PTEN inhibitor VO-OHpic suppresses TSC2\(-/-\) MEFs proliferation by excessively inhibiting autophagy via the PTEN/PRAS40 pathway

WENDA WANG\(^1\), XU WANG\(^1\), HAO GUO\(^1\), YI CAI\(^1\), YUSHI ZHANG\(^1\) and HANZHONG LI\(^1\)

\(^1\)Department of Urology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100730; \(^2\)Department of Urology, Xiangya Hospital, Central South University, Changsha, Hunan 410008, P.R. China

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Abstract. Tuberous sclerosis complex (TSC) is a relatively rare autosomal dominant disease which involves multiple organs, including the brain, kidney, lung, skin and heart. Renal angiomyolipomas (RAML) are the main causes of mortality in patients with TSC. The preferred treatment for RAML is the use of mTOR inhibitors, but the efficacy of these are not satisfactory. Therefore, an alternative treatment is urgently required. Autophagy levels decline in TSC associated cortical tubers, and the inhibition of autophagy in animal or cell models of TSC may suppress tumor development and cell proliferation. PTEN is a protein tyrosine phosphatase and can inhibit the activation of Akt. In the present study, it was indicated that the PTEN inhibitor, hydroxyl(oxo)vanadium 3-hydroxypiridine-2-carboxylic acid (VO-OHpic), suppressed proliferation and growth of TSC\(-/-\) murine embryonic fibroblasts (MEFs) by further inhibiting autophagy of cells. The expression levels of human microtubule-associated protein 1 light chain 3-I (LC3-I) and LC3-II, which are autophagy associated proteins, were demonstrated to decline following VO-OHpic treatment. The expression levels of phosphorylated proline-rich Akt substrate 40 kDa (PRAS40) also decreased in TSC\(-/-\) MEFs treated with VO-OHpic. The PTEN inhibitor may inhibit the proliferation of TSC\(-/-\) MEFs through the PTEN-PRAS40 pathway by excessively inhibiting autophagy, without the dependence of the Ras homolog, mTORC1 binding/mTOR pathway. PTEN may be a potential therapeutic target for the treatment of TSC. Further \textit{in vivo} studies are required to confirm these results.

Introduction

Tuberous sclerosis complex (TSC) is a relatively rare autosomal dominant disorder, which can involve multiple organs, including brain, kidney, lung, skin and heart (1). The typical manifestations of TSC in the kidneys include angiomyolipomas (AMLs), multiple renal cysts and renal carcinomas (2). Renal AML (RAML) is present in \(~\text{70-90\%}\) of patients with TSC, and it is the leading cause of mortality in adult patients with the disorder (3). The recommended first-line therapy for TSC-RAML is the use of mTOR inhibitors. However, \(~\text{30-40\%}\) patients using mTOR inhibitors do not achieve clinical remission, which is defined as a RAML volume reduction \(\geq 50\%\) and severe adverse reactions may also occur in some patients (4,5). Despite these issues, there are currently no other treatment options for RAML (5). The critical role of autophagy in the formation and development of tumors has become increasingly recognized (6). Autophagy influences the rearrangement of the plasma membrane, leading to the sequestration of proteins and organelles intended for delivery into lysosomes for degradation (7). Autophagy is a highly conserved catabolic process of cellular proteins and organelles in which they are sequestered into lysosomes for degradation to maintain cellular homeostasis (8). There are three types of autophagy, including microautophagy, macroautophagy and chaperone-mediated autophagy (7), in which macroautophagy is among the most researched. During macroautophagy, the membrane expands to form phagophores, which expand and bend continuously. The membrane then wraps around the cargo to form complete autophagosomes, which is then delivered into the lysosomes for degradation where the materials are then reused (7). Human microtubule-associated protein 1 light chain 3 (LC3) is an important protein for autophagosome formation (7). LC3 is normally spliced further into LC3-I and LC3-II, which is mainly localized in the cytoplasm and on the autophagosomal double membrane, respectively (9). The levels of LC3-I and II can therefore be used to reflect the degree of autophagy. Miyahara et al (9) previously demonstrated in the cerebral cortical tubers of patients with TSC that the levels of LC3-I and LC3-II were lower compared with those in normal cerebral tissues. Autophagy may promote or
inhibit tumorigenesis, depending on the cellular context (6). Autophagic dysfunction can lead to a variety of human pathologies, including cancer, metabolic diseases and degenerative conditions (10). In Myc-induced lymphomas, chloroquine may inhibit autophagy and the fusion of autophagosomes and lysosomes, resulting in the suppression of lymphoma development (11). Haploinsufficiency of the autophagy gene, Beclin 1, promotes tumor formation in the TSC2-/- mouse model (6) and allelic loss of Beclin 1 may be associated with breast, ovarian and prostate cancers (12).

mTOR also participates in the regulation of autophagy by inhibiting the formation of the autophagosome (13). Ras homolog-enriched in brain (Rheb) is a principal downstream target of the TSC1/TSC2 complex and an upstream regulator of mTOR. When TSC1 or TSC2 mutates, Rheb can be abnormally activated and promote mTOR activation to downregulate autophagy (13). PTEN is an upstream regulator of Akt that regulates the TSC1/TSC2 complex (14,15), but has also been revealed to potentially regulate autophagy via a different signaling pathway independent of TSC1/TSC2 (15). The current study indicated that the PTEN inhibitor, hydroxyl(oxo)vanadium 3-hydroxypiridine-2-carboxylic acid (VO-OHpic), could suppress the proliferation of TSC2-/- murine embryonic fibroblasts (MEFs) by excessively inhibiting autophagy via the PTEN/proline-rich Akt substrate 40 kDa (PRAS40) pathway.

Materials and methods

Cell culture. The TSC2-/- and TSC2+/- MEF cell lines, as aforementioned (16), were donated by Professor Hongbing Zhang, Chinese Academy of Medical Sciences and Peking Union Medical College (Beijing, China). The cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified 5% CO₂ atmosphere. The TSC2-/- MEFs were used as two groups: i) With VO-OHpic treatment at 1 µM as previously described (17); or ii) without VO-OHpic treatment. All cultured TSC2-/- and TSC2+/- MEFs were harvested at 24, 48 and 72 h, respectively, for further analysis.

Cell proliferation analysis. Cell proliferation assays were conducted using a Cell Counting kit-8 (CCK-8; Beijing Solarbio Science & Technology Co., Ltd.; cat. no CA1210) according to the manufacturer’s protocol. The harvested cells (5,000 cells/well) were seeded in 96-well plates at 100 µl per well and cultured in growth medium at 37°C and 5% CO₂. A total of 10 µl of CCK-8 were added to each well at 24, 48 and 72 h. Subsequently, the cells were incubated at 37°C for 3 h and the optical density was measured at 450 nm.

Western blot analysis. The cells were stored at -80°C, then proteins were subsequently extracted with protein lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.; cat. no. R0020). The protein concentration was standardized using a BCA protein assay kit (Beijing Solarbio Science & Technology Co. Ltd.; cat. no. PC0020). A total of 30 µg protein extracts from the various groups (with or without VO-OHpic treatment, at 24, 48 or 72 h, respectively) were loaded into a 15% SDS-PAGE gel and transferred onto PVDF membranes. The membranes were blocked with 5% skimmed milk in Tris-buffered saline with Tween 20 (0.01 M Tris-HCl, pH 7.6; 0.15 M NaCl; 0.05% Tween 20) for 2 h at room temperature. The membranes were incubated with rabbit anti-LC3 antibodies Abs (Sigma-Aldrich; Merck KGaA; cat. no. L8918; 1:2,000), rabbit anti-phosphorylated (p)-PTEN Abs (Abcam; cat. no. ab31392; 1:1,000), rabbit anti-PTEN Abs (Abcam; cat. no. ab32199; 1:1,000), rabbit anti-P-PRAS40 Abs (Abcam; cat. no. ab34084; 1:1,000) or rabbit anti-PRAS40 Ab (Abcam; cat. no. ab151719; 1:1,000) overnight at 4°C. GAPDH antibodies were used as an internal control (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.; cat. no. TA-08; 1:1,000). The bound primary Ab was detected with horseradish peroxidase-linked goat anti-rabbit immunoglobulin G (IgG; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.; cat. no. ZB-2301; 1:10,000). The secondary incubation was conducted at room temperature for 1 h. Subsequently, the antigen-antibody complexes were detected using ECL (Tanon Science & Technology Co. Ltd.). Gray values were calculated using Image J 1.50i software (National Institutes of Health).

Statistical analysis. All of the data were analyzed using SPSS version 19.0.0 (IBM Corp.) and GraphPad Prism version 6.0 (GraphPad Software, Inc.). The results are presented as the mean ± SEM. Differences between groups were analyzed.

Figure 1. The autophagy levels in TSC2-/- and TSC2+/- MEFs. (A) Western blot analysis of LC3-I and LC3-II in TSC2-/- and TSC2+/- MEFs. (B) Average relative gray values of LC3-I at the 24, 48 and 72 h. (C) Average relative gray values of LC3-II at the 24, 48 and 72 h. The stated time point represents the time point at which the MEFs were harvested. *P<0.001. MEF, murine embryonic fibroblasts; TSC, tuberous sclerosis complex; LC3, human microtubule-associated protein 1 light chain 3.
using two-way ANOVAs followed by post-hoc Tukey’s tests. P<0.05 was considered to indicate a statistically significant difference. All experiments were repeated at least three times.

Results

To confirm the autophagic conditions in TSC2⁻/⁻ MEFs, the expression levels of LC3 were analyzed. TSC2⁻/⁻ and TSC2⁺/+ MEFs were harvested at 24, 48 and 72 h, and western blot analysis was performed (Fig. 1). Overall, LC3 expression in TSC2⁻/⁻ MEFs was reduced compared with that in TSC2⁺/+ MEFs at 24 and 72 h. Two-way ANOVA analysis indicated that neither TSC2⁺/+/TSC2⁻/⁻ nor the time point of collection, had a significant effect on LC3-I expression levels (P=0.245 and P=0.291, respectively; data not shown). Considering the effect of TSC2⁺/+/TSC2⁻/⁻, no statistically significant differences were found (All P>0.05; Fig. 1B). By contrast, both TSC2⁺/+/TSC2⁻/⁻ and the collection time had a significant effect on LC3-II expression (both P<0.001). When considering the effect of TSC2⁺/+/TSC2⁻/⁻, except for the cells at 48 h, LC3-II was expressed at a significantly lower level in TSC2⁻/⁻ MEFs (24 h, P<0.001; 72 h, P<0.001; Fig. 1C).
Subsequently, a PTEN inhibitor, VO-OHpic, was added to TSC2−/−MEFs and the effect on cell proliferation and growth was observed. At various time points, the cells that underwent VO-OHpic treatment demonstrated a reduced volume and a decrease in their density (Fig. 2). The MEFs displayed larger cellular bodies and nuclei, with more obvious processes, and the cells grew at a greater density when VO-OHpic was not applied. VO-OHpic treatment led to atrophy of the cell bodies and nuclei of MEFs and a reduction in the size and number of processes. CCK-8 assays were also performed for quantitative analysis. A two-way ANOVA revealed that both the intervention and the time points of collection had a significant effect on the extent of cell viability (both, P<0.001). When considering the effect of the intervention, VO-OHpic led to a reduction in cell viability (with vs. without VO-OHpic treatment: 24 h, P=0.333; 48 h, P=0.005; 72 h, P<0.001; Fig. 3).

To further analyze the effects of the PTEN inhibitor on autophagy of TSC2−/−MEFs, the LC3 levels of cells treated with or without VO-OHpic were examined (Fig. 4). Two-way ANOVA analysis showed that the intervention had a significant effect on LC3-I expression levels (overall, P<0.001; intervention vs. control: 24 h, P=0.001; 48 h, P=0.015; and 72 h, P=0.001); however, this did not change with time (P=0.800). Similar results were indicated for LC3-II expression levels (overall intervention, P<0.001; overall time, P=0.644; intervention vs. control: 24 h, P=0.020; 48 h, P<0.001; and 72 h, P=0.007).

The potential pathways which allowed for this change were subsequently investigated. p-PTEN and PTEN levels were examined in TSC2−/−MEFs under various conditions (with or without VO-OHpic treatment at 24, 48 or 72 h). The relative expression ratios of p-PTEN/PTEN were then calculated. Two-way ANOVA analysis revealed that both interventions and time points had a significant effect on the p-PTEN/PTEN expression ratio (interventions, P<0.001; time points, P=0.013). When considering the effect of the intervention, the relative expression ratio of p-PTEN/PTEN declined in TSC2−/−MEFs treated with VO-OHpic (intervention vs. control: 24 h, P=0.001; 48 and 72 h, P<0.001; Fig. 4). The activity of PRAS40, which is a downstream regulator of PTEN, was also affected by treatment with VO-OHpic. Both interventions and time point measured had a significant effect on p-PRAS40/PRAS40 expression ratio, as indicated by two-way ANOVA analysis (both, P<0.001). When considering the effect of the intervention, the relative expression ratio of p-PRAS40/PRAS40 deceased in TSC2−/−MEFs treated with VO-OHpic (intervention vs. control: 24 h, P=0.137; 48 and 72 h, P<0.001; Fig. 4).

Discussion

TSC-associated RAML is a life-threatening disease in adult patients (3). Recently, mTOR inhibitors such as everolimus are
PTEN may regulate autophagy by allowing for a bypass of PTEN/PRAS40 signaling in TSC2-/- cells and influence cell proliferation. PTEN could suppress the activation of mTOR and promote autophagy not only by inhibiting Akt/Rheb/mTOR pathway, but also by activating the PTEN/PRAS40 pathway.

Figure 5. PTEN may regulate autophagy by allowing for a bypass of PTEN/PRAS40 signaling in TSC2-/- cells and influence cell proliferation. PTEN could suppress the activation of mTOR and promote autophagy not only by inhibiting Akt/Rheb/mTOR pathway, but also by activating the PTEN/PRAS40 pathway.

In conclusion, the PTEN inhibitor, VO-OHpic, may inhibit the development of the tumors (6). It is hypothesized that there is a balance between the inhibition and activation of autophagy in TSC tumors (6). The inhibition of autophagy leads to the accumulation of P62/SQSTM1 in TSC2-/- MEFs, which initiates the development of TSC tumors (6). Autophagy cannot be induced adequately with numerous metabolic defects existing in TSC2-/- MEFs; therefore, further suppression of autophagy may exhibit an inhibitory effect on tumor development. Therefore, the development of TSC tumors may also depend on autophagy.

PTEN is a highly conserved dual-function protein tyrosine phosphatase, which can dephosphorylate both inositol lipids and proteins. PTEN can dephosphorylate the lipid second messengers phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate to produce phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 4-phosphate, respectively, leading to the inhibition of Akt activation (14). VO-OHpic is a specific and reversible small molecule inhibitor of PTEN (14). PRAS40 is a component of the mTORC1 complex and is also a downstream target of Akt (25). However, p70 ribosome S6 kinase and 4E-binding protein 1, substrates of mTORC1, can combine with mTORC1, thereby competing with PRAS40, thereby suppressing autophagy by upregulating mTOR-p70S6K, resulting in further mTORC1 activation and autophagy inhibition (26).

In a study by Nardella et al (15) on prostate cancer, PTEN deficiencies promoted prostate tumor formation in Rheb overexpressing mice. The aforementioned study indicated that PTEN not only regulates autophagy through the Akt/Rheb/mTOR pathway, but also through the Akt/PRAS40 pathway (Fig. 5). In the present study, the effect of PTEN inhibitors on autophagy in TSC2-/- MEFs was investigated. It was revealed that the PTEN inhibitor, VO-OHpic, could suppress autophagy, cell proliferation and cell growth in TSC2-/- MEFs. The expression of p-PTEN and p-PRAS40 also declined, revealing that VO-OHpic may regulate autophagy via the PTEN/PRAS40 pathway. The results are based on in vitro experiments, and further in vivo studies are required to validate these results. The further downstream proteins also require investigation.

In conclusion, the PTEN inhibitor, VO-OHpic, may suppress the proliferation of TSC2-/- MEFs, potentially through the excessive inhibition of autophagy via the PTEN/PRAS40 pathway, and may be a potential therapeutic target for the treatment of TSC-RAML.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author's contributions

YZ and HL conceived and designed the experiments. WW, XW and HG performed the cellular experiments. YC provided and performed the cell culture. WW produced the manuscript. WW and YZ conducted data analysis. All the authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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