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Original Article

Overexpression of PTEN enhanced gemcitabine-induced apoptosis by promoting autophagy in osteosarcoma cell line MG-63

Yuanliang Xue[1], Zhiqiang Guan[2], Xiangqi Meng[3]

[1]Department of Orthopedics, Clinical Medical College of Shandong University of Traditional Chinese Medicine, Jinan, Shandong, P. R. China; [2]Department of Orthopedics, People's Hospital of Zhucheng City, Zhucheng, Shandong, P. R. China; [3]Department of Orthopedics, Suzhou Hospital of Traditional Chinese Medicine, Suzhou, Jiangsu, P. R. China

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Abstract: Osteosarcoma is the most common form of primary bone cancer in adolescent with a male predominance. Down-regulation of PTEN is a common event in osteosarcoma progression. In this study, we found that overexpression of PTEN in osteosarcoma derived cell lines MG-63 could sensitize cell for gemcitabine-induced apoptosis. Further experiment demonstrated PTEN overexpression could induce autophagy in MG-63 cell and PTEN-mediated autophagy was required for enhancing apoptosis induced by Gemcitabine, which suggested that PTEN could enhance apoptosis induction via autophagic cell death. Moreover, the upregulated of Autophagy related genes (ATGs) were observed in PTEN overexpressed cell as well. In conclusion, our data suggested that dysregulation of PTEN may not only contributing to the osteosarcoma progression but also drug resistance of chemotherapy. PTEN-mediated autophagic cell death may serve as a new pathway for osteosarcoma treatment.

Keywords: Osteosarcoma, PTEN, apoptosis, autophagy, autophagic cell death

Introduction

Osteosarcoma is the most common form of primary bone cancer and most prevalent in children and young adults with a male predominance [1-3]. Osteosarcoma is considered as aggressive malignant neoplasm which originated from cells with mesenchymal origin (and thus a sarcoma) [4]. Therefore, osteosarcoma generally exhibits osteoblastic differentiation and produces malignant osteoid [4]. Moreover, osteosarcoma is the most common bone tumor in cats and dogs as well [5].

Although osteosarcoma can occur in any bone, most malignancy sites are frequent found in the metaphyseal regions of the distal femur, proximal tibia and proximal humerus [3]. Osteosarcoma is generally characterized by a local invasion of bone and soft tissues, loss of function in the affected extremity and distant metastasis [3]. Without metastases during diagnosis, osteosarcoma patients have a five-year survival rate from 60% to 70% if aggressive surgical resection and chemotherapy were combined together for therapy [6]. However, in the cases with metastasis or recurrence, the chance of long-term survival always below 20% [4]. Therefore, elucidating the etiology of osteosarcoma is needed for novel therapy development.

The phosphatase and tensin homolog (PTEN) belongs to a family of genes called Protein tyrosine phosphatase (PTP) [7]. PTEN is found in almost all tissues in the body and modifies other proteins and fats (lipids) by removing phosphate groups [8]. PTEN is one of critical negative regulator of the PI3K-Akt signaling pathway via catalyzing the dephosporylation of the 3'phosphate of the inositol ring in PIP_3, resulting in the biphosphate product PIP_2 [9]. The PI3K-Akt signaling pathway is considered as one of the most important oncogenic pathways which was involving in almost all kinds of human cancers [10]. Therefore, PTEN is consid-

Table 1. Primers and their sequence used in this study

Primer name		Sequence (5' to 3')
BCL2	F	CCGATCAGTGGAGCTGAAGAA
	R	GCCACAGGATGTTCTCGTCA
CyclinD1	F	CAAGGCCTGAACCTGAGGAG
	R	CTTGGGGTCCATGTTCTGCT
C-FLIP	F	GAGTGCCGGCTATTGGACTT
	R	GCGCTTCTCTCCTACACCTC
Caspase-3	F	GCGGTTGTAGAAGTTAATAAAGGT
	R	TACCAGACCGAGATGTCATTCC
Caspase-7	F	CGTGGGAACGGCAGGAAGT
	R	CGGGTGGTCTTGATGGATCG
ATG4	F	GGAATTGGCCCAGGATGACA
	R	AGCATACATCCCCAACCAGC
ULK1	F	TCTGCCTTGTCTTCAGGTCC
	R	GCTCAGGGATGGTTCCAACT
LAMP1	F	CTCACGTGACAAGCGCTGC
	R	TTAGAGACAGCGGCGTTACC
ATG5	F	GGGTCCCTCTTGGGGTACAT
	R	ACCACACATCTCGAAGCACA
ATG12	F	CTGTGTAATTGCGTCCCCCT
	R	GAAGCTGCAACACAGACTG
GAPDH	F	CAGCCTCAAGATCATCAGCA
	R	TGTGGTCATGAGTCCTTCCA

ered as a tumor suppressor gene and PTEN's function includes in regulation of proliferation, cell growth, migration, genomic stability and stem cell self-renewal [9, 11].

As a conserved gene, PTEN orthologs have been identified from the other mammalian species as well and may play the same role as human [5]. It has been reported that mutated PTEN has been identified in dogs with osteosarcoma [5]. In human, down-regulation of PTEN is a common event in osteosarcoma tumors [12]. *In vitro* studies also demonstrated that targeting PTEN by microRNAs could promote the proliferation of osteosarcoma-derived cell lines [13, 14], which suggesting the important role of PTEN in osteosarcoma. In this study, we found that overexpression of PTEN in osteosarcoma-derived cell lines MG-63 could sensitize cell for Gemcitabine-induced apoptosis. This apoptosis enhancement is autophagy depended and qualified for the term "autophagic cell death". Our data suggested that dysregulation of PTEN may not only contribute to the osteosarcoma progression but also to drug resistance of chemotherapy.

Materials and methods

Cells, plasmids and chemicals

Osteosarcoma derived cell line MG-63 (ATCC® CRL-1427™) was purchased from ATCC and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA). The plasmids pHR-SIN-PTEN-WT (Addgene plasmid#30370) and pQCXI Neo DsRed-LC3-GFP (Addgene plasmid #31183) were purchased from Addgene. Transfection of MG-63 cell with plasmids was conducted by using Lipofectamine™ 2000 (Invitrogen, Grand Island, NY, USA), according to the instructions of manufacturer. Generation of MG-63 cell line with stable expression of DsRed-LC3-GFP was carried out by adding G418 (Sigma-Aldrich, St. Louis, MO, USA) at 500 μg/mL to pQCXI Neo DsRed-LC3-GFP transfected MG-63 cells. The surviving cells with G418 resistance were subjected to sub-cloning by limited dilution to obtain the MG-63 cell stable expressing DsRed-LC3-GFP. Gemcitabine (Sigma-Aldrich) and 3-methyladenine (3MA, Sigma-Aldrich) were used to treat the cell at 50 μg/mL and 5 μMol, respectively.

Reverse transcription and real-time PCR (qPCR)

The RNA isolation from MG-63 cells was conducted by using TRizol (Invitrogen) according to manufacturer's instruction. Synthesis of cDNA was conducted by using AMV reverse transcriptase (Promega, Madison, WI, USA) with according manufacturer's instruction. Real-time PCR (qPCR) detection with SYBR Green Mix (Life technologies) for the targeting genes was described previously [15, 16]. Transcripts of GAPDH were also amplified from the same sample to serve as an internal control for normalization of cellular gene expression. Gene expression was quantified by $2^{-\Delta\Delta CT}$ method as described previously [17]. Primers for qPCR detection were listed as **Table 1**.

Flow cytometry based cell apoptosis assay

Cells from indicated groups were treated accordingly. The trypsinized cells were fixed with 70% ethanol and permeabilized by PBS containing 1% TritonX100 (Sigma-Aldrich). A totally 1×10^6 cells were stained with FITC labeled Annexin V and Propidium iodide. Then the stained cells were analyzed via flow cytom-

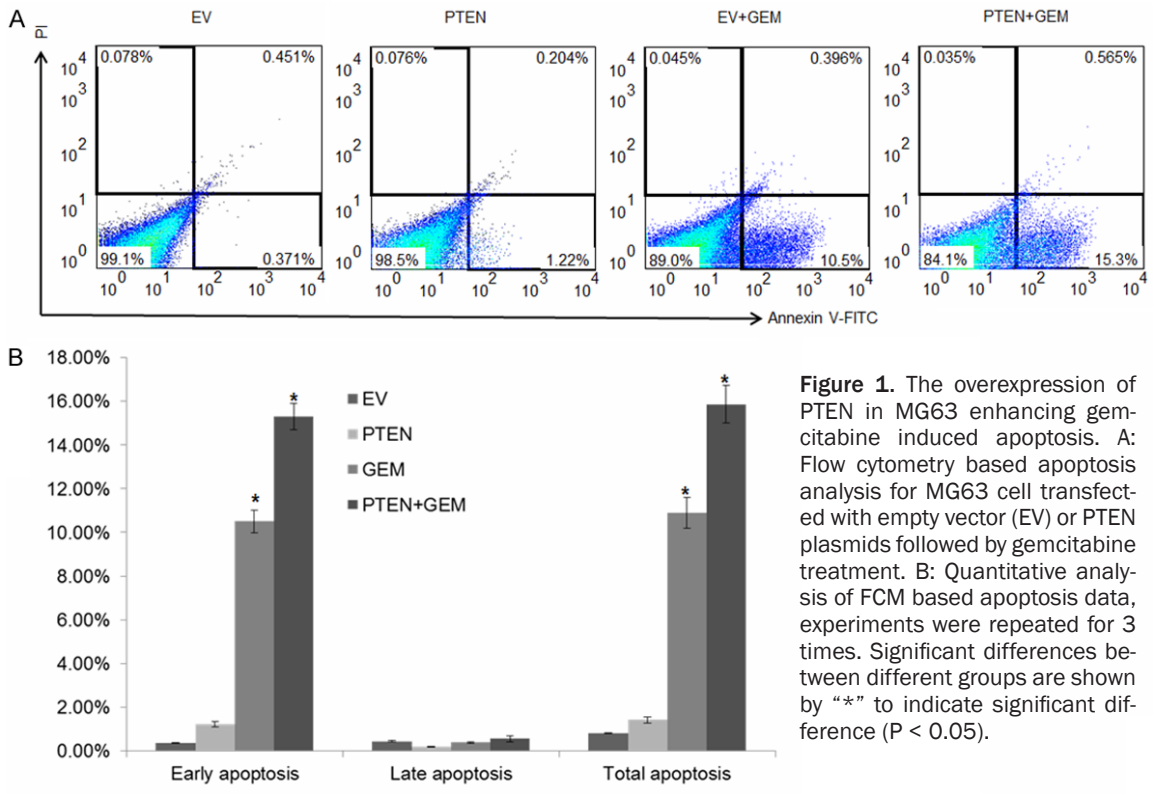

Figure 1. The overexpression of PTEN in MG63 enhancing gemcitabine induced apoptosis. A: Flow cytometry based apoptosis analysis for MG63 cell transfected with empty vector (EV) or PTEN plasmids followed by gemcitabine treatment. B: Quantitative analysis of FCM based apoptosis data, experiments were repeated for 3 times. Significant differences between different groups are shown by "*" to indicate significant difference ($P < 0.05$).

etry machine (FACSCalibur, BD Biosciences, San Jose, CA, USA) for apoptosis analysis.

Western blotting

The SDS-PAGE was conducted as previously described [16, 18]. The SDS-PAGE separated proteins were transferred into PVDF membrane, followed by blocking with SuperBlock™ (PBS) Blocking Buffer (ThermoFisher Scientific, Waltham, Massachusetts, USA) for 15 mins. Then the membrane was probed by rabbit anti-PTEN (SC-9145, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-LC-3 (Sigma-Aldrich) as well as mouse monoclonal anti-Caspase-3 (SC-56053, Santa Cruz Biotechnology) antibodies. Specific reactions between antibodies and corresponding proteins were detected by using goat anti-rabbit or anti-mouse antibodies conjugated with horseradish peroxidase (Sigma-Aldrich) and revealed by a SuperSignal Chemiluminescence Substrate (ThermoFisher Scientific). The antibody targeting GAPDH (Santa Cruz) was also included to normalize the total protein loading. The chemiluminescence signal was digitally recorded and analyzed by the ChemiDoc XRS imaging system (Bio-Rad, Hercules, CA, USA) with Quantity One Program (Version 4.6, Bio-Rad).

Statistical analysis

Statistical analysis was conducted in Excel program (Microsoft, Seattle, WA, USA). Data are represented as the Mean ± SEM. Difference in indicators between samples were subjected to the Student's *t* test. A two tailed *P*-value of less than 0.05 was considered significant.

Results

Overexpression of PTEN in MG-63 cell enhanced gemcitabine-induced apoptosis

PTEN plays crucial role in regulating cell proliferation, migration, genomic stability and stem cell self-renewal [9, 11], therefore it is considered as a tumor suppressor gene. However, in our preliminary study for PTEN's function in osteosarcoma, our data suggested that dysregulation of PTEN not only contributed to cell transformation, but also chemo-drug resistance. Based on FACS based apoptosis analysis, transient expression of PTEN-WT could enhance gemcitabine (GEM)-induced apoptosis in osteosarcoma derived cell line MG-63 (**Figure 1A**). Based on our data, in empty vector (EV) or PTEN coding plasmid transfected MG-63 cells, there were minimum cells under-

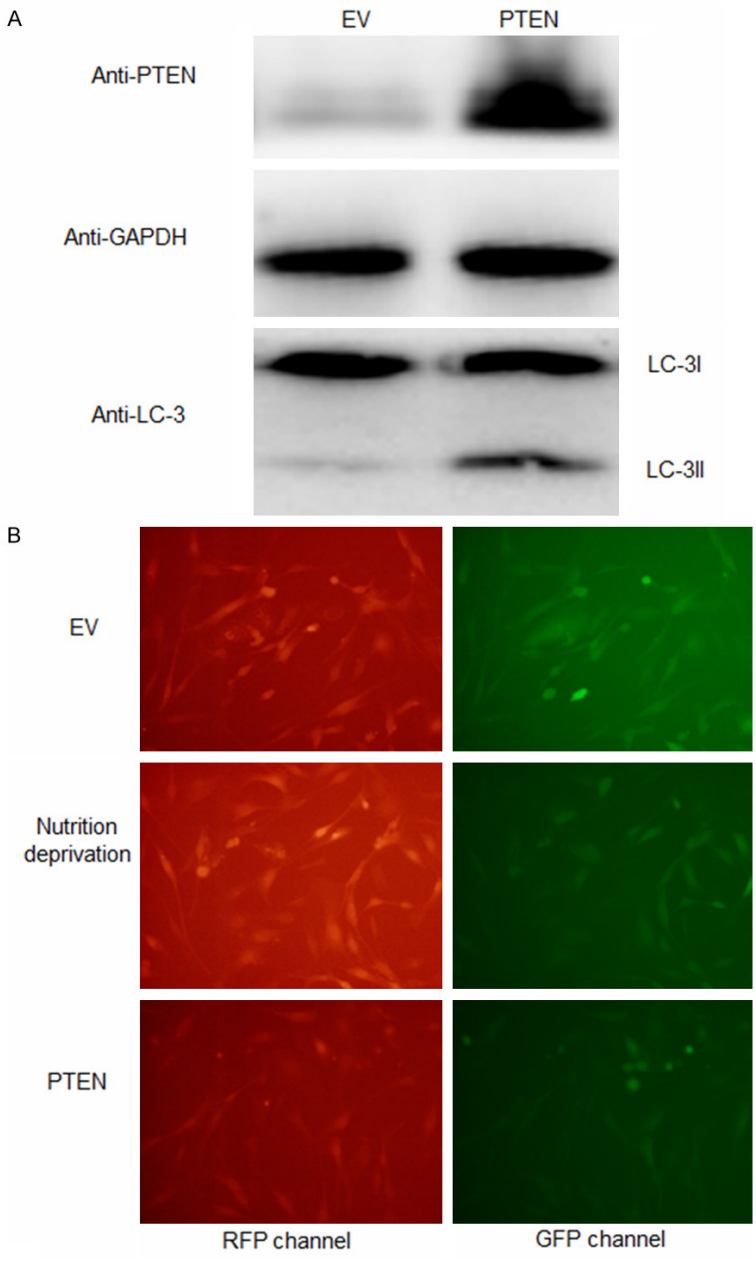

Figure 2. Overexpression of PTEN inducing autophagy in MG63. A. Western blot for LC-3 level in MG-63 cell transfected with empty vector or PTEN plasmids indicated a high level of LC-3II conversion level. B. PTEN overexpression in DsRed-LC3-GFP stable expressing MG-63 cell, the reduced green fluorescence signal to similar level of cells with nutrition deprivation, suggesting a strong autophagy occurring in cells.

going apoptosis (**Figure 1A**). However, significant amount (10%) of GEM treated MG-63 cells with EV transfection experienced apoptosis, while overexpression of PTEN along with GEM treatment in MG-63 cells resulted in more cells (15.3%) undergoing apoptosis. Statistical analysis for repeating experiments also demonstrated similar results, which confirmed our observation (**Figure 1B**). Taken together, our data suggested PTEN could enhance GEM induced apoptosis in MG-63 cell.

Overexpression of PTEN induced autophagy in MG-63 cell

As our data suggested that PTEN overexpression sensitized cells for apoptosis induction by GEM, it is interesting to know the mechanisms behind this apoptosis enhancement. Recently, several reports demonstrated that PTEN could promote autophagy in normal cell or cell underwent apoptosis [19, 20]. Therefore, the autophagy status in PTEN expressed cell was examined by investigating the conversion of Microtubule-associated protein 1A/1B-light chain 3 (LC-3), which is a hall marker for autophagy [21]. During the formation of autophagesome, a cytosolic form of LC-3 (LC-3I) is conjugated to phosphatidylethanolamine to form LC-3-phosphatidylethanolamine conjugate (LC-3II) [21]. In MG-63 cell with overexpression of PTEN, the conversion of LC-3II was significantly increased (**Figure 2A**). Moreover, to further confirm the enhanced autophagy in PTEN expressing cell, we utilized a fluorescence protein tagged LC-3 reporter system as previously described [22]. Briefly, RFP and GFP were fused to LC-3 at N terminal and C terminal, respectively. During the autophage some formation, the fused GFP in C terminal will be cleaved and degraded, which results reduced green fluorescence signal but keeps the same red fluorescence. By generating DsRed-LC3-GFP stable expressing MG-63 cell, PTEN plasmid was transfected to

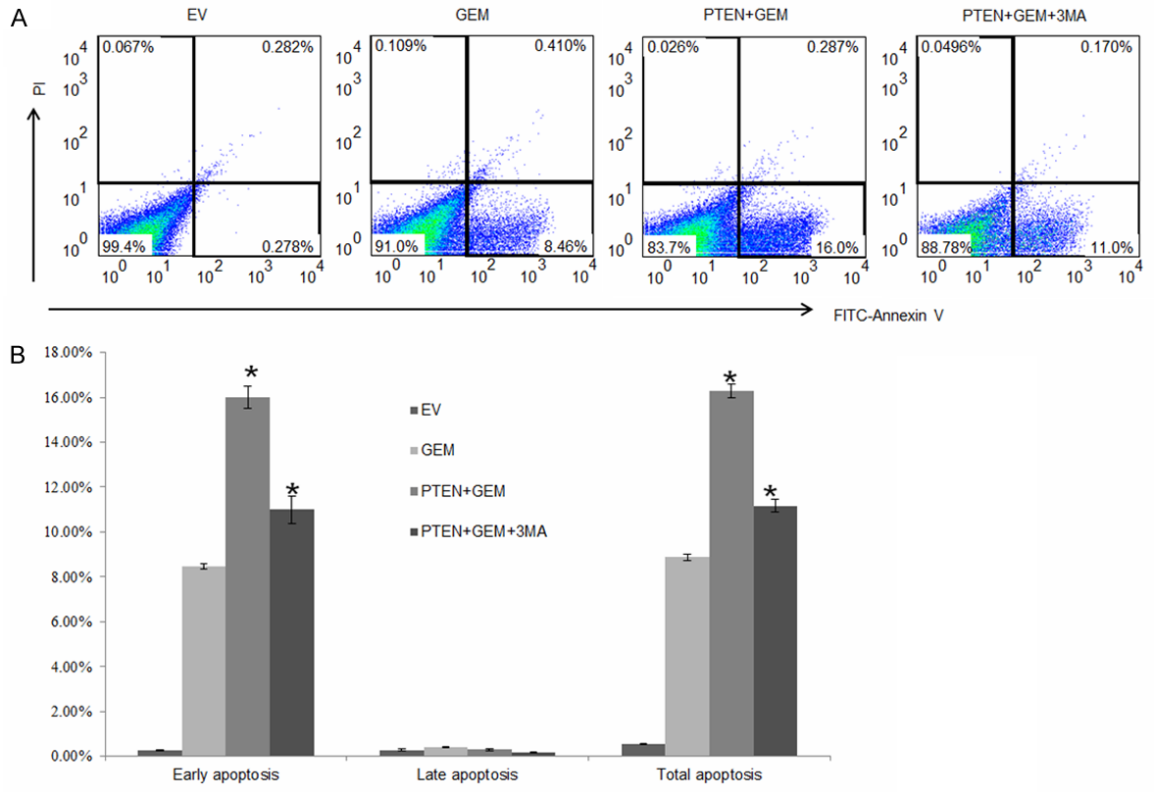

Figure 3. The enhanced apoptosis induction via PTEN is autophagy depended. (A) Flow cytometry based apoptosis analysis for MG63 cell transfected with empty vector (EV) or PTEN plasmids followed by gemcitabine treatment. 3MA was used to inhibit PTEN induced autophagy which indicated the in indicated enhanced apoptosis is autophagy depended group (B): Quantitative analysis of FCM based apoptosis data among indicated groups, experiments were repeated for 3 times. Significant differences between different groups are shown by "*" to indicate significant difference ($P < 0.05$).

the stable cells to induce autophagy, while normal cells and cells undergoing nutrition deprivation were included as controls. Based on our data, PTEN expression could reduce the green fluorescence signal to similar level of cells with nutrition deprivation, suggesting a strong autophagy occurring in cells (**Figure 2B**). Therefore, these data confirmed the PTEN could induce autophagy in osteosarcoma cell line.

PTEN enhancing gemcitabine-induced apoptosis via autophagic cell death and was autophagy depended

Autophagy was originally identified as an intracellular degradation system that plays an important role for homeostasis. However, current data also suggested that autophagy confers both protective role as well as resulting autophagic cell death in transformed tumor cells [23-25]. Therefore, we speculated the enhanced apoptosis induction in MG-63 cell is autophagy depended. To confirm this, 3MA, an autophagy inhibitor, was used to inhibit autophagy induced by PTEN. Based on the apoptosis analysis, by inhibiting autophagy via 3MA, only 11% of the total cells were undergoing apoptosis compared with PTEN overexpressing cell with GEM treatment (**Figure 3A** and **3B**). However, with the treatment of GEM, compared with EV transfected cell which had 8.46% of cells experienced apoptosis, inhibition of autophagy in PTEN transfected still demonstrated higher level of apoptosis (**Figure 3B**). This may suggest that other pathways rather than autophagy which affected by PTEN overexpression also played role in sensitizing tumor cell for apoptosis induction.

PTEN overexpression led to upregulation of ATGs and pro-apoptotic genes

As PTEN is involved in the regulation of the cell cycle, preventing cells from over proliferation andpromoting autophagy [9, 19, 26], we also

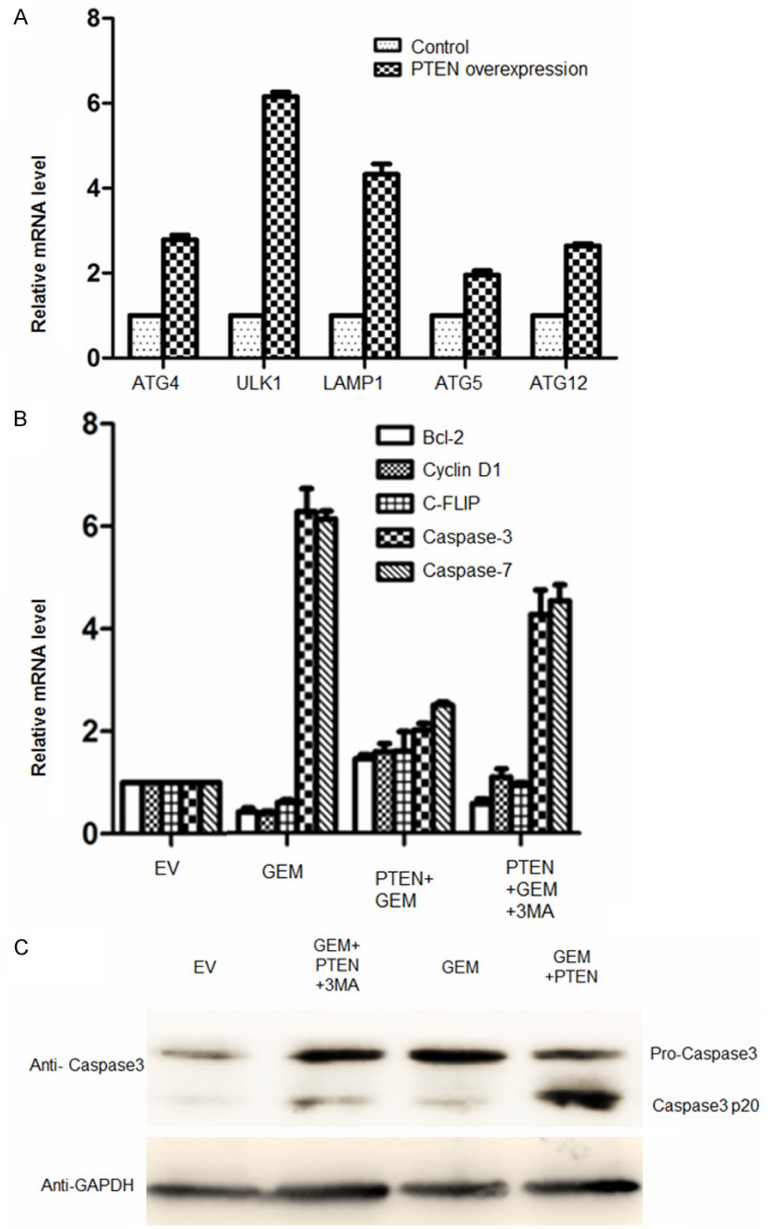

Figure 4. Overexpression of PTEN inducing autophagy in MG63. A. qPCR for transcripts level of autophagy related gene (ATG), MG-63 cells were transfected with PTEN plasmids for 48 hours, then cells were harvest for RNA extraction, Reverse transcription and qPCR, empty plasmids transfected cells were used as control in here. B. qPCR for transcripts level of pro-apoptotic genes. Significant differences between different groups are shown by "*" to indicate significant difference ($P < 0.05$). C. Western blot for Caspase-3 activation of MG-63 cell with indicated treatments. A strong cleavage of Caspase 3p20 indicated enhanced activation of Caspase-3.

examined transcripts level of certain genes related with cell cycle, apoptosis and autophagy. Based on qPCR screening for the transcripts level of indicated genes, compared with control, ATG4, ATG5 and ATG12, who are members from autophagy related genes (ATG) family, were significantly upregulated in PTEN overexpressed cells (**Figure 4A**). Moreover, Unc-51-like kinase (ULK1) and Lysosomal-associated membrane protein-1 (LAMP-1) other two protein involved in autophagy pathways, also up-regulated as well. These data may give a possible explanation of increased autophagy observed in MG-63 cell with PTEN overexpression.

On the other hand, the anti-apoptotic genes B-cell lymphoma 2 (Bcl-2), Cellular FLICE-inhibitory Protein (cFLIP) were down-regulated in GEM treated group and GEM+ PTEN+3MA groups (**Figure 4B**). However, their expression increased slightly in GEM+PTEN groups. Cyclin D1, which is required for progression through the G1 phase of the cell cycle and overexpressed in some cancer [27, 28], also decreased in GEM treated group but increased slightly in PTEN+GEM group and PTEN+GEM+3MA group (**Figure 4B**). Moreover, Caspase 3 and Caspase 7, two mediators of apoptosis [29], were up-regulated in all three groups but demonstrated highest level in GEM treated cells (**Figure 4B**). Out of surprise, the Caspase 3 and 7 mRNA level only indicated slightly increasing in GEM+ PTEN groups, while cell undergoing apoptosis was highest in the same groups (**Figure 4B**). This may be explained partially that dysregulation of cellular transcription machinery in apoptosis cell may disrupt the accuracy of qPCR as half-life for mRNAs are different. Moreover, Western blot was conducted to investigate the Caspase 3 protein level. As Caspase 3 were up-regulated in all groups except control, the cleaved caspase 3 (activate form of Caspase 3) was highest in

GEM+PTEN group, which is consisted with the highest apoptosis status in this group (**Figure 4C**). Taken together, our data demonstrated that PTEN could promote apoptosis during the GEM treatment via inducing autophagy.

Discussion

PTEN was firstly identified in 1997 as the relevant gene in a region of chromosome 10 that is often lost in late-stage human tumors [3]. Dysregulation of PTEN expression and activation was reported in many cancers [30, 31]. For osteosarcoma, earlier report demonstrated that the chromosome arm on which PTEN is located was deleted in a subset of human osteosarcoma tumors [32]. Clinical samples from osteosarcoma excision indicated that expression of PTEN was negatively correlated to the histological grade of osteosarcoma [33]. Another report also demonstrated that PTEN slicing via a Sleeping Beauty transposase system could accelerate osteosarcoma development and metastasis in mice model [34]. These reports consisted with observation that activating Akt signaling was observed in majority of osteosarcoma derived cell lines [35], which suggests an indispensable role of PTEN played during the progression of osteosarcoma. In this study, our result demonstrated overexpression of PTEN could sensitize tumor cell for apoptosis induction, which suggested that down-regulation of PTEN may involve in chemo-drug resistance as well.

On the other hand, our data also suggested enhanced apoptosis by PTEN overexpression may rely on its capability to induce autophagy. Although the role of autophagy played during the program cell death is exclusively investigated, there are still strong arguments whether autophagic activity in dying cells might actually be a survival mechanism or not [36]. Indeed, data from different cancer cell lines had demonstrated that autophagy induction could protect cancer cell from apoptosis and promote cell survival [37, 38]. However, autophagic cell death which occurs via the activation of autophagy was also confirmed in cancer cells as well as Bax/Bak-deficient cells which do not undergo apoptosis after apoptosis induction [39, 40]. Reports have already shown that mutations of Atg5 and LC-3 promote myeloma and glioblastoma, respectively [41], suggesting that cells deficient in autophagy pathway leads to tumor progression as well. In our study, the enhancement of GEM induced apoptosis via PTEN could be inhibited by autophagy inhibitor 3MA, indicating enhanced apoptosis induction is autophagy depended and qualified for the definition of the term “autophagic cell death (ACD)” according to a previous review by Shimizu et al [39]. Moreover, a recent study had demonstrated that survival and lethal autophagy can be distinguished by the type and degree of regulatory signaling during stress such as viral infection [42]. Therefore, it is interesting to further investigated whether same regulator signaling also applies to PTEN induced ACD in osteosarcoma cell lines, as well as other ACD occurring in cancer cells.

In this study, our data demonstrated that overexpression of PTEN along resulted little cell death although autophagic activity increased in PTEN overexpressed cells. However, PTEN overexpression combined with GEM treatment could cause significant cell death based on apoptosis analysis. This observation was also consisted with previous report that activation of autophagy is necessary but not sufficient for ACD and imitation of ACD requires additional death signals [43]. Combined together, these data further supported that PTEN enhanced GEM induced apoptosis via autophagic cell death and PTEN induced autophagy do not confer survival advantage in osteosarcoma cell line MG-63. However, whether the same role of PTEN also applied to other osteosarcoma derived cell lines still need further investigation. In conclusion, our data demonstrated that PTEN could promote GEM induced apoptosis in osteosarcoma derived MG-63 cell via autophagic cell death.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xiangqi Meng, Department of Orthopedics, Suzhou Hospital of Traditional Chinese Medicine, Suzhou 215000, Jiangsu, P. R. China. E-mail: mengxiangqi223@sina.com

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