MiR-4465 directly targets PTEN to inhibit AKT/mTOR pathway-mediated autophagy

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Abstract

Autophagy plays an important role in maintaining cell function. Abnormal autophagy leads to cell dysfunction and is associated with many diseases such as tumors, immunodeficiency diseases, lysosomal storage disorders, and neurodegenerative diseases. Autophagy is precisely regulated, and PTEN plays an important role in regulating autophagy. As noncoding small RNAs, miRNAs play an important role in the fine regulation of cellular processes. However, the mechanism of the miRNA regulation of PTEN-related autophagy has not been fully elucidated. In this study, our results showed that miR-4465 significantly inhibited the expression of PTEN, upregulated phosphorylated AKT, and thereby inhibited autophagy by activating mTOR in HEK293, HeLa, and SH-SY5Y cells. Further studies indicated that miR-4465 reduced PTEN mRNA levels through posttranscriptional regulation via directly targeting the 3'-UTR. Our novel findings provide useful hints for the comprehensive elucidation of the molecular mechanism of miRNA-regulated PTEN-related autophagy and may also provide some new insights for the exploration of miRNAs in the treatment of PTEN-related diseases.

Keywords Autophagy · miR-4465 · PTEN · mTOR

Abbreviations

PTEN	Phosphatase and tensin homolog deleted on chromosome ten
mTOR	Mammalian target of Rapamycin
MAPK	Mitogen-activated protein kinase
Baf A1	Bafilomycin A1

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Introduction

Autophagy is necessary to maintain normal cellular function, cell self-renewal, and homeostasis (Mizushima and Komatsu 2011). When a cell scavenges a target protein or organelle through autophagy, it first forms a bilayer membrane structure of the autophagosome in the cytoplasm, thereby recognizing the target protein or organelle and finally fusing with the lysosome for degradation (Kroemer et al. 2010), and this process is precisely regulated. Numerous studies show that abnormal autophagy is closely related to various diseases, such as immunodeficiency diseases, tumors, and neurodegenerative diseases (Menzies et al. 2015; Zhong et al. 2016).

PTEN (phosphatase and tensin homolog deleted on chromosome ten) consists of 403 amino acids and is necessary for the maintenance of normal cellular functions. PTEN was identified as a candidate tumor-suppressor gene (Li et al. 1997). PTEN is also the second most common tumor suppressor, after P53, and is closely related to tumorigenesis (Nakamura et al. 2000). Overexpressing PTEN in lung cancer cells significantly inhibits proliferation (Kim et al. 1998), and tumor growth and migration are significantly inhibited in PTEN-deficient model animals (Chen et al. 2005). Furthermore, PTEN is also widely expressed in the central nervous system

(Song et al. 2012), and not only plays an important role in the development of the nervous system and maintenance of normal functions but is also closely associated with neuropsychiatric disorders, such as Alzheimer's disease (Sonoda et al. 2010) and autism (Zhou and Parada 2012). Rapamycin, as an mTOR (mammalian target of rapamycin) inhibitor, reverses neuronal hypertrophy and polarity (Zhou et al. 2009). The mTOR signaling pathway plays a central role in the regulation of autophagy (Sancak et al. 2008). MTOR negatively regulates autophagy, and the mTOR inhibitor rapamycin, amino acid deprivation, and starvation can activate autophagy (Sancak et al. 2008). The above studies suggest that autophagy is closely related to PTEN-related diseases.

MicroRNAs (miRNAs) are a class of noncoding single-stranded small RNAs that are approximately 22 nucleotides in length and are involved in transcriptional and posttranscriptional regulating gene expression (Gregory et al. 2005b). MiRNAs target messenger RNA (mRNA) molecules by directly binding their 3' untranslated regions (UTRs), resulting in mRNA degradation and/or translational inhibition (Bartel 2009). Interestingly, miRNAs are reported to regulate autophagy through various mechanisms, and targeting miR-21 induces autophagy and chemosensitivity in leukemia cells (Seca et al. 2013). Although many studies report that miRNAs affect autophagy, the mechanism by which miRNAs regulate PTEN-related autophagy is not fully elucidated. In this study, we attempted to find novel miRNAs that regulate PTEN in addition to the reported miRNAs and explored their molecular mechanism.

Materials and methods

Plasmid constructs

FLAG-p62 and Mcherry-EGFP-LC3 were described previously (Guo et al. 2015; Zhou et al. 2013). Mcherry-LC3 was a gift from David Rubinsztein (Addgene plasmid no. 40827) (Jahreiss et al. 2008). To construct the plasmids and measure the effects of miR-4465 on PTEN 3'-UTR luciferase activity, the partial PTEN 3'-UTR oligonucleotides were searched and synthesized according to GenBank (gene ID, 5728). Briefly, the PTEN 3'-UTR-1 sequences, containing 322 bp from positions 10 to 331 of the PTEN 3'-UTR; the PTEN 3'-UTR-2 sequences, containing 235 bp from 1194 to 1428 of the PTEN 3'-UTR; the PTEN 3'-UTR-3 sequences, containing 316 bp from 2531 to 2846 of the PTEN 3'-UTR; and the PTEN 3'-UTR-4 sequences, containing 46 bp from 3728 to 4072 of the PTEN 3'-UTR were synthesized and cloned into the psi-CHECK2 vector separately, via the XhoI and NotI sites. Those sequences contained miR-4465 binding sites. For the construct mutants, the sequence mutations, as

indicated, were synthesized and cloned into the psi-CHECK2 vectors. All the constructs were confirmed via DNA sequencing.

Cell culture, transfection, and treatment

Human embryonic kidney 293 (HEK293) and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) at 37 °C in the presence of 5% CO₂. SH-SY5Y cells were cultured in DMEM/F12 (1:1) medium containing 10% FBS. All the medium contained penicillin (100 U/ml) and streptomycin (100 µg/ml). Bafilomycin A1 (Baf A1) was purchased from CST, and rapamycin was purchased from Sigma. For starvation, cells were cultured with Earle's balanced salt solution (Gibco) for 2 h and re-fed with the RPMI-1640 medium (Gibco) supplemented with 10% FBS.

Small RNA transfection

The RNA oligonucleotides, such as the miRNA mimics, the miRNA control, the si-RNA control, the si-PTEN (GAGCGUGCAGAUAAUGACA) that targets PTEN (Ma et al. 2014), and the miR-4465 inhibitor were purchased from GenePharma (Shanghai, China). The mutant miR-4465 was mutated several bases in the seed sequence region of miR-4465 and synthesized according to the classical mutation method. These RNAs were transfected into HEK293 cell, HeLa, and SH-SY5Y cells with RNAiMAX (Invitrogen, USA). Briefly, the RNA oligonucleotides were mixed with Opti-MEM that contained RNAiMAX, and then, these were mixed and incubated for 15–20 min at room temperature before transfection. Twenty-four hours later, the medium was replaced with fresh complete medium, and 72 h after transfection, the cells were harvested and subjected to further analysis.

Cell viability assay

The cell viability was assessed with an MTT assay. The HEK293 cells and SH-SY5Y cells were treated with condition media and were incubated with 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at 37 °C for 2 h. Then, 150 µL of dimethylsulfoxide was added to stop the reaction. The absorbance was measured at 570 nm to determine cell viability.

Immunoblot analysis and antibodies

The cells were collected at the time indicated after transfection and were lysed in cell lysis buffer containing 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, and protease inhibitor cocktail (Roche, Mannheim, Germany). Approximately 20 µg of the cell

lysates were separated by SDS-PAGE and was then transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The immunoblot analysis was performed with the following primary antibodies: the p62 antibody and anti-LC3 antibody were purchased from Abcam; the FLAG antibody was purchased from Sigma; and the GAPDH antibody was purchased from Millipore. The phospho-p38 antibody, p38 antibody, phospho-AKT antibody, AKT antibody, phospho-mTOR antibody, mTOR antibody, phospho-p70S6K antibody, p70S6K antibody, phospho-ERK1/2 antibody, and anti-ERK1/2 antibody were purchased from CST. The anti-mouse IgG (H + L) and anti-rabbit IgG (H + L) secondary antibodies, coupled to horseradish peroxidase, were from Jackson ImmunoResearch (West Grove, PA, USA). The proteins were visualized using an ECL detection kit (Thermo).

Quantitative RT-PCR

The total RNA was extracted from the cells using TRIzol Reagent (Invitrogen) and was subsequently reverse-transcribed into cDNA for the PCR assays with a TransScript First-Strand cDNA Synthesis Kit (Takara, Otsu, Shiga, Japan). The real-time PCR analysis was carried out for the quantitative measurement of the target RNA abundance with the SYBR Green Real-Time PCR Master Mix (Takara) in a PCR detection system (Applied Biosystems). The following primers were used: 5' AATCCCATCACCATTCTTCCA 3' and 5' TGGACTCCACGACGTACTCA 3' for human GAPDH and 5' TTTGAAGACCATAACCCACCAC 3' and 5' ATTACACCAGTTCGTCCCTTTC 3' for human PTEN. The levels of PTEN mRNA were normalized to those of GAPDH. For miRNA detection, the total RNA was extracted from cells as above, and then total RNA was reversely transcribed and amplified using TaqMan MicroRNA assay kit (Invitrogen, USA) according to the manufacturer's instructions. U6 snRNA was used as an internal loading control.

Immunofluorescence

The HEK293 cells were washed with PBS and were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 5 min. Then, the cells were treated with 0.1% Triton-X100 for 5 min and blocked with 4% FBS in PBST (PBS containing 0.1% tween-20). Immunocytochemical staining was performed using monoclonal anti-FLAG antibodies or anti-LC3 antibodies. The cells were then incubated with Alexa Fluor 488 donkey anti-mouse secondary antibodies or donkey anti-rabbit secondary antibodies (Thermo). The cells were observed by using fluorescence microscopy (Nikon) or using LIEKA sp8 confocal microscopy TCS SP8 (Leica). The HEK293 cells that were transfected with Mcherry-EGFP-LC3 were fixed with 4% paraformaldehyde in PBS and were

then observed using LIEKA sp8 confocal microscopy TCS SP8 (Leica).

Luciferase reporter assay

The psi-CHECK2-PTEN 3'-UTR-1, psi-cherk2-PTEN 3'-UTR-2, psi-cherk2-PTEN 3'-UTR-3, psi-cherk2-PTEN 3'-UTR-4 or psi-cherk2-PTEN 3'-UTR-1-mut, psi-cherk2-PTEN 3'-UTR-2-mut, psi-cherk2-PTEN 3'-UTR-3-mut, and psi-cherk2-PTEN 3'-UTR-4-mut plasmids were transfected into the HEK293 cells using Lipofectamine 2000 (Invitrogen), and 24 h later, the luciferase activity was detected according to the manufacturer's protocol using a Dual-Luciferase Reporter Assay System (Promega).

Statistical analysis

The statistical analyses were performed through a two-tailed Student's *t* test for the comparison of the two groups. The comparison of multiple groups of more than two were analyzed using a one-way ANOVA or a two-way ANOVA test, depending on comparison variables, with Tukey's post hoc analysis as indicated (GraphPad Prism 6). The data are shown as the mean $\pm$ S.D., and a *P* value < 0.05 is considered statistically significant.

Results

MiR-4465 significantly decreases PTEN expression

The miRNAs targeting PTEN 3'-UTR were predicted by the database (TargetScan, miRanda, and PITA) as indicated, and the top 13 miRNAs (Fig. 1a, b) were selected. These miRNA mimics were transfected into the HEK293 cells. After 48 h, the immunoblotting results showed that miR-4465 significantly inhibited the expression of PTEN, and the effect was similar to the knockdown of PTEN (Fig. 1b). Similar results were observed in the HeLa and SH-SY5Y cells (Fig. 1c, d). Transfection of the miR-4465 mutants into the HEK293 cells did not reduce PTEN expression (Fig. 1e). Additionally, the transfection of the miR-4465 mimics into the HEK293 cells revealed that miR-4465 significantly reduced PTEN mRNA levels, whereas the transfected miR-4465 inhibitor increased the expression of PTEN mRNA (Fig. 1f, g). Similar results were also observed in HeLa cells (Fig. 1f, g). The above results indicated that miR-4465 significantly reduced the expression of PTEN in many different types of cells.

MiR-4465 inhibits autophagy

PTEN plays an important role in regulating autophagy. PTEN deficiency in neurons significantly inhibits autophagy, leading

Fig. 1 MiR-4465 inhibits PTEN expression. **a** Thirteen candidate miRNAs that target PTEN were predicted through the indicated database. **b** The candidate miRNAs were transfected to HEK293 cells. **c, d** MiR-4465 was transfected into HeLa cells or SH-SY5Y cells. The cells were harvested for immunoblotting. **e** MiR-4465 or miR-4465 mutants were transfected into the HEK293 cells, and 72 h later, the cells were harvested for immunoblotting. **f** MiR-4465 or mi-con (negative control) groups were transfected into the HEK293 cells or HeLa cells for 72 h, and then, the cells were subjected to qRT-PCR to detect the PTEN mRNA. **g** Similarly, the miR-4465-inhibitors or con-inhibitors were transfected into the HEK293 cells or HeLa cells for 72 h to detect the PTEN mRNA. * $P < 0.05$; ** $P < 0.01$

to abnormal cellular function (Liu et al. 2010). To explore whether miR-4465 affects cell autophagy, miR-4465 mimics were transfected to HeLa cells, which were a cell model used to study autophagy. The results showed that the level of LC3-II was significantly upregulated (Fig. 2a), which was consistent with the results of the knockdown of PTEN. The adaptor protein p62/SQSTM1 is an autophagy substrate that binds to LC3 selectively to induce degradation by autophagy (Komatsu et al. 2010). The results showed that p62 was also significantly upregulated (Fig. S1-A). Similar results were

also observed in the HEK293 cells (Fig. S1-B) and SH-SY5Y cells (Fig. S1-C). The co-transfection of FLAG-p62 or Mcherry-LC3 with the miR-4465 mimics in the HEK293 cells also showed that LC3-II and p62 accumulation significantly increased (Fig. S1-D, E). To further observe the effect of miR-4465 on autophagy, miR-4465 was transfected into the HEK293 cells, and the cells were treated with the autophagy inhibitor Baf A1 (Bafilomycin A1), a vacuolar-type H^+ -ATPase inhibitor that blocks the fusion of autophagosomes with lysosomes. The results showed that miR-4465 significantly increases LC3-II even without Baf A1 treatment (Fig. 2b). However, when treated with Baf A1, the LC3-II levels in the HEK293 cells were increased to a large extent, but the levels of LC3-II of the miR-4465 group were decreased compared to those of the miRNA control group (mi-con) (Fig. 2b). Mcherry-EGFP-LC3 is often used to indicate autophagic flux. In the cells expressing Mcherry-EGFP-LC3, when the autophagosome is fused with the lysosome because of the quenching of the pH-sensitive EGFP, the autophagosome, autophagosome fusion, and degradation of autophagosome are indicated (Pankiv et al. 2007). MiR-

Fig. 2 MiR-4465 inhibits autophagy. **a** MiR-4465, si-PTEN, or mi-con (negative control) was transfected into the HEK293 cells for 72 h, and then, the LC3 level was detected. **b** MiR-4465 was transfected into the HEK293 cells for 62 h, and then, it was treated with 100 nM Baf A1 for 10 h or not. The cell lysates were subjected to an immunoblot analysis using the indicated antibodies. **c** The HEK293 cells were transfected with miR-4465 or con for 32 h and then were transfected with Mcherry-EGFP-LC3 for 24 h. The cells were treated with or without 100 nM Baf A1 for 6 h, and the EGFP-LC3 and mCherry-LC3 punctate aggregates were detected using confocal microscopy. The number of green/red merged punctate aggregates (yellow), and Mcherry-LC3 punctate aggregates (red) was statistically analyzed in 30 positive cells. $n = 30$; * $P < 0.05$; ** $P < 0.01$. The scale bars indicate 5 μm

4465 mimics were transfected into HEK293 cells expressing Mcherry-EGFP-LC3, and the results showed that the yellow fluorescence increased and the red fluorescence significantly decreased compared to that of the mi-con. When the two groups were treated with Baf A1, miRNA-4465 significantly increased the yellow fluorescence ratio compared with that of the mi-con (Fig. 2c). In conclusion, miR-4465 significantly inhibited intracellular autophagy.

MiR-4465 induces cytotoxicity in certain types of cells by inhibiting autophagy is related to reduced PTEN

To further observe the effect of miR-4465 on cells, miR-4465 mimics were transfected into HEK293 cells, and cell viability was observed at 24 h, 48 h, and 72 h. The results showed that miR-4465 significantly induced cytotoxicity similar to the knockdown of PTEN (Fig. 3a–d) and that the effect was time dependent (Fig. 3a). In addition, the cytotoxicity induced by miR-4465 was concentration dependent (Fig. 3b). Similar results were observed in SH-SY5Y cells (Fig. S2-A). Rapamycin is an mTOR inhibitor that significantly activates autophagy (Harrison et al. 2009). To determine whether miR-4465-induced cytotoxicity is mediated by autophagy, HEK293 cells were transfected with miR-4465 and treated with rapamycin for 4 h, and then, the cells were replaced with new medium. The results showed that rapamycin partly rescued the miR-4465-induced cytotoxic effects (Fig. 3d). The above results indicated that the downregulation of PTEN by

miR-4465 resulted in cytotoxicity that was mediated by autophagy.

MiR-4465 decreases PTEN expression by posttranscription and inhibits autophagy through the AKT/mTOR signaling pathway

The classical regulatory mechanism of miRNA is through binding to the 3'-UTR of the mRNA of the target gene resulting in mRNA degradation and/or translational inhibition (Bartel 2009). In this study, the molecular mechanism of miR-4465 in regulating PTEN was further explored. MiR-4465 decreased PTEN mRNA levels, suggesting that miR-4465 inhibits PTEN expression possibly through posttranscriptional regulation (Fig. 1f, g). Four binding sites existed between miR-4465 and the PTEN 3'-UTR through TargetScan bioinformatics tools (Fig. S2-B). To further explore whether miR-4465 binds to the PTEN 3'-UTR, luciferase reporter constructs, containing either wild-type (wt) or mutated-type (mut) miR-4465 binding sites at the 3'-UTR of PTEN, were designed (Fig. S2-B). The luciferase reporter assay showed that miR-4465 significantly reduced the luciferase activity of wt PTEN 3'-UTR-A and D, and the wt PTEN 3'-UTR-B revealed a decline in activity as well, but to a lesser extent than the other two. However, there was no significant effect on the luciferase activity of PTEN 3'-UTR-mut and wt PTEN 3'-UTR-C (Fig. 4a). These results suggested that miR-4465 inhibited PTEN expression by targeting the PTEN 3'-UTR. In many reports, PTEN is a negative

Fig. 3 MiR-4465 induces cytotoxicity in HEK293 cells. **a** HEK293 cells were transfected with miR-4465 mimics or con for 24, 48, or 72 h, and cell viability was detected and normalized to that of con. **b** HEK293 cells were transfected with miR-4465 mimics at concentrations of 0, 5, 10, 20, 50, and 100 nM for 72 h. Cell viability was detected and normalized to that of con. **c** HEK293 cells were transfected with con, miR-4465 mimics, or si-PTEN for 72 h, and then, the cells were observed by microscopy. The scale bars indicate 20 μ m. **d** The HEK293 cells were transfected with miR-4465 mimics or con, and 36 h later, they were treated with rapamycin 100 nM for 4 h and then replaced with new medium. Thirty-two hours later, the cell viability was detected. * $P < 0.05$; ** $P < 0.01$

Fig. 4 MiR-4465 directly targets the 3'-UTR of PTEN mRNA and inhibits autophagy mediated by the AKT/mTOR signaling pathway. **a** The PTEN 3'-UTR reporters and PTEN 3'-UTR mutant reporters were co-transfected with miR-4465 into HEK293 cells for 24 h, and the luciferase reporter assay was performed. $*P < 0.05$; $**P < 0.01$. **b** MiR-4465 mimics or con was transfected into HEK293 cells for 72 h, and the cell lysates were detected using the indicated antibodies. **c** MiR-4465 or con was transfected to HEK293 cells, and the samples were detected using antibodies as indicated. **d** MiR-4465 or mi-con was transfected to HEK293 cells, and 48 h later, the cells were incubated with EBSS for 2 h. Then the cells were fixed and stained with LC3 antibody (green) and DAPI (blue). Cells were visualized by a confocal microscopy. scale bar, 10 μ m. **e** HEK293 cells were incubated with EBSS for 2 h, then the total RNA was extracted; the miR-4465 expression were detected by Quantitative real-time PCR. $*P < 0.05$. **f** The model of miR-4465 induced cell cytotoxicity by inhibiting autophagy

regulator of PI3K phosphatase (Camero et al. 2008), and PI3K/AKT significantly affects mTOR and further regulates autophagy (Saiki et al. 2011). Here, we further investigated whether miR-4465 also has an effect on the AKT/mTOR signaling pathways. The results revealed that miR-4465 significantly upregulated the phosphorylated AKT levels after the inhibition of PTEN, and phosphorylated mTOR was also significantly increased (Fig. 4b). Meanwhile, the mTOR substrate protein P70S6K was significantly increased, suggesting that mTOR activity increased (Fig. 4b). However, miR-4465 showed no significant change to other autophagy-related signaling pathways, such as MAPK/p38 or MAPK/ERK signaling (Fig. 4c). These results suggest that miR-4465 decreased PTEN inhibiting autophagy through AKT/mTOR signaling specificity. To further determine the effect of miR-4465 on autophagy, we transfected miR-4465 into HEK293 cells and activated autophagy through the starvation stress with EBSS (Korolchuk et al. 2011). miR-4465 significantly inhibited the formation of

LC3 aggregates induced by starvation stress when compared with mi-con. These results suggest that miR-4465 can significantly inhibit starvation-induced autophagy. Interestingly, we examined the expression of miR-4465 under starvation and found that the expression of miR-4465 was significantly reduced (Fig. 4d, e). Therefore, we speculated that starvation stress might feedback to regulate miR-4465 expression.

Discussion

It is important to eliminate large amounts of abnormal proteins and old organelles. When the accumulation of a large number of abnormal proteins or damaged organelles cannot be eliminated due to abnormal autophagy, this contributes to the pathogenesis of many diseases (Menzies et al. 2015; Sasaki et al. 2010; Shirakabe et al. 2016; Zhong et al. 2016). PTEN-deficient prostate cancer cells significantly enhance the sensitivity of tumor

cells to radiotherapy after treatment with mTOR inhibitors, which activate autophagy (Cao et al. 2006). PTEN-associated autophagy plays an important role in the pathogenesis of many diseases, and these regulatory mechanisms naturally attract much attention. MiRNAs are important noncoding RNA molecules that function in intracellular posttranscriptional regulation (Chendrimada et al. 2005). Their classic regulatory mechanism mainly involves the mature form of the miRNA binding to the 3'-UTR of the target mRNA through seed sequence complementary base pairing in the cytoplasm with the assistance of AGO2, TRBP, and other proteins (Chendrimada et al. 2005; Gregory et al. 2005a). MiRNAs play a critical role in a wide range of biological functions, such as cell growth, differentiation, apoptosis, and autophagy (Frankel and Lund 2012; Sethupathy and Collins 2008). In this study, we found that miR-4465 significantly inhibited both the protein and mRNA expression of PTEN and upregulated the key autophagic proteins LC3-II and p62. Similar results were observed in other cell lines, such as HeLa and SH-SY5Y cells. MiR-4465 suppresses tumor proliferation and metastasis in non-small cell lung cancer by directly targeting the oncogene EZH2 (Sun et al. 2017), but other functions remain unknown. To further explore the effect of miR-4465 on autophagy, we manipulated the autophagic flow by treating the cells with the autophagy inhibitor Baf A1, and the results showed that miR-4465 inhibited autophagy (Fig. 2a, b; Fig. S1-B, C). Considering that PTEN has an important role in regulating the PI3K/AKT signaling pathway (McLoughlin et al. 2018) and that AKT positively regulates mTOR (Qin et al. 2010), the effect of miR-4465 on these signaling pathways was observed. In fact, miR-4465 inhibited PTEN to further activate the AKT/mTOR signaling pathway. PTEN-knockout mice die during embryonic development, indicating that PTEN is extremely important in normal function (Di Cristofano et al. 1998). Indeed, miR-4465 induced cytotoxicity by decreasing PTEN in HEK293 and SH-SY5Y cells. We speculate two reasons for this result. First, PTEN is important for maintaining normal cell function in nontumor cell lines. Second, the downregulation of PTEN leads to excessive activation of autophagy-induced cytotoxicity. This fact is also the reason why the rapamycin treatment only partially restored cell viability. Of course, the characteristics of the miRNA multitargets should also be considered (Bartel 2009). At the same time, further studies showed that miR-4465 can inhibit starvation-induced autophagy, and starvation is likely to reduce the expression of miR-4465 through feedback regulation (Fig. 4d, e). In summary, our study discovered a novel target gene and function of miR-4465, specifically showing that it significantly downregulated PTEN and activated autophagy. These results provide some helpful hints for the exploration of miRNAs in the treatment of PTEN-related disease.

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Authors' contributions All the authors contributed significantly to the study. M.W. and Z.T. designed the study, conducted the experiments, and wrote the manuscript. The immunoblotting, immunofluorescence, cell transfection, and statistical analysis were performed by Z.T., C.F., and C.M. The cell culture and viability detection were performed by Y.T. and H.G. The plasmids were constructed by M.P. and Y.Z., and G.W. and J.R. revised the manuscript. All the authors are in agreement with the content of the manuscript for publication.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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