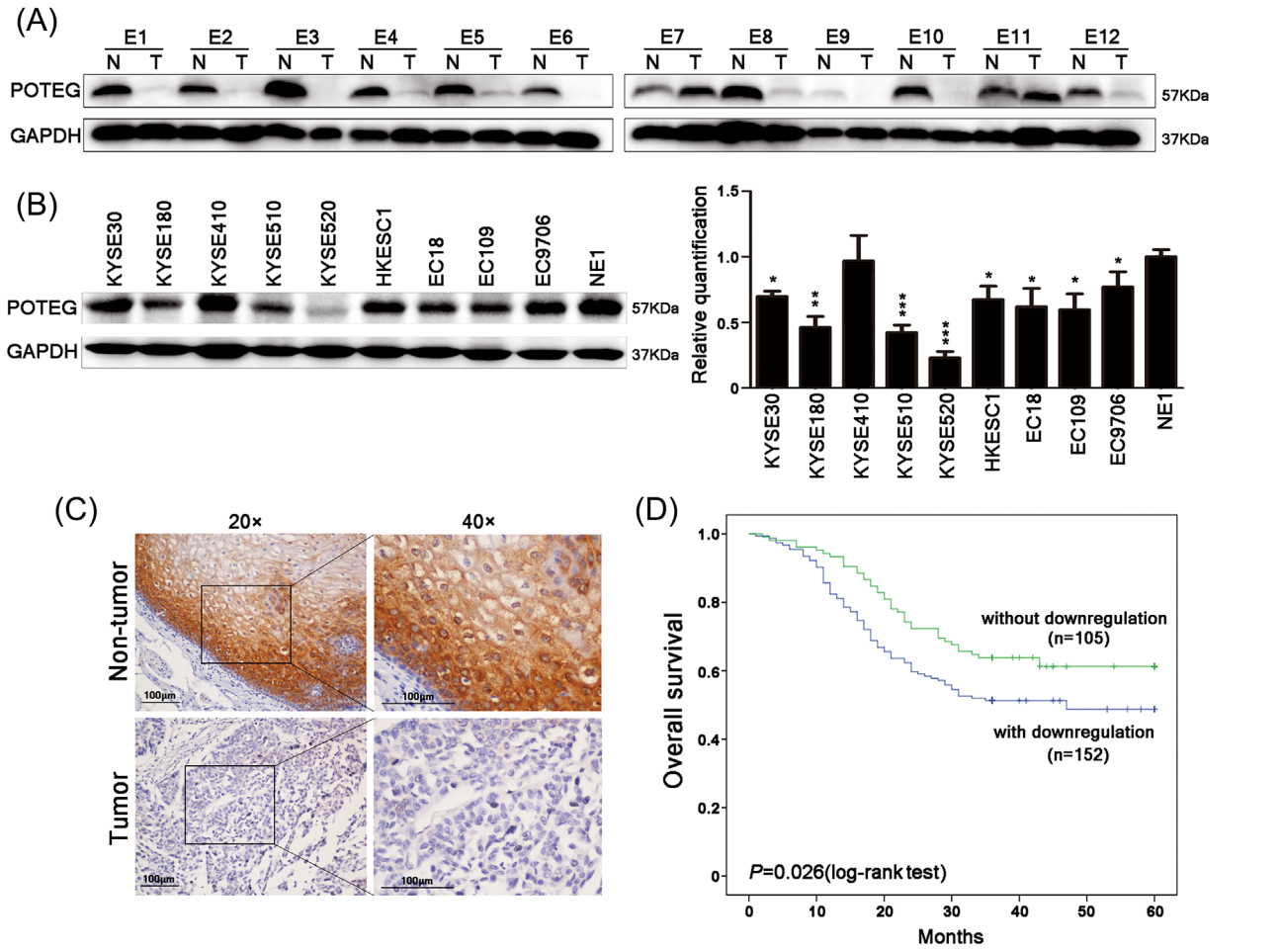


**FIGURE 1** POTEG is frequently down-regulated in ESCC primary tumor tissues. A, Down-regulation of POTEG was frequently detected in primary ESCCs by Western blotting assay (N, non-tumor tissue; T, tumor tissue). GAPDH was set as internal control. B, POTEG protein was detected in ESCC cell lines and an immortalized esophageal epithelial cell line (NE1). GAPDH was set as internal control. Western blot data was quantified using Image J software (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). C, Representatives of POTEG staining of a pair of tumor tissue and adjacent non-tumor tissue. The boxed regions were amplified as images (right). D, Kaplan-Meier plots of overall survival of ESCC patients, stratified by down-regulation of POTEG (log-rank test)

BALB/c mice ($n = 4$). The nude mice were executed 8 weeks later and metastatic nodules on the livers and lungs were examined.

### 2.10 | Statistical analyses

Statistical Package for Social Sciences (SPSS) standard Version 20.0 software (SPSS, Inc., Chicago, IL) was used for statistical analysis. Kaplan-Meier method was used for survival analysis. The correlation between POTEG down-regulation and clinicopathological features was examined by Pearson Chi-square test. Cox proportional hazard regression model was used to identify the independent prognostic factors. $P < 0.05$ was considered statistically significant. Data was expressed as mean ± SEM from at least three independent determinations.

## 3 | RESULTS

### 3.1 | Down-regulation of POTEG is frequently detected in ESCC and correlated with poorer prognosis of ESCC

Western blot results demonstrated that POTEG was down-regulated in 9 out of 12 (75.0%) ESCC tumor tissues compared with paired non-tumor tissues (Figure 1A). POTEG was down-regulated in most ESCC cell lines compared with NE1, an immortalized esophageal epithelial cell line (Figure 1B).

**TABLE 1** Association analysis of POTEG downregulation in tumor with clinicopathologic characteristics of ESCC patients

| Clinicopathologic characteristics | Total | POTEG down-regulation (%) | *P*-value |
|---|---|---|---|
| Gender | | | 0.251 |
| Female | 109 | 60 (55) | |
| Male | 148 | 92 (62.2) | |
| Age | | | 0.357 |
| < 60 | 138 | 78 (56.5) | |
| > = 60 | 119 | 74 (62.2) | |
| Differentiation | | | **0.018** |
| Well | 30 | 19 (63.3) | |
| Moderate | 169 | 90 (53.3) | |
| Poor | 58 | 43(74.1) | |
| Tumor infiltration | | | 0.243 |
| $T_{0\text{-}1}$ | 45 | 25 (55.6) | |
| $T_2$ | 103 | 56 (54.4) | |
| $T_3$ | 109 | 71 (65.1) | |
| LN Metastasis | | | **0.021** |
| $N_0$ | 202 | 112 (55.4) | |
| $N_1$ | 55 | 40 (72.7) | |
| TNM Stage | | | **0.014** |
| Early (0 + I + II) | 224 | 126 (56.3) | |
| Advance (III + IV) | 33 | 26 (78.8) | |

Bold values indicate $P < 0.05$.

A 73 pairs of ESCC tumor and paired non-tumor specimens and an ESCC tissue microarray (300 pairs) were used to detect POTEG by immunohistochemistry (IHC). Down-regulation of POTEG was detected in 50 out of 270 (18.52%) informative adjacent non-tumor tissues; in 257 informative tumor tissues, POTEG down-regulation was detected in 152 cases (59.14%) ($P < 0.05$) (Figure 1C and Supplementary Table S1).

The correlation between POTEG down-regulation and clinicopathological characters of ESCC was examined and summarized in Table 1. POTEG down-regulation was significantly correlated with tumor cell differentiation (Pearson $\chi^2$ test, $P = 0.018$), lymph node metastasis (Pearson $\chi^2$ test, $P = 0.021$) and advanced clinical staging (Pearson $\chi^2$ test, $P = 0.014$). Kaplan-Meier analysis showed that the overall survival rate was significantly lower in patients with POTEG down-regulation ($n = 152$, with a median of 39 months) than that in patients without POTEG down-regulation ($n = 105$, with a median of 45 months, $P = 0.026$, log-rank test; Figure 1D). By univariate analysis, down-regulation of POTEG ($P = 0.029$), tumor infiltration ($P = 0.024$), lymph node metastasis ($P = 0.002$), and advanced clinical staging ($P < 0.001$) were negative factors for predicting overall survival in ESCC patients (Table 2). Multivariate analysis (Cox regression) indicated that down-regulation of POTEG was an independent prognostic predictor for ESCC patients ($P = 0.046$, Table 2).

### 3.2 | POTEG suppresses tumor cell growth in vitro and in vivo

Because POTEG down-regulation was frequently detected in ESCC tumor tissues, we were wondering, whether POTEG enforced expression could affect tumor cell growth. Two ESCC cell lines, EC109 and KYSE510, were stably transfected with POTEG (Figure 2A). Cell grow rates were significantly decreased in 109-POTEG and 510-POTEG cells compared with their corresponding vector control cells (Figure 2B). POTEG could also inhibit the cells' anchorage-independent and -dependent growth ability significantly ($P < 0.01$, Figures 2C and 2D). To further validate the effect of POTEG on tumor growth, in vivo growth assay was also performed. Cells with POTEG overexpression (510-POTEG) and vector control were injected subcutaneously into nude mice, respectively. The xenograft growth was significantly inhibited in POTEG overexpressed cells ($P < 0.01$, Student's $t$-test, Figures 2E and 2F). IHC staining results confirmed the POTEG overexpression in 510-POTEG cells (Figure 2G).

### 3.3 | POTEG arrests cell cycle at G1/S checkpoint and induces apoptosis

In order to explore the mechanism of POTEG inhibiting tumor cell growth, we used flow cytometry to compare cell distribution in cell cycle between POTEG overexpressing cells and vector cells. The results suggested that 109-POTEG cells was arrested at G1/S checkpoint, displayed as cell accumulation in G1-phase (average 61.47%) and a decrease in S-phase (average 25%), compared to 109-Vec cells (G1-phase: 55.21% on average; S-phase: 29.63% on average) (Figure 3A). Similar G1/S arrest was also observed in 510-POTEG cells (Figure 3B).

**TABLE 2** Univariate and multivariate analysis of different prognostic variables in ESCC patients

| | Univariable analysis[a] | | Multivariable analysis[a] | |
|---|---|---|---|---|
| **Clinicopathologic characteristics** | **HR (95%CI)** | ***P*-value** | **HR (95%CI)** | ***P*-value** |
| Gender | 1.151 (0.790 ~ 1.676) | 0.463 | | |
| Age | 0.895 (0.618 ~ 1.297) | 0.559 | | |
| Differetiation | 1.117 (0.812 ~ 1.537) | 0.498 | | |
| Tumor infiltration | 1.507 (1.056 ~ 2.151) | **0.024** | 1.061 (0.729 ~ 1.544) | 0.756 |
| LN metastasis | 1.919 (1.280 ~ 2.879) | **0.002** | 1.111 (0.731 ~ 1.689) | 0.622 |
| TNM stage | 5.657 (3.105 ~ 10.308) | **<0.001** | 5.303 (2.812 ~ 10.000) | **<0.001** |
| POTEG down-regulation | 1.539 (1.045 ~ 2.267) | **0.029** | 1.488 (1.007 ~ 2.198) | **0.046** |

HR, hazards ratio; CI, confidence interval.
Bold values indicate $P < 0.05$.
[a]Cox regression model.

Because POTE family was reported to play a role in apoptosis,[12,18] we tested whether POTEG could affect apoptosis in ESCC cells. The apoptotic index increased in 109-POTEG cells compared with vector cells (Figures 3C and 3D). When cells were treated with STS, a broad-spectrum kinase inhibitor that can induce apoptosis, apoptotic index increased significantly in both 109-POTEG and 510-POTEG compared with corresponding control cells ($P < 0.01$, Student's *t*-test, Figures 3C and 3D).

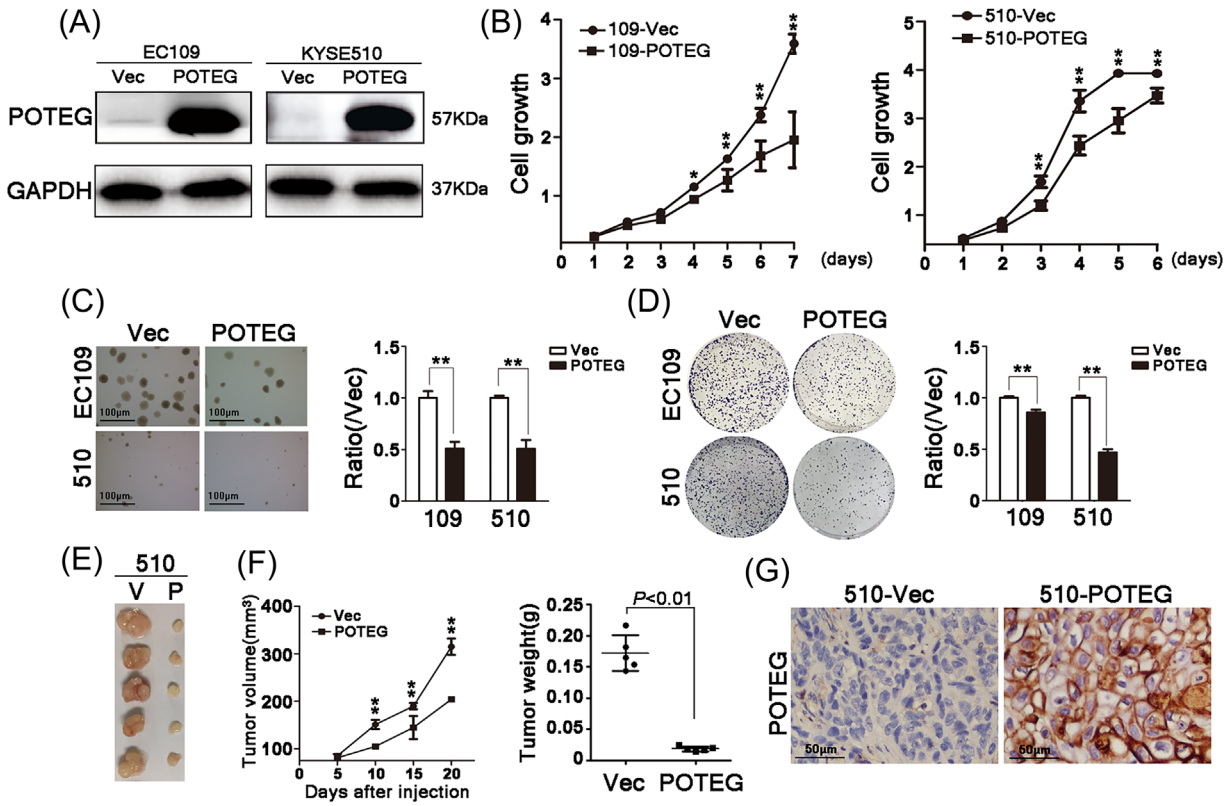


**FIGURE 2** POTEG suppresses ESCC tumor cell growth in vitro and in vivo. A, Expression of POTEG in POTEG-transfected EC109 and KYSE510 cells was confirmed by Western blotting. B, XTT assay was used to compare cell growth between POTEG overexpressed cells and vector control cells. (EC109 [left]; KYSE510 [right]) (Student's *t*-test; $^{*}P < 0.05$, $^{**}P < 0.01$). C, The frequency of colony formation in soft agar (left, representative; right, summary) was significantly lower in POTEG overexpressed cells (109-POTEG and 510-POTEG) compared with vector control cells. (Student's *t*-test; $^{**}P < 0.01$). D, Representatives (left) and summary (right) of foci formation in monolayer culture in POTEG overexpressed cells and control cells. (Student's *t*-test; $^{**}P < 0.01$). E, Images of xenografts induced by 510-POTEG and vector control cells (V: vector; P: POTEG). F, Xenografts growth curves and weight were compared between 510-POTEG and vector control cells. (Student's *t*-test; $^{**}P < 0.01$) G) POTEG expression was confirmed by IHC in 510-POTEG xenografts. (original magnification: 20×)

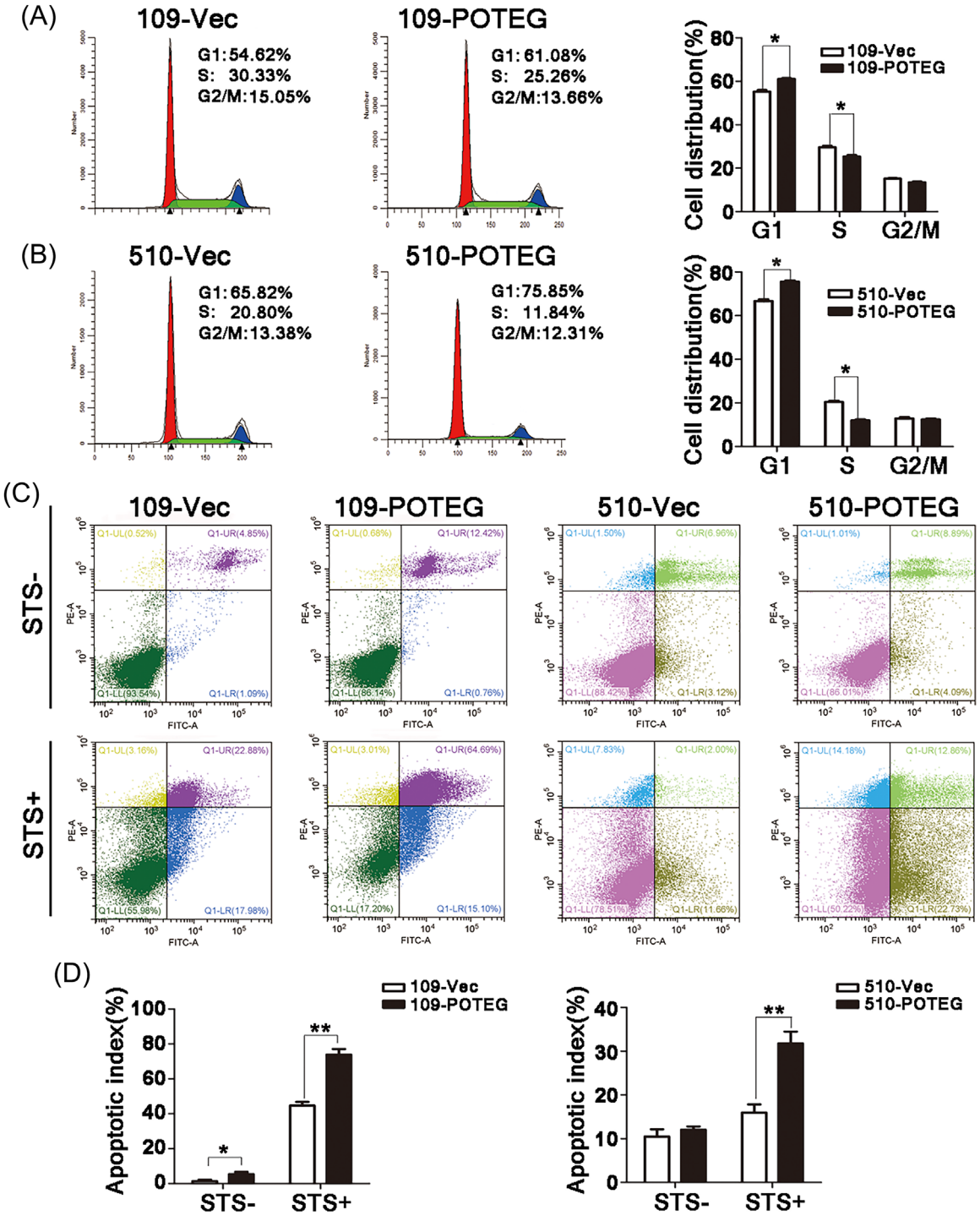


**FIGURE 3** POTEG arrests cell cycle at G1/S checkpoint and induces apoptosis. A and B, Representatives and summary of DNA content of 109-POTEG (A) and 510-POTEG (B) and corresponding vector control cells detected by flow cytometry. Triplicate independent assays were repeated. (Student's *t*-test; $^{*}P < 0.05$). C and D, POTEG overexpressed cells and corresponding control cells were treated with STS (STS +) or without STS (STS-), and apoptosis was analyzed by FACS(C). Triplicate independent assays were repeated and summarized (D). (Student's *t*-test; $^{*}P < 0.05$, $^{**}P < 0.01$)

### 3.4 | POTEG arrests cell cycle by decreasing Rb phosphorylation

In order to identify potential molecules responsible for G1/S arrest induced by POTEG overexpression, Western blotting was processed. CDK6, cyclin D1, and CDK2 decreased significantly in the POTEG overexpressed cells (Figure 4A and Supplementary Figure S1A). Whereas, p18 INK4C and p15 INK4B, which selectively inhibit CDK4/6 activity, increased in the POTEG overexpressed cells (Figure 4A and Supplementary Figure S1A). Although no significant changes were observed in total Rb (retinoblastoma), the

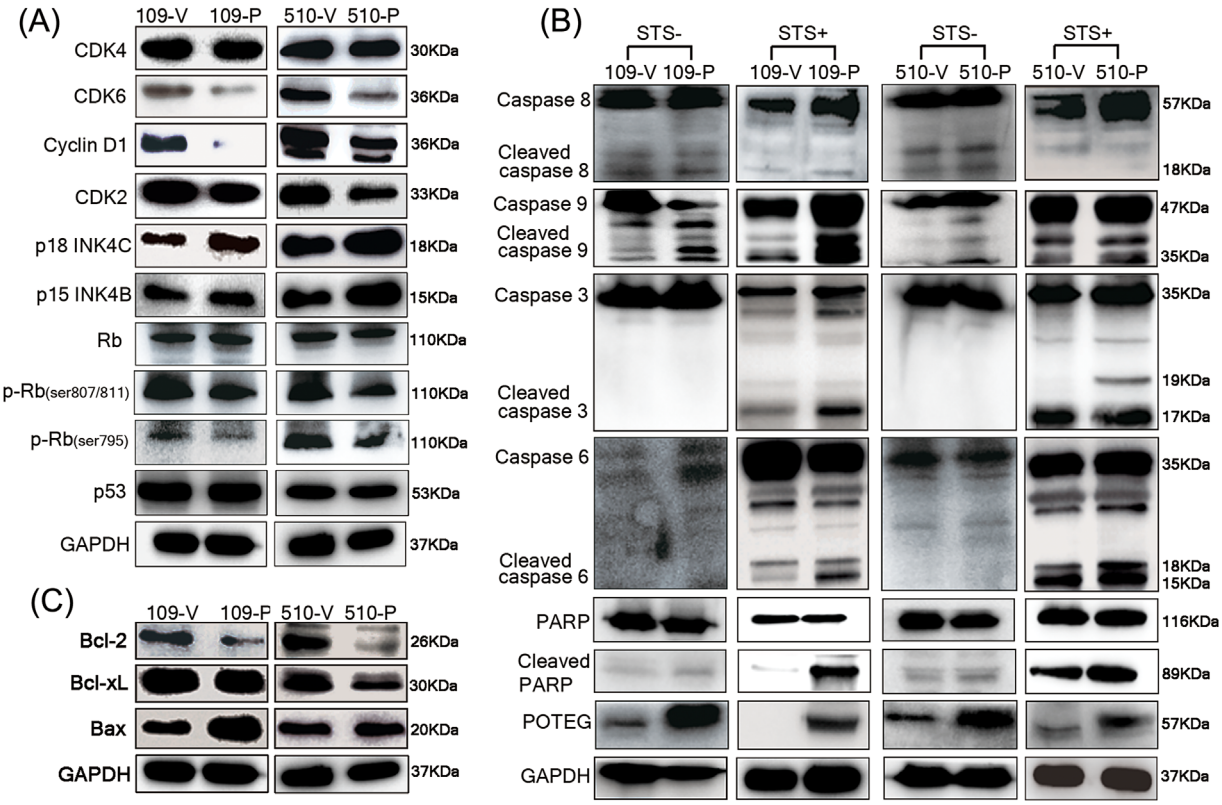


**FIGURE 4** POTEG down-regulates Rb phosphorylation and promotes apoptosis by regulating canonical mitochondria apoptotic signaling pathways. A, The decrease of CDK6, cyclin D1, CDK2, and increase of p18 INK4C and p15 INK4B were observed in POTEG overexpressed cells. Phosphorylation of Rb decreased in POTEG overexpressed cells. (V, vector control; P, POTEG). B, Caspase-8, 9, 3, 6, PARP, and cleaved PARP were detected in POTEG overexpressed cells and control cells with or without STS treatment. C, Bcl-2, Bcl-xL, and Bax were detected in POTEG-overexpressed cells compared with vector control cells. GAPDH was set as loading control

phosphorylation of Rb (Ser807/811 and Ser795) decreased significantly in both 109-POTEG and 510-POTEG cells (Figure 4A and Supplementary Figure S1A). The key cell cycle regulator p53 was also tested and no obvious change was observed (Figure 4A and Supplementary Figure S1A).

## 3.5 | POTEG promotes apoptosis by regulating canonical mitochondria apoptotic signaling pathways

Western blotting results revealed that POTEG overexpression did not increase the cleavages of caspase-8, caspase-3, and caspase-6 except caspase-9 (Figure 4B and Supplementary Figure S1B). When cells were treated with STS (STS+), POTEG overexpressed cells showed significant increase on cleaved caspase-9, caspase-3, and caspase-6 compared with vector control cells (Figure 4B and Supplementary Figure S1B). Cleaved PARP also increased with STS treatment. Consistently, the protein levels of anti-apoptotic proteins Bcl-2, Bcl-xL were down-regulated, and pro-apoptotic protein Bax increased in POTEG overexpressed cells (Figure 4C and Supplementary Figure S1C).

## 3.6 | POTEG inhibits cell motility in vitro and in vivo

Because POTEG down-regulation was correlated with lymph node metastasis ($P < 0.05$) in ESCC by IHC analysis, we further investigated the expression of POTEG in metastasized lymph nodes. POTEG staining was negative in four out of five metastasized lymph nodes (Figure 5A). We therefore used migration and invasion assays to test the effects in POTEG overexpressed cells. in vitro assays revealed that cells' ability of migration and invasion decreased significantly in POTEG overexpressed EC109 and KYSE510 cells ($P < 0.01$, Student's *t*-test, Figures 5B and 5C). 109-POTEG and vector control cells were inoculated into nude mice via tail vein ($n = 4$ for each group). After 8 weeks, tested mice were executed and livers and lungs were isolated and examined. No metastatic nodule was detected in mice livers. As shown in Figure 5D, the number of pulmonary metastatic nodules induced by 109-POTEG cells was lower than that induced by control cells ($P < 0.05$, Student's *t*-test). The similar results were repeated with KYSE510 derivative cells (Supplementary Figure S2A). Taken together, our results demonstrated that POTEG overexpression could inhibit ESCC tumor cell's motility in vitro and in vivo.

As epithelial-mesenchymal transition (EMT) is believed to be utilized by cancer cells to gain motility and invasiveness during metastasis,[19] the EMT related markers were examined by Western blotting. Up-regulation of E-cadherin, β-catenin, claudin-1, and ZO-1 were observed in POTEG overexpressed cells (Figure 5E and Supplementary Figure S2B), reduced expression of mesenchymal marker Vimentin was also detected (Figure 5E and Supplementary Figure S2B).

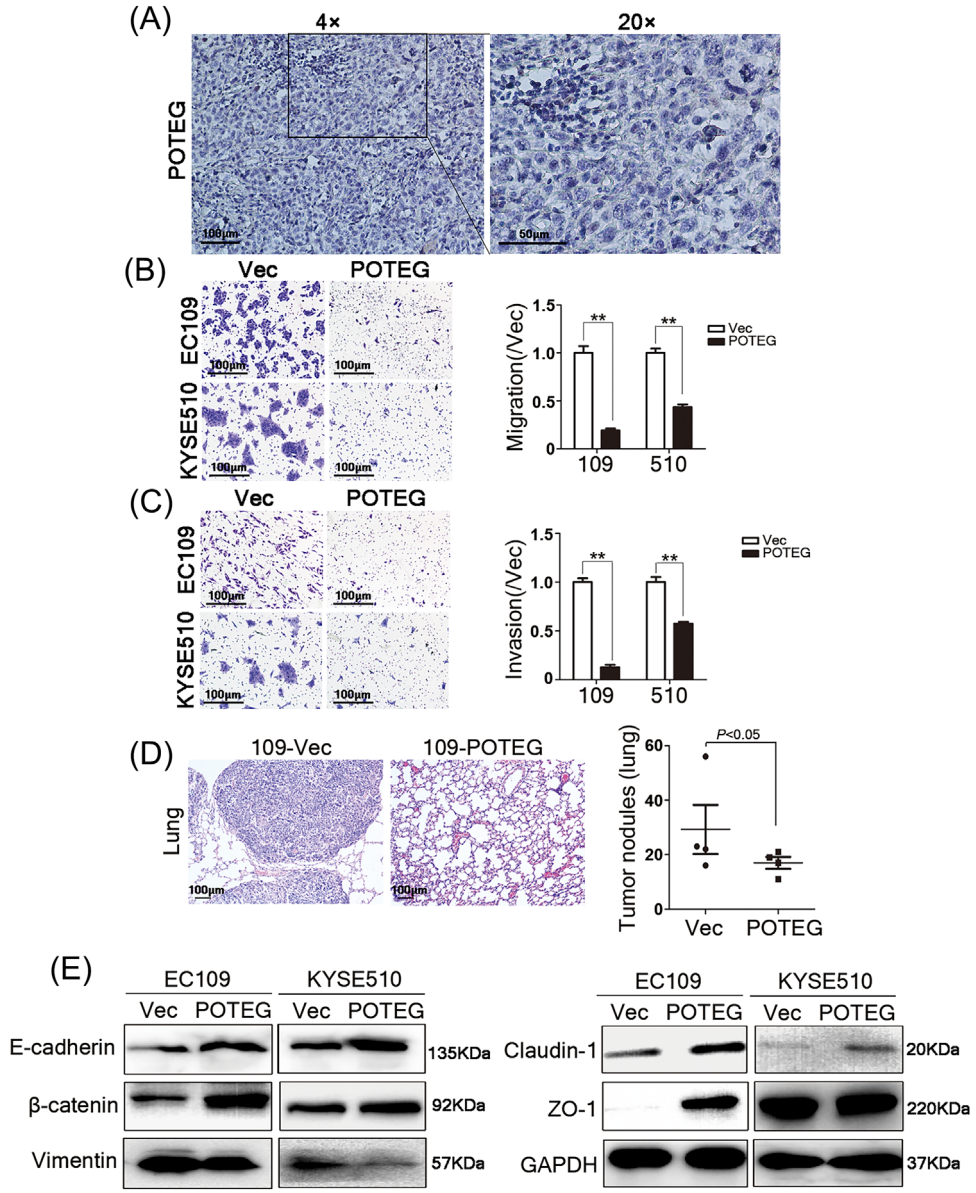


**FIGURE 5** POTEG inhibits cell motility in vitro and in vivo. A, Representative image of metastatic lymph node stained with POTEG antibody. The boxed region was amplified as right picture. B and C, Representatives and summary of cell migration (B) and invasion (C) assays performed with POTEG enforced expression cells or vector control cells (original magnification: 20×) (Student's *t*-test, $^{**}P < 0.01$). D, HE staining of pulmonary sections derived from mice injected via tail vein with 109-POTEG and control cells (original magnification: 20×). Visible tumor nodules of lungs were counted and summarized. E, E-Cadherin, β-catenin, vimentin, claudin-1, and ZO-1were detected in POTEG overexpressed cells and vector controls. GAPDH was set as loading control

## 4 | DISCUSSION

POTE family is a large family and composed of 13 paralogs dispersing among eight chromosomes (2, 8, 13, 14, 15, 18, 21, and 22). The ORFs and splice junctions were preserved in the gene family.[9,20] It has been reported that POTE was expressed in prostate, ovary, testis and placenta.[9] The POTE 2 paralogs were reported to be predominantly expressed in undifferentiated human embryonic stem cells.[21] Previous studies showed that several POTE paralogs (POTE-2α, POTE-2β, and POTE-2γ) were predominantly expressed in prostate, breast, ovary, and lung cancers.[8] However, only cDNA samples were used in the study and sample size was limited (about 10 cases). It was also reported that low serum POTEE was a positive prognostic factor for progression-free survival in patients with non-small-cell lung cancer.[22] In this study, we evaluated POTEG protein level in 257 ESCC tumor specimens. Our results revealed that POTEG down-regulation was detected in about 60% ESCC tissues. The multivariate analysis suggested that POTEG was an independent prognostic maker in ESCC development and progression.

We used in vitro and in vivo studies to investigate whether POTEG could affect ESCC tumor cell growth. Our results revealed that POTEG enhanced expression could suppress ESCC tumor cell

growth significantly in vitro and in vivo. POTEG overexpression blocked G1/S transition by decreasing CDK6, cyclin D1, CDK2, and further decrease Rb phosphorylation. G1/S phase transition is a major checkpoint for cell cycle progression,[23] and Rb plays a key role in regulating G1/S transition and controls cell progression through the late G1 check point.[24] Rb phosphorylation induces Rb to dissociate from transcription factor E2F, accelerating the transcription of S-phase-promoting genes which stimulate cell growth.[25,26] When POTEG was overexpressed in ESCC cells, the decrease of Rb phosphorylation activated the blocking activity of E2F transcription.

Because POTE gene family is highly expressed in primary spermatocytes, many of which are experiencing programmed cell death, indicates potential role in inducing apoptosis.[12,18] Our results demonstrated that the tumor-suppressive role of POTEG was also closely associated with its pro-apoptotic effect. POTEG promoted apoptosis by regulating canonical mitochondria apoptotic signaling pathways by activation of caspase-9, caspase-3, caspase-6, and PARP. The anti-apoptotic proteins Bcl-2 and Bcl-xL were decreased by POTEG overexpression, while the pro-apoptotic protein Bax was increased.

The paralog of POTE, POTEF, was reported to play a key role in mediating Ricinus communis agglutinin I (RCA-I) inhibiting metastatic capacities of triple-negative breast cancer cells.[27] The IHC results indicated that down-regulation of POTEG correlated significantly with lymph nodes metastasis in ESCC patients ($P < 0.05$). POTEG orver-expression could inhibit ESCC cells' motility in vitro and in vivo. EMT is a critical process in embryonic development, allowing epithelial cells to gain mesenchymal phenotypes while losing cell-cell contacts and apical-basal polarity.[19] The hallmark of EMT is the functional loss of E-cadherin, while additional cellular changes of epithelial and mesenchymal markers are also frequently observed. POTEG overexpression induced increase of E-cadherin, β-catenin, claudin-1, and ZO-1. On the other hand, vimentin was decreased in POTEG overexpressed cells. In aggregate, our results suggested that POTEG inhibited EMT, thereby inhibited ESCC tumor cell metastasis.

In summary, our findings show that POTEG plays a critical role in the ESCC tumorgenesis by promoting tumor cell apoptosis and inhibiting metastasis. Our study extended the understanding of POTE family and suggested a new therapeutic strategy for ESCC patients.

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## CONFLICTS OF INTERESTS

The authors declare that they have no conflicts of interest.

## AUTHORS' CONTRIBUTIONS

WL performed the experiments, interpreted the results, and wrote the draft. LM, ZY, BX, and ZT performed the experiments. ZY and YJ provided samples and clinical information. GXY and LY designed, supervised the study, and wrote the final manuscript.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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