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Original Research

TBRG4 silencing promotes progression of squamous cell carcinoma via regulation of CAV-1 expression and ROS formation

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Abstract: Esophageal cancer is the eighth most common cancer globally. Transforming growth factor β regulator 4 (TBRG4) and caveolin-1 (CAV-1) are implicated in tumor progression. The aim of this study was to investigate the expressions of TBRG4 and CAV-1 in esophageal squamous cell carcinoma (ESCC), and their relationship with reactive oxygen species (ROS) formation. Human ESCC cell lines (EC9706, TE-1, and Eca109), and normal esophageal mucosal cell line (Het-1) were used in this study. The silencing of TBRG4 and/or CAV-1 by sh-RNA or overexpression of CAV-1 after TBRG4 knockdown was used to assess ROS levels. The results showed that down-regulation of TBRG4 reduced CAV-1 expression, and promoted ROS formation in ESCCs ($p < 0.01$). However, CAV-1 overexpression increased the expression level of TBRG4, but decreased ROS level in EC9706 cells ($p < 0.01$). Similarly, TBRG4 knockdown significantly reduced CAV-1 expression, promoted ROS formation, and caused cell cycle arrest at G0/G1 phase ($p < 0.01$). Caveolin-1 (CAV-1) knockdown also promoted cell apoptosis, cellular ROS formation and cell cycle arrest at G0/G1 phase ($p < 0.01$). However, CAV-1 overexpression in sh-TBRG4-treated EC9706 cells significantly upregulated TBRG4 expression, but significantly reduced the level of ROS, and inhibited cell-cycle arrest and apoptosis ($p < 0.01$). The enhancements in bcl-2/bax ratio, cytochrome c expression, and ROS levels by sh-TBRG4 were significantly reversed by CAV-1 overexpression in EC9706 cells. These results show that the upregulated expression of TBRG4 or CAV-1 promotes ESCC progression via regulation of intracellular ROS levels and inhibition of mitochondria-dependent apoptotic pathway.

Key words: Esophageal cancer; Caveolin-1; Reactive oxygen species; Cell apoptosis; Expression.

Introduction

Esophageal squamous cell carcinoma (ESCC) is the most common type of esophageal cancer, with 79 % incidence in Asia alone (1). Transforming growth factor β regulator 4 (TBRG4) gene is located on chromosome 7p12.3–13 and encodes a regulator for transforming growth factor β (TGFβ) which is implicated in many types of cancer (2, 3). In addition to stabilizing the expressions of cyclins 1 and 2, it has been demonstrated that TBRG4 interacts with pleiotrophin protein, and its silencing affects the stability of certain mitochondrial mRNAs (4-6). Caveolin-1 (CAV-1) is highly expressed in tumors such as prostate, pancreatic, and lung cancers. High CAV-1 levels have been shown to be correlated with tumor progression, invasion and metastasis, while seriously affecting prognosis (7-9). Therefore, CAV-1 functions as a proto-oncogene in ESCC. In lung cancer cell line (H1299), TBRG4 knockdown downregulates the expression of CAV-1, thereby inhibiting proliferation of the cells via induction of apoptosis (10). However, the roles of TBRG4 and CAV-1 in ESCC remain unknown. An understanding of their interaction may offer a novel strategy for treatment of ESCC and improvement of clinical outcomes. The accumulation of ROS regulates the proliferation and apoptosis of tumor cells (11). Caveolin-1 (CAV-1) promotes the proliferation and migration of lung cancer cells via a mechanism involving the PI3K/Akt pathway. Reactive oxygen species (ROS)-induced degradation of CAV-1 inhibits the growth of cancer cells (12). However, the relationship between ROS and CAV-1 expression in ESCC remains largely unknown. The aim of this study was to investigate the expressions of TBRG4 and CAV-1 in ESCC, and their relationship with ROS formation.

Materials and Methods

Materials and reagents

Human ESCC cell lines (EC9706, TE-1, and Eca109), and normal esophageal mucosal cells (Het-1) were purchased from Shanghai Institute of Cell Resource Center Life Science (China). Bicinchoninic acid (BCA) protein kit was obtained from Beyotime Biotechnology (China). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Hyclone (USA). Enhanced chemiluminescence and annexin V-FITC apoptosis kits were products of Thermo scientific (USA). Polyvinylidene fluoride membrane was obtained from Millipore, (USA). Propidium iodide

(PI) was obtained from BD Biosciences (USA). The SYBR® Premix Ex Taq™ II (Tli RNaseH Plus), and PrimeScript™RT reagent kits were products of Takara Bio Inc. (China), and ROS assay kit was obtained from Abnova (Taiwan). Anti-bax, bcl-2, CaV1.3, cytochrome c, TBRG4, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and horseradish peroxidase (HRP)-conjugated secondary antibodies were products of Abcam (UK). Radio-immunoprecipitation assay (RIPA) buffer was obtained from Sangon Biological Engineering Co. Ltd. (China). ViraDuctin™ Lentivirus transduction kit was *purchased from Cell Biolabs (USA).*

Cell culture

The EC9706, TE-1, Eca109 and Het-1 cells were cultured in DMEM supplemented with 10 % FBS, and 1 % penicillin/streptomycin solution at 37 °C for 24 h in a humidified atmosphere of 5 % CO_2 and 95 % air. Cells in logarithmic growth phase after attaining 80-90 % confluency were used in this study.

Construction of recombinant lentiviral expression vectors and sh-RNA transduction

The sequences of sh-RNA for targeting TBRG4 and CAV-1 sequences (5′-GTT CTT CAG CCT GGT ACA T-3′ and 5′-AGA CGA GCT GAG CGA GAA GCA-3′) were designed by Shanghai GeneChem Co. Ltd. The Sh-RNA without homology to TBRG4 or CAV-1 mRNA when incorporated into the lentiviral vector, served as control (sh-NC, 5'-TTC TCC GAA CGT GTC ACG T-3'). Recombinant pLKO.1-shTBRG4 and pLKO.1-shCAV-1 vectors were then constructed. In addition to the overexpression of CAV-1 in TBRG4 knockdown cells, CAV-1 primers (forward: 5'-AAC GAT GAC GTG GTC AAG ATT G-3', reverse: 5'-GCA GAC AGC AAG CGG TAA AAC-3') were designed, and the amplified products of CAV-1 and sh-TBRG4 were simultaneously integrated into the pLKO.1-TRC vector. For cellular transduction of sh-TBRG4 lentiviral, sh-CAV-1 lentiviral, sh-TBRG4 plus CAV-1 lentiviral or sh-NC lentivirus, 293T cells were used. Recombinant vectors were subjected to transduction using ViraDuctin™ Lentivirus transduction kit, and lentiviral particles were obtained 48 h post-transfection. The isolated lentiviral particles were used to infect ECA109 cells with MOI at ratio of 1:100. After a 2-week selection period with puromycin, the cells were identified, and infection efficiency was determined using real-time quantitative polymerase chain reaction (qRT-PCR).

Western blotting

The expressions of TBRG4, CAV-1, bcl-2, bax and cytochrome c were determined using Western blotting. The cells were washed with phosphate-buffered saline (PBS), and lysed with ice-cold RIPA containing protease inhibitor. The resultant lysate was centrifuged at 12, 000 rpm for 10 min at 4 °C, and the protein concentration of the supernatant was determined using BCA assay kit. A portion of total cell protein (30 µg) from each sample was separated on 12 % sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred to a fixed polyvinylidene fluoride membrane at 110 V and 90 °C for 120 min. Subsequently, non-fat milk powder (3 %) in Tris-buffered saline containing 0.2 % Tween-20 (TBS-T) was added with gentle shaking at 37 °C and incubated to block non-specific binding of the blot. Incubation of the blots was performed overnight at 4 °C with primary antibodies for bax, bcl-2, CaV1.3, cytochrome c, TBRG4, and GAPDH, each at a dilution of 1 to 1000. Then, the membrane was washed thrice with TBS-T and further incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody for 1.5 h at room temperature. The blot was developed using an X-ray film. Grayscale analysis of the bands was performed using Bio-rad gel imaging system Respective protein expression levels were normalized to that of GAPDH which was used as a standard.

qRT-PCR

The levels of expression of TBRG4, CAV-1, bcl-2, bax and cytochrome c mRNAs were determined using qRT-PCR. Trizol RNA extraction reagent was used to extract total RNA from the cells, while cDNA synthesis kit was used to perform cDNA synthesis reaction according to the instructions of the manufacturer. Light Cycler 1536 RT-PCR detection system was used for estimation of the mRNA expressions of the genes. Variation in the cDNA content was normalized using GAPDH. The PCR reaction mixture (20 µL) consisted of 6.4 µL of dH_2O, 1.6 µL of gene-specific primer (10 µM), 2 µL of synthesized cDNA and 10 µL of SYBR Premix Ex Taq™ II. The Ct value of U6 was taken as the internal parameter, and $2^{-\Delta\Delta Ct}$ was used to calculate the relative expression levels of the proteins (13). The primers used for the qRT-PCR are shown in Table 1.

Table 1. Primer sequences used for the qRT-PCR.

Gene		Sequence
TBRG4	Forward	5'-CCT ATT TCC CAT GAT TCC TTC ATA-3'
	Reverse	5'-GTA ATA CGG TTA TCC ACG CG-3'
Caveolin-1	Forward	5'-AAC GAT GAC GTG GTC AAG ATT G-3'
	Reverse	5'-GCA GAC AGC AAG CGG TAA AAC-3'
Bax	Forward	5'-TCC ACC AAG AAG CTG AGC GAG-3'
	Reverse	5'-GTC CAG CCC ATG ATG GTT CT-3'
Bcl-2	Forward	5'-TTC TTT GAG TTC GGT GGG GTC-3'
	Reverse	5'-TGC ATA TTT GTT TGG GGC AGG-3'
GAPDH	Forward	5'-GTC GTA TTG GGC GCC TGG TCA CC-3'
	Reverse	5'-CAC ACC CAT GAC GAA CAT GGG GGC-3'

Determination of ROS level

The levels of ROS in EC9706, TE-1, Eca109 and Het-1 cells were determined using DCFH-DA assay. The cells were washed with PBS after an initial incubation for 72 h. Then, 100 µL of DCFH-DA (10 µM) solution was added to the plates, followed by incubation for another 20 min at 37°C. Thereafter, the cells were washed with PBS and injected into the flow cytometer for analysis at 520 nm/605 nm.

Cell cycle analysis

The distribution of the cells among the different phases of the cell cycle was determined using a flow cytometer. The treated cells were seeded in 6-well plates and incubated for 72 h. The cells were then washed with PBS, and fixed with 70 % ethyl alcohol at 4°C overnight. Tris-hydrochloride buffer (pH 7.5) containing 1 % RNase A was thereafter added to the plates. The cells were subsequently stained with propidium iodide and injected into the flow cytometer for analysis.

Cell apoptosis assay

The cells were seeded at a density of 2.5 x 10^6 cells/well in 6-well plates and cultured for 24 h. Then, the cells were washed with PBS, and thoroughly mixed with 300 µL binding buffer. The cells were then stained with annexin V-fluorescein isothiocyanate and propidium iodide (5 µL each) within 25 min at room temperature in the dark. Cell apoptosis was assessed using a flow cytometer fitted with argon laser operated at 485 nm.

Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was performed using SPSS (22.0). Groups were compared using Tukey and Student *t*-tests. Values of $p < 0.01$ were considered statistically significant.

Results

Basal expressions of TBRG4 and CAV-1, and ROS levels in ESCC and normal cells

The expressions of TBRG4 and CAV-1 were significantly higher in ESCCs (EC9706, TE-1, and Eca109 cells) than in Het-1 cells (3-fold higher in EC9706 cells; $p < 0.01$). However, the levels of ROS were significantly reduced in human ESCCs relative to Het-1 cells, and was least in EC9706 cells ($p < 0.01$). These results are shown in Figure 1.

Flow cytometric analysis of cell cycle and apoptosis in EC9706 cells

In control (sh-NC) group, the cell cycle distribution curve revealed that 61.94 and 63.13 % of the cells were in G1 phase, 13.03 and 11.77 % were in G2/M phase, and 25.03 and 25.10 % were in S phase. However, TBRG4 knockdown induced G0/G1 phase arrest in EC9706 cells; 75.98 % of the cells were in G1 phase, 3.03 % were in G2/M phase, and 20.99 % were in S phase. In addition, the silencing of TBRG4 significantly promoted cell apoptosis up to 5 folds. These results are shown in Figure 2.

Expressions of TBRG4, CAV-1, and apoptosis-related proteins in EC9706 cells

As shown in Figure 3, sh-TBRG4 treatment significantly upregulated the expressions of bax and cytochrome c, but down-regulated the expressions of bcl-2 mRNA and protein in EC9706 cells ($p < 0.01$). The sh-TBRG4 treatment enhanced bcl-2/bax ratio. The mRNA and protein expressions of CAV-1 were also significantly down-regulated after TBRG4 knockdown. However, sh-TBRG4 treatment caused a significant increase in ROS level up to 1.5 folds.

Effect of CAV-1 knockdown on the distribution of EC9706 cells among phases of the cell cycle

Sh-CAV-1 treatment significantly induced G0/G1 phase arrest in EC9706 cells; 73.30 % of the cells were in G1 phase, 5.16 % were in G2/M phase, and 21.54 % were in S phase. In addition, sh-CAV-1 treatment significantly increased the number of apoptotic cells ($p < 0.01$; Figure 4).

Effect of CAV-1 knockdown on the expressions of bcl-2, bax, and cytochrome c

Caveolin-1 (CAV-1) knockdown in EC9706 cells

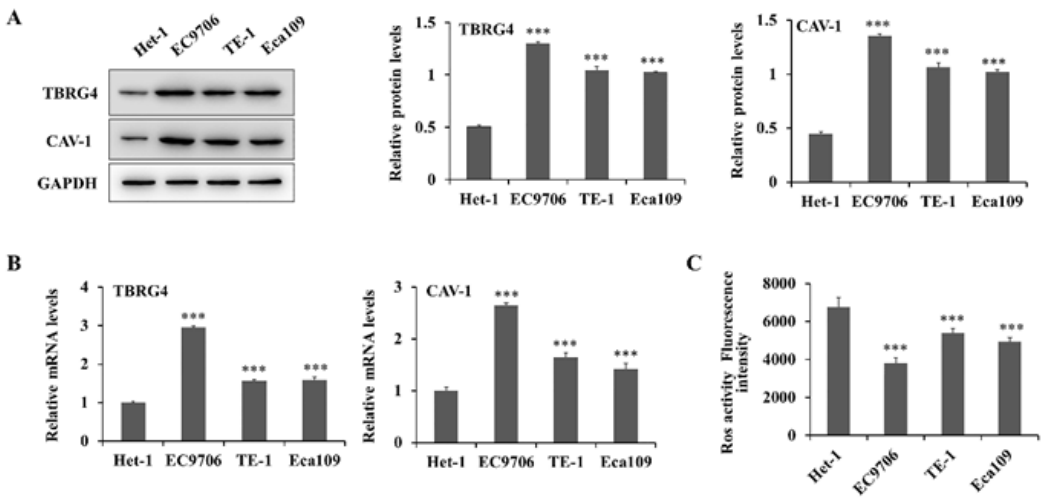

Figure 1. Expressions of TBRG4 and CAV-1, and ROS levels in ESCC and normal cells. (A): The expressions of TBRG4 and CAV-1 were measured using Western blotting; (B): Expressions of TBRG4 and CAV-1 mRNA as measured using qRT-PCR; and (C): Levels of ROS in ESCC and Het-1 cells. $^{**}p < 0.01$; $^{***}p < 0.001$, when compared with Het-1 cells.

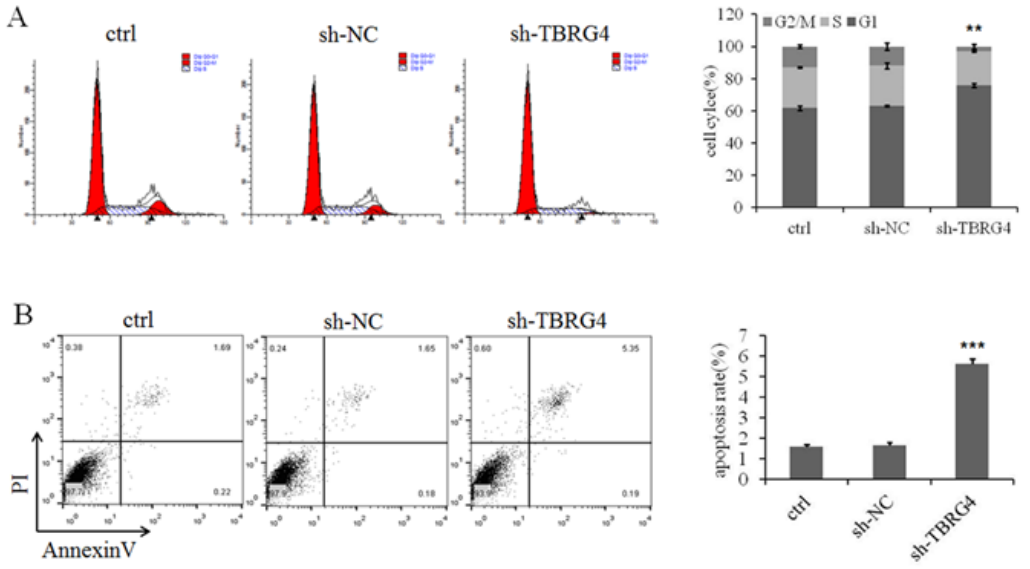

Figure 2. Distribution of EC9706 cells among phases of the cell cycle. (A): Flow cytometric analysis of cell cycle; and (B): Flow cytometric analysis of cell apoptosis. $^{**}p < 0.01$; $^{***}p < 0.001$, when compared with control group.

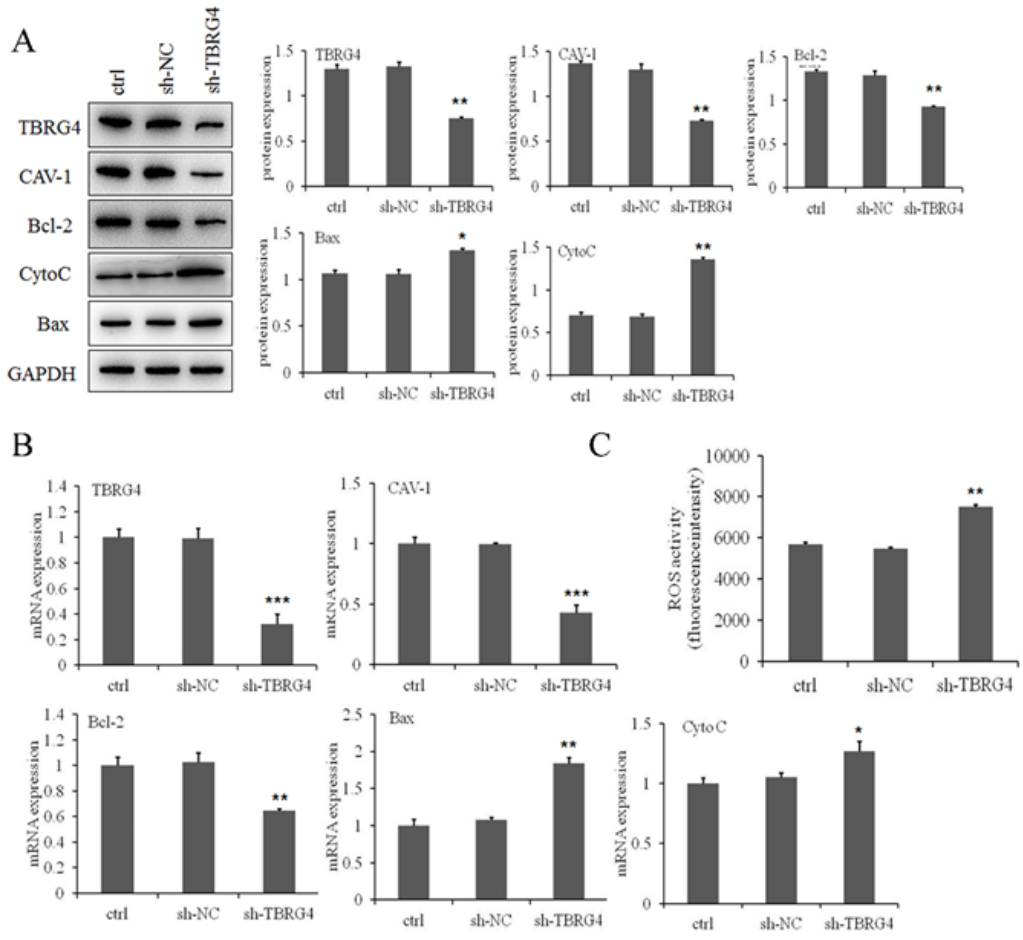

Figure 3. Expressions of TBRG4, CAV-1, and apoptosis-related proteins in EC9706 cells. (A): Expressions of TBRG4, CAV-1, bcl-2, bax, and cytochrome c, as measured using Western blotting; (B): Expressions of TBRG4, CAV-1, bcl-2, bax, and cytochrome c, as measured using qRT-PCR; and (C): levels of ROS in ESCC cells after TBRG4 silencing. Sh-NC group refers to cells transfected with sh-scramble sequence; $^{**}p < 0.01$; $^{***}p < 0.001$, when compared with control group.

significantly upregulated the expressions of bax and cytochrome c, but down-regulated bcl-2 expression ($p < 0.01$). It also significantly increased the level of ROS ($p < 0.01$; Figure 5).

Effect of CAV-1 overexpression on sh-TBRG4-induced G0/G1 phase arrest and apoptosis in EC9706 cells

As shown in Figure 6, CAV-1 overexpression significantly reduced the proportion of G0/G1 phase cells

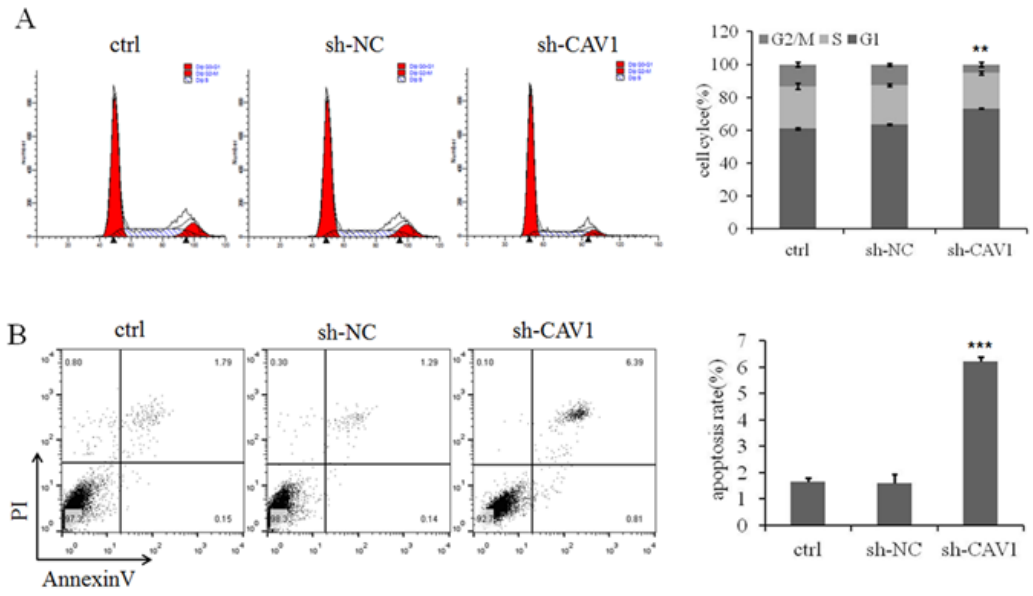

Figure 4. Effect of CAV-1 knockdown on the distribution of EC9706 cells among phases of the cell cycle. (A): Flow cytometric analysis of EC9706 cell cycle; and (B): Flow cytometric analysis of EC9706 apoptosis. $^{**}p < 0.01$; $^{***}p < 0.001$, when compared with control group.

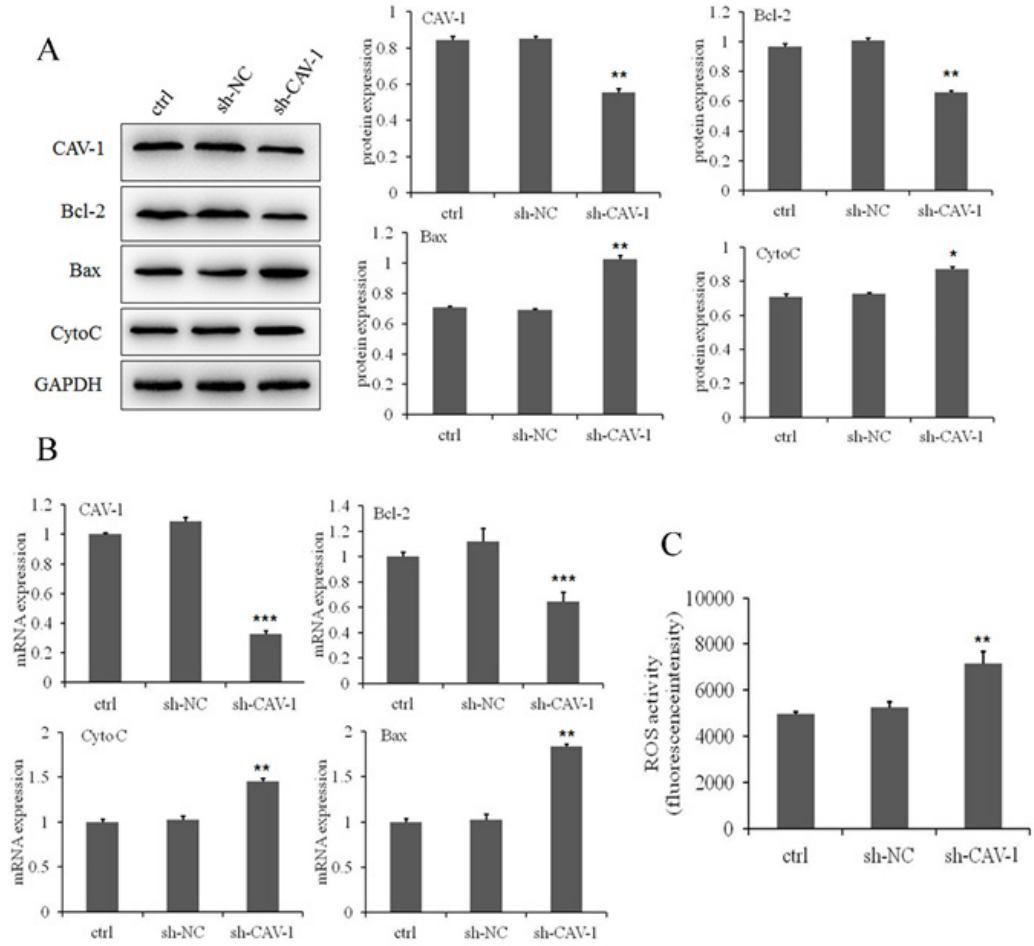

Figure 5. Effect of CAV-1 knockdown on the expressions of bcl-2, bax, and cytochrome c, and ROS levels. (A): Expressions of bcl-2, bax, and cytochrome c as, measured using Western blotting; (B): Expressions of bcl-2, bax, and cytochrome c, as measured using qRT-PCR; and (C): Level of ROS in ESCC cells after CAV-1 knockdown. $^{**}p < 0.01$; $^{***}p < 0.001$, when compared with control group.

(63.73 %), but increased the proportion of G2/M and S phase cells (10.17 and 26.10 %, respectively) ($p < 0.01$). It also significantly inhibited EC9706 cell apoptosis ($p < 0.01$). $^{**}p < 0.01$; $^{***}p < 0.001$, when compared with control group.

Effect of CAV-1 overexpression on the expressions of bcl-2, bax and cytochrome c

The bcl-2/bax ratio, cytochrome c expression, and ROS level which were enhanced by sh-TBRG4 were significantly reversed by CAV-1 overexpression in EC9706 cells. These results are shown in Figure 7.

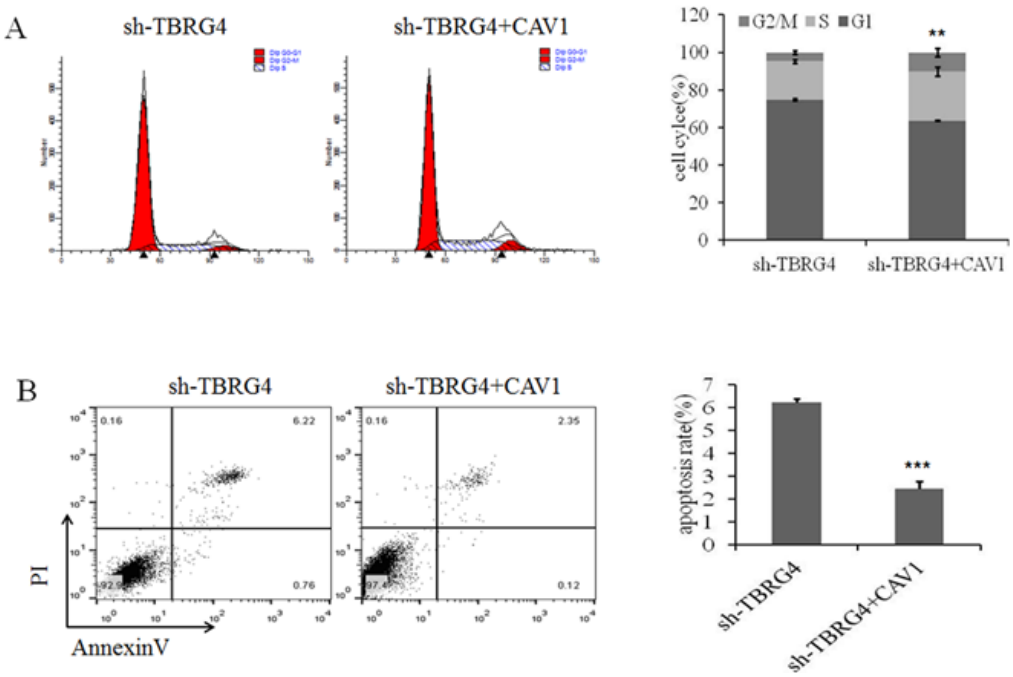

Figure 6. Effect of CAV-1 overexpression on sh-TBRG4-induced G0/G1 phase arrest and apoptosis in EC9706 cells. $^{**}p < 0.01$; $^{***}p < 0.001$, when compared with control group.

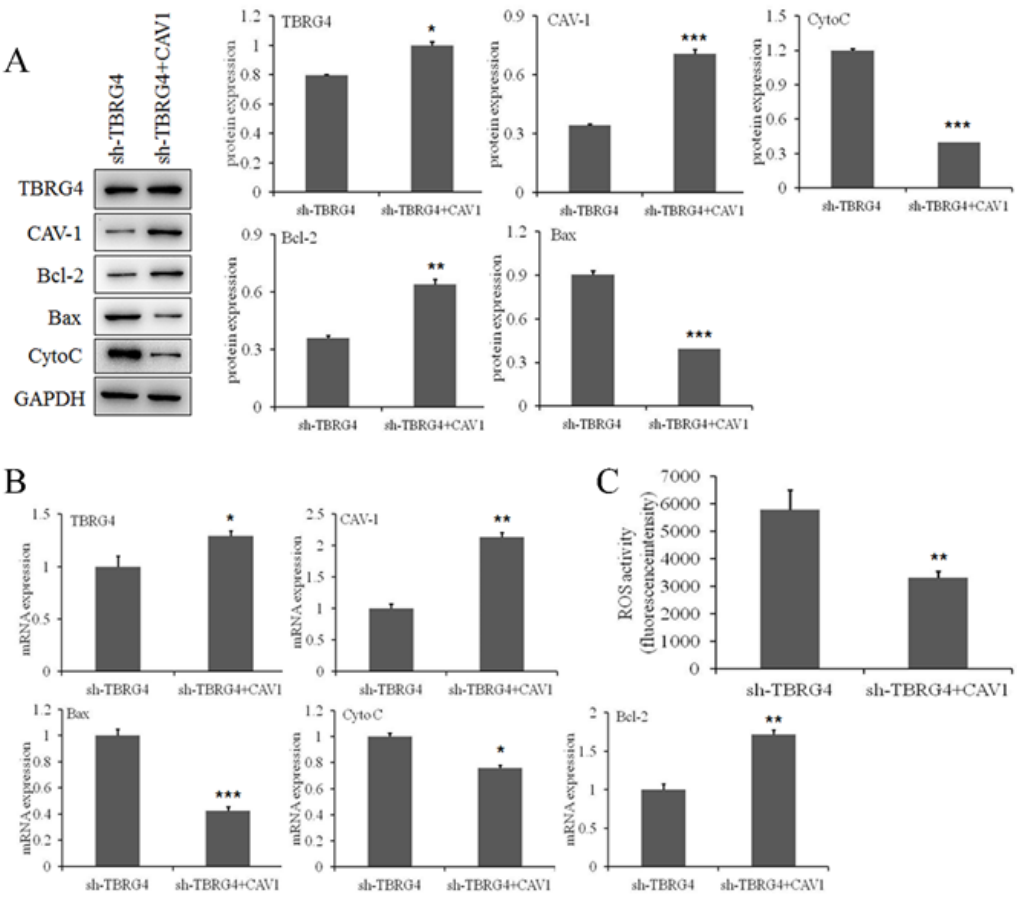

Figure 7. Effect of CAV-1 overexpression on the expressions of bcl-2, bax and cytochrome c, and levels of ROS. (A): Expressions of bcl-2, bax and cytochrome c, as measured using Western blotting; (B): Expressions of bcl-2, bax and cytochrome c, as measured using qRT-PCR; and (C): Effect of CAV-1 overexpression on ROS levels in EC9706 cells. $^{**}p < 0.01$; $^{***}p < 0.001$, when compared with control group.

Discussion

Esophageal squamous cell carcinoma (ESCC) is one of the most common types of esophageal cancer, and the sixth leading cause of cancer-related deaths worldwide (14-19). The disease is usually detected in its advanced stage due to its insidious onset. Hence, it is necessary to find potential molecular markers and novel therapeutic strategies for ESCC. Transforming growth factor β regulator 4 (TBRG4) has been implicated in the pathogenesis of many cancers (7).

In the present study, the basal levels of TBRG4

and CAV-1 were significantly higher in human ESCCs (EC9706, TE-1 and Eca109 cells) than in normal esophageal mucosal cells (Het-1), and was 3 folds higher in EC9706 cells at both protein and mRNA levels. In addition, on silencing TBRG4, the mRNA and protein expressions of CAV-1 were significantly reduced in EC9706 cells, an indication that the complete absence of TBRG4 may weaken the transcription of CAV-1 and affect the stability of its mRNA. It has been reported that CAV-1 expression is upregulated in many tumors, and high CAV-1 levels are correlated with tumor progression, invasion and metastasis (7). The results of this study are in agreement with those reported in a previous study (9). The protein and mRNA of CAV-1 were overexpressed in ESCC cells. In this study, in order to evaluate the pathological significance of CAV-1 expression in ESCC development, CAV-1 was overexpressed in TBRG4 knockdown EC9706 cells and the results indicate that CAV-1 overexpression may upregulate the expression of TBRG4. These results suggest that TBRG4 down-regulation may lead to a decrease in CAV-1 level, while CAV-1 overexpression may result in an increased level of CAV-1.

Reactive oxygen species (ROS) play important roles in living systems: they reduce mitochondrial membrane potential (MMP) via activation of mitochondrial permeability transition. They also promote cell apoptosis via the release of apoptosis-related proteins, including cytochrome c into the cytosol (20). Sensitivity to ROS-induced oxidative stress vary from one cancer cell to another (21, 22). Therefore, elevated ROS levels stimulated by anticancer drugs leads to the induction of apoptosis in the cell (11). It has been reported that ROS promote degradation of CAV-1 via the proteasome system which in turn enhances the proliferation and migration of lung cancer cells (12). The upregulation of ROS results in mitochondrial dysfunction and regulation of cellular bioenergy metabolism which affects the survival of tumor cells (14). In this study, the relationship between ROS level and CAV-1 expression in ESCC was assessed. The results showed that TBRG4 and CAV-1 were significantly overexpressed, while the level of ROS was significantly reduced in ESCC cells (especially in EC9706 cells). In addition, the expression of bcl-2 was significantly upregulated, while the expressions bax and cytochrome c were significantly downregulated in EC9706 cells. However, TBRG4 or CAV-1 knockdown significantly promoted ROS formation. It also significantly upregulated bax and cytochrome c expressions, but downregulated bcl-2 expression, an indication that elevated ROS levels due to TBRG4 silencing may be reversed by the overexpression of CAV-1 in EC9706 cells.

The upregulated expression of TBRG4 or CAV-1 promotes ESCC progression via regulation of intracellular ROS levels and inhibition of mitochondria-dependent apoptotic pathway.

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Conflict of interest
No conflict of interest is associated with this work.

Author's contribution
All work was done by the authors named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Xiaowei Fu; Junqi Wang, Qing Luo, Mingwei Liu, Chao Zhang, Yong Jia, Ruifeng Tong, Litao Yang, Xiaowei Fu and Qing Luo collected and analysed the data; Junqi Wang wrote the text and all authors have read and approved the text prior to publication.

References

1. Arnold M, Soerjomataram I, Ferlay J, Forman D. Global incidence of oesophageal cancer by histological subtype in 2012. Gut 2015; 64: 381-387.
2. Simarro M, Gimenez-Cassina A, Kedersha N, Lazaro JB, Adelmant GO, Marto JA. Fast kinase domain-containing protein 3 is a mitochondrial protein essential for cellular respiration. Biochem Biophys Res Commun 2010; 401: 440-446.
3. Sevcikova S, Paszekova H, Besse L, Sedlarikova L, Kubaczkova V, Almasi M, et al. Extramedullary relapse of multiple myeloma defined as the highest risk group based on deregulated gene expression data. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 2015; 159: 288-293.
4. Edwards MC, Liegeois N, Horecka J, DePinho RA, Sprague GF, Tyers M, et al. Human CPR (cell cycle progression restoration) genes impart a Far- phenotype on yeast cells. Genetics 1997; 147: 1063-1076.
5. Wolf AR, Mootha VK. Functional genomic analysis of human mitochondrial RNA processing. Cell Rep 2014; 7: 918-931.
6. Rolland T, Tasan M, Charloteaux B, Pevzner SJ, Zhong Q, Sahni N, et al. A proteome-scale map of the human interactome network. Cell 2014; 159: 1212-1226.
7. Burgermeister E, Liscovitch M, Rocken C, Schmid RM, Ebert MP. Caveats of caveolin-1 in cancer progression. Cancer Lett 2008; 268: 187-201.
8. Hu YC, Lam KY, Law S, Wong J, Srivastava G. Profiling of differentially expressed cancer-related genes in esophageal squamous cell carcinoma (ESCC) using human cancer cDNA arrays: overexpression of oncogene MET correlates with tumor differentiation in ESCC. Clin Cancer Res 2001; 7: 3519-3525.
9. Kato K, Hida Y, Miyamoto M, Hashida H, Shinohara T, Itoh T, et al. Overexpression of caveolin-1 in esophageal squamous cell carcinoma correlates with lymph node metastasis and pathologic stage. Cancer 2002; 94: 929-933.
10. Wang A, Zhao C, Liu X, Su W, Duan G, Xie Z, et al. Knockdown of TBRG4 affects tumorigenesis in human H1299 lung cancer cells by regulating DDIT3, CAV1 and RRM2. Oncol Lett 2018; 15: 121-128.
11. Lei K, Tan S, Du W, Xu Y, Lin S, Zheng Y, et al. 3B, a novel of photosensitizer, exhibited anti-tumor effects via mitochondrial apoptosis pathway in MCF-7 human breast carcinoma cells. Tumour Biol 2015; 36: 5597-5606.
12. Luanpitpong S, Talbott SJ, Rojanasakul Y, Nimmannit U, Pongrakhananon V, Wang L, et al. Regulation of lung cancer cell migration and invasion by reactive oxygen species and caveolin-1. J Biol Chem 2010; 285: 38832-38840.
13. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25: 402-408.
14. Zhang X, Lan L, Niu L, Lu J, Li C, Guo M, et al. Oxidative stress regulates cellular bioenergetics in esophageal squamous cell carcinoma cell. Biosci Rep 2017; 37: 20171006.

15. Robertson JD, Orrenius S, Zhivotovsky B. Review: nuclear events in apoptosis. J Struct Biol 2000; 129: 346-358.
16. Zhang Y. Epidemiology of esophageal cancer. World J Gastroenterol 2013; 19: 5598-5606.
17. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016, CA. Cancer J Clin 2016; 66: 7-30.
18. Zeng H, Zheng R, Guo Y, Zhang S, Zou X, Wang N, et al. Cancer survival in China, 2003-2005: a population-based study. Int J Cancer 2015; 136: 1921-1930.
19. DeAngelis R, Sant M, Coleman MP, Francisci S, Baili P, Pierannunzio D, et al. Cancer survival in Europe 1999-2007 by country and age: results of EUROCARE--5-a population-based study. Lancet Oncol 2014; 15: 23-34.
20. Zaccagnino P, Saltarella M, D'Oria S, Corcelli A, Saponetti MS, Lorusso M, et al. N-arachidonylglycine causes ROS production and cytochrome c release in liver mitochondria. Free Radic Biol Med 2009; 47: 585-592.
21. Lee JH, Won YS, Park KH, Lee MK, Tachibana H, Yamada K, et al. Celastrol inhibits growth and induces apoptotic cell death in melanoma cells via the activation ROS-dependent mitochondrial pathway and the suppression of PI3K/AKT signaling. Apoptosis 2012; 17: 1275-1286.
22. Xie CM, Chan WY, Yu S, Zhao J, Cheng CH. Bufalin induces autophagy-mediated cell death in human colon cancer cells through reactive oxygen species generation and JNK activation. Free Radic Biol Med 2011; 51: 1365-1375.