

PTEN enhances G2/M arrest in etoposide-treated MCF-7 cells through activation of the ATM pathway

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Abstract. As an effective tumor suppressor, phosphatase and tensin homolog (PTEN) has attracted the increased attention of scientists. Recent studies have shown that PTEN plays unique roles in the DNA damage response (DDR) and can interact with the Chk1 pathway. However, little is known about how PTEN contributes to DDR through the ATM-Chk2 pathway. It is well-known that etoposide induces G2/M arrest in a variety of cell lines, including MCF-7 cells. The DNA damage-induced G2/M arrest results from the activation of protein kinase ataxia telangiectasia mutated (ATM), followed by the activation of Chk2 that subsequently inactivates CDC25C, resulting in G2/M arrest. In the present study, we assessed the contribution of PTEN to the etoposide-induced G2/M cell cycle arrest. PTEN was knocked down in MCF-7 cells by specific shRNA, and the effects of PTEN on the ATM-Chk2 pathway were investigated through various approaches. The results showed that knockdown of PTEN strongly antagonized ATM activation in response to etoposide treatment, and thereby reduced the phosphorylation level of ATM substrates, including H2AX, P53 and Chk2. Furthermore, depletion of PTEN reduced the etoposide-induced phosphorylation of CDC25C and strikingly compromised etoposide-induced G2/M arrest in the MCF-7 cells. Altogether, we demonstrated that PTEN plays a unique

role in etoposide-induced G2/M arrest by facilitating the activation of the ATM pathway, and PTEN was required for the proper activation of checkpoints in response to DNA damage in MCF-7 cells.

Introduction

Cellular DNA is constantly challenged by either endogenous [reactive oxygen species (ROS)] or exogenous (UV) factors. To properly transmit the genetic information to the next generation, multiple delicate mechanisms underlying this event have been evolved in eukaryotic cells (1,2). DNA damage response (DDR) is such a crucial mechanism to ensure that genetic information is faithfully transmitted between generations. In response to DNA damages, eukaryotic cells activate a complex signaling cascade to arrest the cell cycle for DNA repair. Dysregulation of DDR may result in severe disorders, such as tumorigenesis (1,3-5). Therefore, it is critical to elucidate the mechanism of DDR in order to treat human diseases, such as cancer. The major regulators of DDR are phosphoinositide 3-kinase (PI3K)-related protein kinases (PIKKs), which include ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and RAD3-related (ATR) (3,6,7). ATM and ATR respond to different types of DNA damage; ATM mainly responds to double-strand breaks (DSBs) while ATR mainly responds to replication stress and UV-induced pyrimidine dimers. According to previous studies (1,4,6-9), Ser/Thr kinase checkpoint kinase-1 (Chk1) and -2 (Chk2) are the two most thoroughly investigated substrates of ATR and ATM, respectively. In the situation of DNA damage, these pathways are able to activate *p53*, phosphorylate H2AX and other downstream pathways to activate the checkpoints of DNA damage, which eventually causes cell cycle arrest for DNA repair.

Defects in the ATR/Chk1 and ATM/Chk2 pathways are known to increase cancer risk (1,3). Once DSBs occur, ATM kinases can phosphorylate histone H2AX to form γ H2AX foci at the DNA break sites. The formation of γ H2AX foci subsequently promotes the gathering of repair factors on damaged

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DNA sites and activates the DNA damage arrest. This indicates that γ H2AX foci play an important role in cellular DDR. Besides activating the formation of γ H2AX nuclear foci, ATM can also induce cell cycle arrest through phosphorylation of its targets, p53 and Chk2. Numerous studies have demonstrated that phosphorylation of Chk2 can induce G2/M arrest by phosphorylating CDC25C, which prevents CDC25C from dephosphorylating CDC2 (or CDK1) (1-3,6,9-11).

The phosphatase and tensin homolog (PTEN) gene encodes a major plasma membrane lipid and protein phosphatase. Scientists have reported that PTEN functions as a highly effective tumor suppressor in various tissues and is frequently mutated in a wide variety of cancers, including endometrial, breast and prostate cancer and glioblastomas. Previous studies revealed that PTEN is a central negative regulator of PI3K/AKT-mediated signaling cascade. The loss of function of PTEN in various human cancers enhances AKT activation, resulting in dysregulated cell proliferation, survival, migration and spreading, which are all important factors in tumor development and progression (12,13). Recent findings have demonstrated various novel functions of PTEN in several cellular processes. For instance, PTEN plays a critical role in DNA damage repair and response. In 2007, Shen *et al* demonstrated that cells deficient in PTEN have defective DNA DSB repair, possibly due to the lack of or downregulation of Rad51 and the lack of PTEN at centromeres (14). In addition, several studies have indicated that PTEN is able to promote the formation of γ -H2AX foci, a marker of DNA damage, which suggests that PTEN functions to reduce DSB levels (15-17). Furthermore, previous studies have shown that PTEN plays a unique role in DDRs through its interaction with the Chk1 and p53 pathways (10,18-21). In the present study, we report that PTEN is required for the proper checkpoint activation in etoposide-treated MCF7 cells. While etoposide treatment induces G2/M arrest in MCF-7 cells by activating the ATM-Chk2-CDC25C pathway, knockdown of PTEN compromises this process, thereby reducing etoposide-induced G2/M arrest in MCF-7 cells.

Materials and methods

Materials and reagents. PTEN shRNA and related products were purchased from Invitrogen. ECL Western Blotting kit, anti-p53, anti- β -actin, anti- γ H2AX, anti-H2AX and anti-CDC25C antibodies were purchased from Abcam. Anti-phospho-ATM, anti-Chk2, anti-phospho-Chk2 (T68), anti-phospho-p53 (S15) and anti-phospho-CDC25C (S216) antibodies were purchased from Cell Signaling. Anti-phospho-ATM (S1981) and anti-ATM antibody, FITC-donkey anti-rabbit IgG and Rhodamine-donkey anti-mouse IgG were purchased from Sigma. Immobilon-P membranes were purchased from Millipore.

Cell lysis and western blotting. The cells were lysed with a buffer containing 20 mM Tris (pH, 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 25 mM sodium pyrophosphate, 1 mM NaF, 1 mM β -glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM PMSF, 2 μ g/ml leupeptin and 10 μ g/ml aprotinin. Total cell lysate (50 μ g) was separated on SDS-PAGE gel and transferred onto Immobilon-P membranes.

The membranes were first blocked with 5% skim milk, and then incubated with primary antibodies at room temperature for 2 h. The ECL Western Blotting kit was utilized to detect the signals (22). The concentrations of antibodies were as follows: anti-p53 (FL-353 at 1 μ g/ml), anti-phospho-p53 (S15) (1:1,000), anti-ERK (1:500), anti-H2AX (1:1,000), anti- γ H2AX (1:1,000), anti-phospho-ATM (S1981) (1:500), anti-ATM (1:500), anti-phospho-Chk2 (T68) (1:500), anti-Chk2 (1:1,000), anti-phospho-CDC25C (S216) (1:500), anti-CDC25C (1:1,000) and anti- β -actin (1:1,000).

Immunofluorescence staining. Cells were treated as described in the figure legends. The cells were fixed with pre-chilled (-20°C) acetone-methanol for 15 min. The primary antibodies anti- γ H2AX (0.5 μ g/ml; Upstate) and anti-phospho-ATM (S1981) (1:100; Cell Signaling) were then added onto the slides and incubated at 4°C overnight. The slides were gently washed with 1X phosphate-buffered saline (PBS) twice, and the FITC-conjugated secondary antibodies (donkey anti-rabbit IgG and Rhodamine-donkey anti-mouse IgG; 1:200; Jackson ImmunoResearch Laboratories) were added onto the slides and incubated at room temperature for 1 h. Afterwards the slides were covered with Vectashield mounting medium containing DAPI (CA94010; Vector Laboratories, Inc., Burlingame, CA, USA). Images were captured with a fluorescence microscope (Axiovert 200; Carl Zeiss) (22).

Cell cycle assessment. Cells in the logarithmic growth phase were cultured in 6-well plates for 24 h and different concentrations of etoposide were added into the plates. After 18 h of incubation, cells were trypsinized and transferred into a 15-ml tube, followed by centrifugation at 1,000 rpm in a 5840R cooled centrifuge (Eppendorf, Germany) for 5 min. The cells were then washed with 1X PBS and the centrifugation was repeated twice. The supernatant was discarded and 1 ml propidium iodide (PI) solution (0.05 mg/ml) was added into each tube. The samples were kept in the dark at room temperature for 30 min, and then the phases of the cell cycle were analyzed using a flow cytometer (22).

Statistical analysis. Data were analyzed using one-way ANOVA followed by a Tukey's test. Statistical significance was achieved if the p-value was ≤ 0.05 . Data values are expressed as the mean \pm standard error (SE).

Results

PTEN promotes etoposide-induced p53 phosphorylation. PTEN was initially identified as a tumor suppressor, which has been found to be mutated in a large number of cancers at a high frequency (23). Mutations of PTEN are a critical step in the development of many cancers and this gene may play an important role in DDRs (12,14,20,21). In order to investigate how PTEN acts on the etoposide-induced DDR, we knocked down this gene in MCF-7 cells (Fig. 1A). As observed, depletion of PTEN did not significantly affect proliferation of the MCF-7 cells (data not shown). It has been widely recognized that p53 phosphorylation is a crucial event of DDR by affecting MDM2, 14-3-3 σ , DADD45 and p21^{Cip1} (24-27). Therefore, we first investigated the impact of PTEN deple-

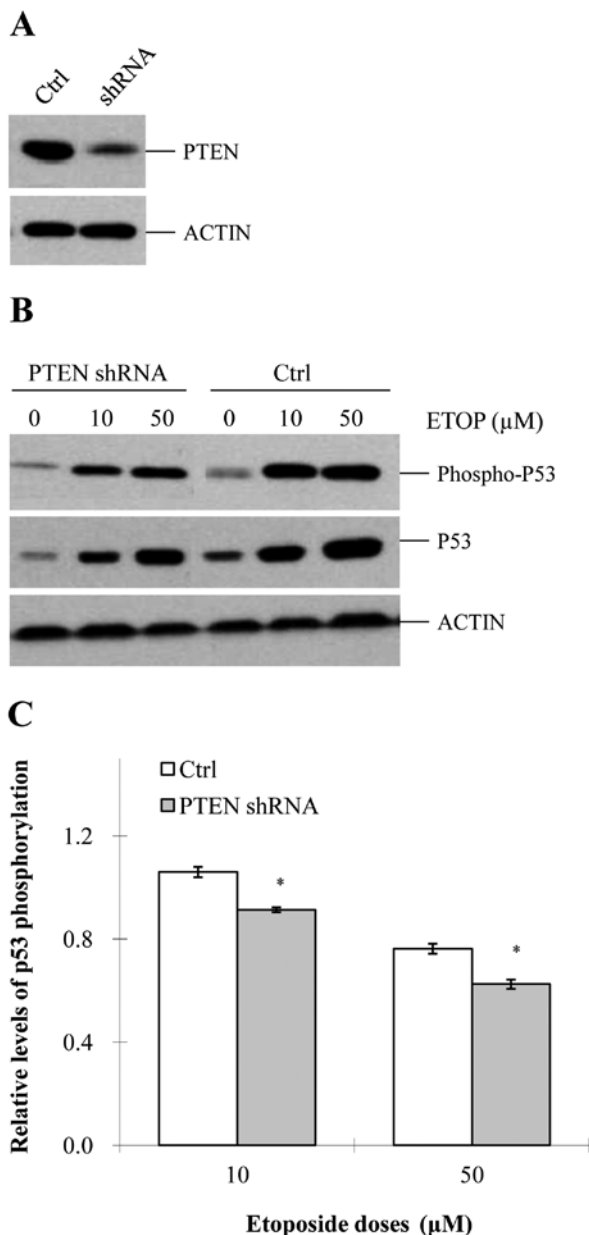


Figure 1. Depletion of *PTEN* reduces etoposide-induced p53 phosphorylation. (A) MCF-7 cells were stably infected with an empty vector (control), or *PTEN* shRNA, followed by investigation of the expression level of *PTEN* and actin by western blotting using specific antibodies. (B) The expression levels of p53 phosphorylation (phospho-p53), total p53 and actin were detected by western blotting (n=3) in the *PTEN*-knockdown and control cells treated with different doses of etoposide (ETOP) for 8 h. (C) The relative level of phospho-p53 against β -actin was quantified in both the control and *PTEN*-knockdown MCF-7 cells as determined in B. Data are shown as means \pm SE (n=3). * p <0.05 in comparison to the respective expression level of phospho-p53 in the control.

tion on etoposide-induced p53 phosphorylation. In both the control and *PTEN*-knockdown cells, etoposide induced p53 phosphorylation in a dose-dependent manner (Fig. 1B). However, at each specific concentration of etoposide, the level of p53 phosphorylation was significantly reduced in the *PTEN*-knockdown cells, compared with that in the control cells (Fig. 1B and C). These data suggest that *PTEN* is able to promote etoposide-induced p53 phosphorylation.

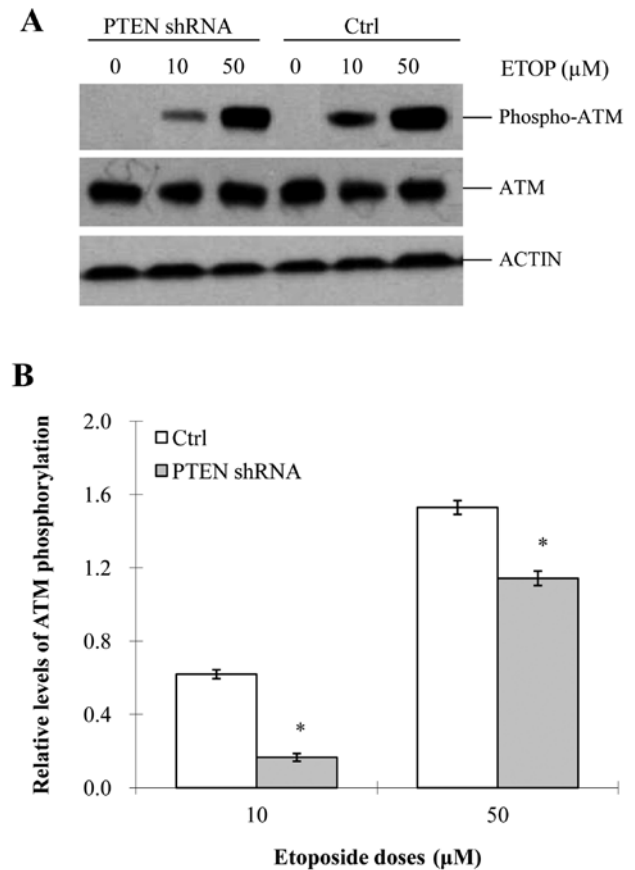


Figure 2. *PTEN* promotes etoposide-induced ATM activation. (A) The control and *PTEN*-knockdown MCF-7 cells were treated with different doses of etoposide (ETOP) for 8 h followed by western blotting for phosphorylated ATM and total ATM. Representative images of western blotting from the control and knockdown group are presented (n=3). (B) The relative expression level of phospho-ATM was quantified against total ATM and shown as means \pm SE (n=3). * p <0.05 in comparison to the respective level of phosphorylated ATM in the control group.

PTEN promotes etoposide-induced ATM activation. In cells, one of the key responses to DNA damage is ATM activation, which occurs via auto-phosphorylation of ATM (S1981). ATM activation can facilitate the activation of p53 given that S15 of p53 is the substrate of ATM (28-30). As previously reported (31,32), etoposide activates ATM and thereby promotes a series of downstream responses leading to G2/M arrest. This finding was further supported by our results which showed that etoposide was able to enhance the phosphorylation level of ATM (Fig. 2A and B). In addition, the phosphorylated level of ATM was increased in an etoposide dose-dependent manner. However, the activation of ATM was largely compromised in the *PTEN*-knockdown cells at any specific concentration of etoposide (Fig. 2A and B), which indicated that *PTEN* is needed for the etoposide-induced activation of ATM/ATR.

PTEN enhances the etoposide-induced production of γ H2AX. Histone H2A is one of the main histone proteins involved in the structure of chromatin in eukaryotic cells and is responsible for maintaining the shape and structure of the nucleosome (33). H2AX is a variant of H2A and its C-terminus is involved in DNA repair. A known response of H2AX to DNA damage

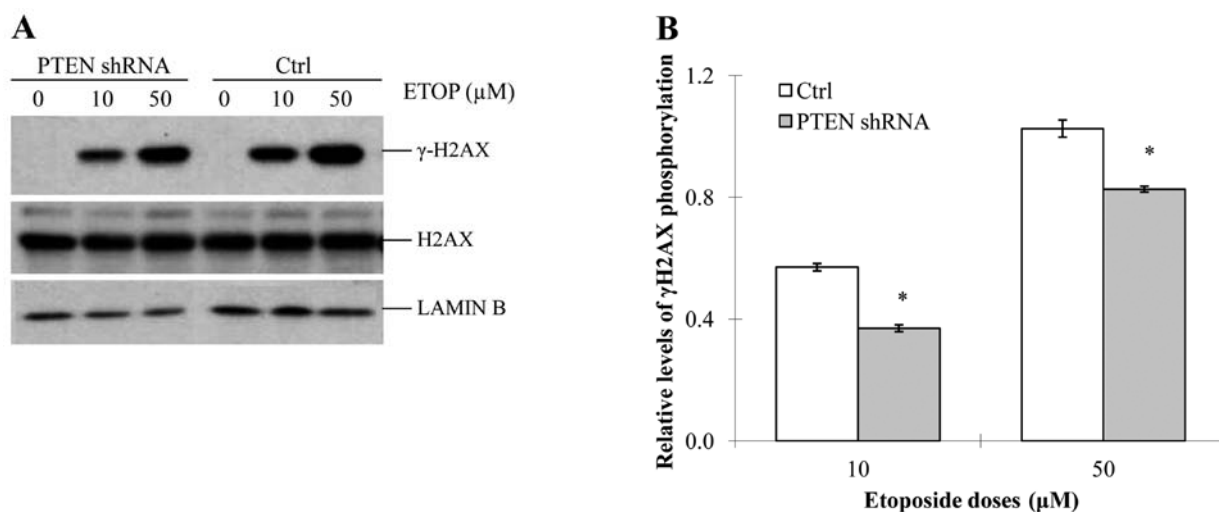


Figure 3. PTEN enhances etoposide-induced production of γ H2AX. (A) The control and *PTEN*-knockdown MCF-7 cells were treated with different doses of etoposide (ETOP) as indicated for 8 h. The expression levels of γ H2AX, H2AX and lamin B were analyzed by western blotting (n=3). Representative images of western blotting are presented. (B) The relative expression level of γ H2AX was quantified against the total level of H2AX that was first normalized by lamin B. Data are shown as means \pm SE (n=3). *p<0.05 in comparison to the respective phosphorylated H2AX (γ H2AX) in the control group.

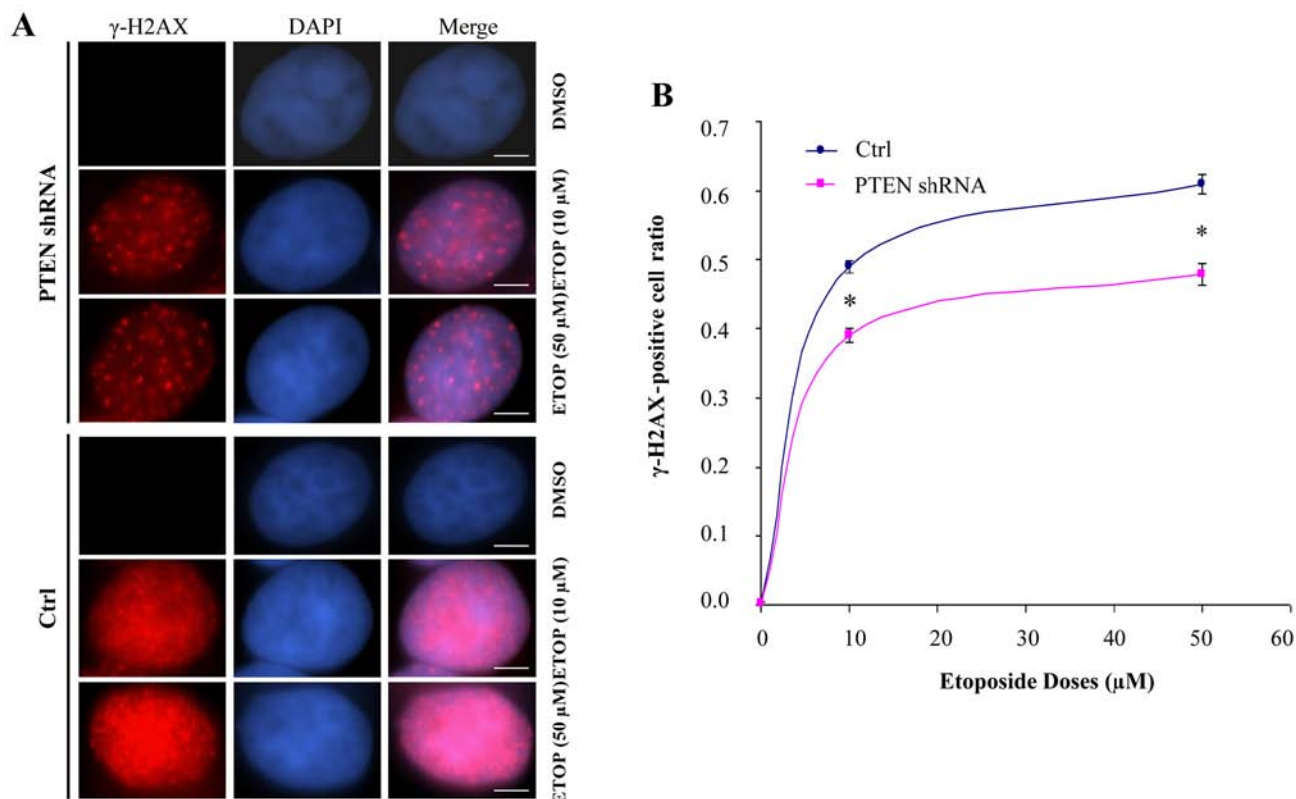


Figure 4. Knockdown of *PTEN* inhibits the formation of γ -H2AX nuclear foci in etoposide-treated MCF-7 cells. (A) The control and *PTEN*-knockdown MCF-7 cells were treated with different doses of etoposide or DMSO for 8 h followed by immunostaining for γ -H2AX (red). Nuclei were stained with DAPI (blue). Scale bar, 5 μ m. (B) Quantification of γ -H2AX-positive cells in both *PTEN*-knockdown and control cells. More than 200 cells in four randomly selected fields were counted to assess the ratio of γ -H2AX-positive cells. Data are presented as means \pm SE (n=3). *p<0.05 in comparison to the control cells.

is the formation of γ H2AX (34). According to previous studies (35-37), ATM activation promotes the phosphorylation of H2AX, and this phosphorylated form, named γ H2AX, may bind to SDBs and form nuclear γ H2AX foci, which are critical for DNA repair or G2/M arrest (38). Given the role of γ H2AX in DDRs, in the present study, we also investigated whether

PTEN contributes to etoposide-induced production of γ H2AX. As shown in the control cells, the higher the dose of etoposide, the more γ H2AX was produced in the MCF-7 cells. However, γ H2AX was significantly reduced in the *PTEN*-knockdown MCF-7 cells (Fig. 3A and B). In addition, the formation of γ H2AX was dose-dependent on etoposide in both the control

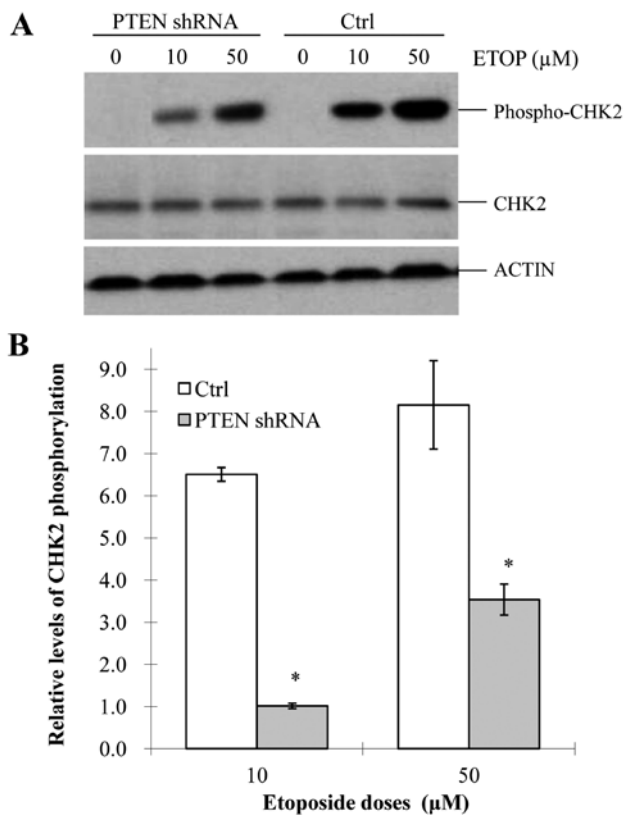


Figure 5. PTEN facilitates etoposide-induced Chk2 activation. (A) The control and *PTEN*-knockdown MCF-7 cells were treated with different doses of etoposide (ETOP) for 8 h followed by evaluation of Chk2 activation using western blotting. Representative images of western blotting are presented (B). The relative expression level of phospho-Chk2 was quantified against the total Chk2 that was first normalized by β -actin. Data are presented as means \pm SE. * $p < 0.05$ in comparison to the respective phospho-Chk2 in the control group.

and *PTEN*-depleted cells (Fig. 3A and B). In order to confirm these results, we performed immunostaining to examine the formation of γ H2AX nuclear foci in the etoposide-treated *PTEN*-knockdown MCF-7 cells. The γ H2AX nuclear foci were strikingly reduced in the *PTEN*-knockdown etoposide-treated cells, compared with that in the control cells (Fig. 4A). In addition, the production of γ H2AX nuclear foci was dose-dependent on etoposide in both groups. Furthermore, the number of γ H2AX-positive cells was significantly reduced in the *PTEN*-knockdown MCF-7 cells, compared with that in the control cells. This quantitative difference between the control and *PTEN*-knockdown cells was also dose-dependent on etoposide (Fig. 4B). These data demonstrated that *PTEN* facilitated etoposide-induced γ H2AX formation. Given that γ H2AX foci not only functions in DNA damage repair but also activates DNA damage checkpoints, together with the data we presented above, we concluded that *PTEN* plays an important role in etoposide-induced G2/M arrest.

PTEN facilitates etoposide-induced G2/M arrest in MCF-7 cells. During DDR, ATM/ATR can be activated and thereby influences their substrates such as *p53* and *Chk2* (29). Similar with *p53*, phosphorylated Chk2 modulates downstream CDC25C and CDC2 (39-41). As a typical substrate of ATM, Chk2 kinase can be phosphorylated and activated by ATM (42).

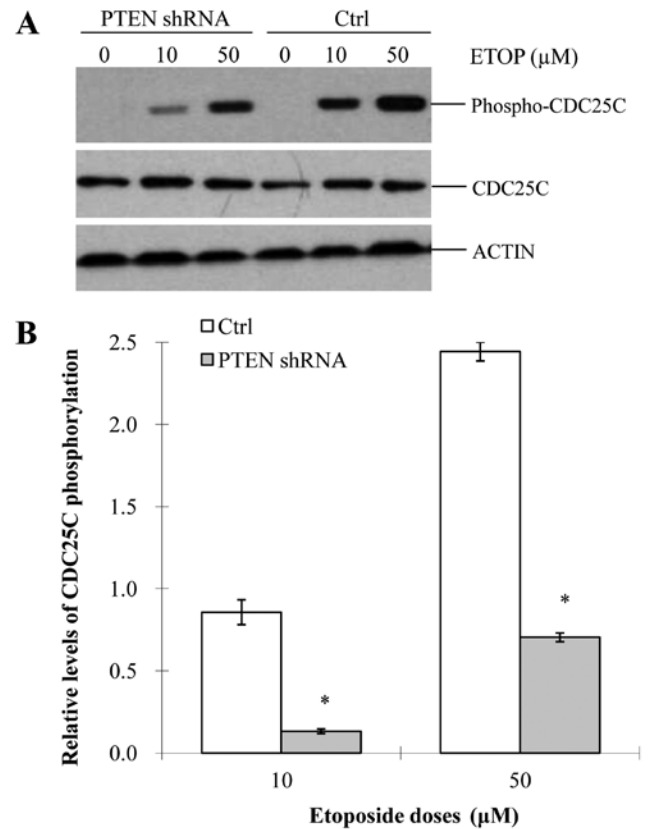


Figure 6. PTEN promotes etoposide-induced inactivation of CDC25C. (A) The control and *PTEN*-knockdown MCF-7 cells were treated with different doses of etoposide (ETOP) for 8 h and phospho-CDC25C (inactive form) was detected by western blotting. Representative images of western blotting are presented for both control and *PTEN*-knockdown groups. (B) The relative expression level of phosphorylated CDC25C was quantified against total CDC25C that was first normalized by β -actin. Data are presented as means \pm SE. * $p < 0.05$ in comparison to the respective phospho-CDC25C (S216) in the control group.

To further confirm our results and examine the role of *PTEN* in the DNA damage pathway, we investigated whether *PTEN* regulates Chk2 in the etoposide-treated MCF-7 cells. Our data showed that phospho-Chk2 (T68) was strikingly reduced in the *PTEN*-knockdown cells, compared with that in the control cells (Fig. 5A and B). In addition, the activation of Chk2 was dose-dependent on etoposide in both groups (Fig. 5A and B). These results demonstrated that *PTEN* promoted etoposide-induced Chk2 phosphorylation, which is consistent with our above finding that *PTEN* facilitated etoposide-induced ATM activation. It is known that the activation of CDC2 is needed for cells to pass G2/M during a cell cycle. Normally, CDC2 is dephosphorylated and activated by CDC25C, which can be phosphorylated and activated by Chk2. Once CDC25C is phosphorylated, cells cannot proceed through G2/M and thus G2/M arrest occurs. Therefore, in addition to assessing etoposide-induced Chk2 activation, we examined whether *PTEN* could activate CDC25C in the MCF-7 cells. Similar to our previous results, phosphorylated CDC25C was significantly reduced in the *PTEN*-knockdown MCF-7 cells, compared with that in the control cells (Fig. 6A and B). We also observed that etoposide induced the phosphorylation of CDC25C in a dose-dependent manner. To further confirm the role of *PTEN* in

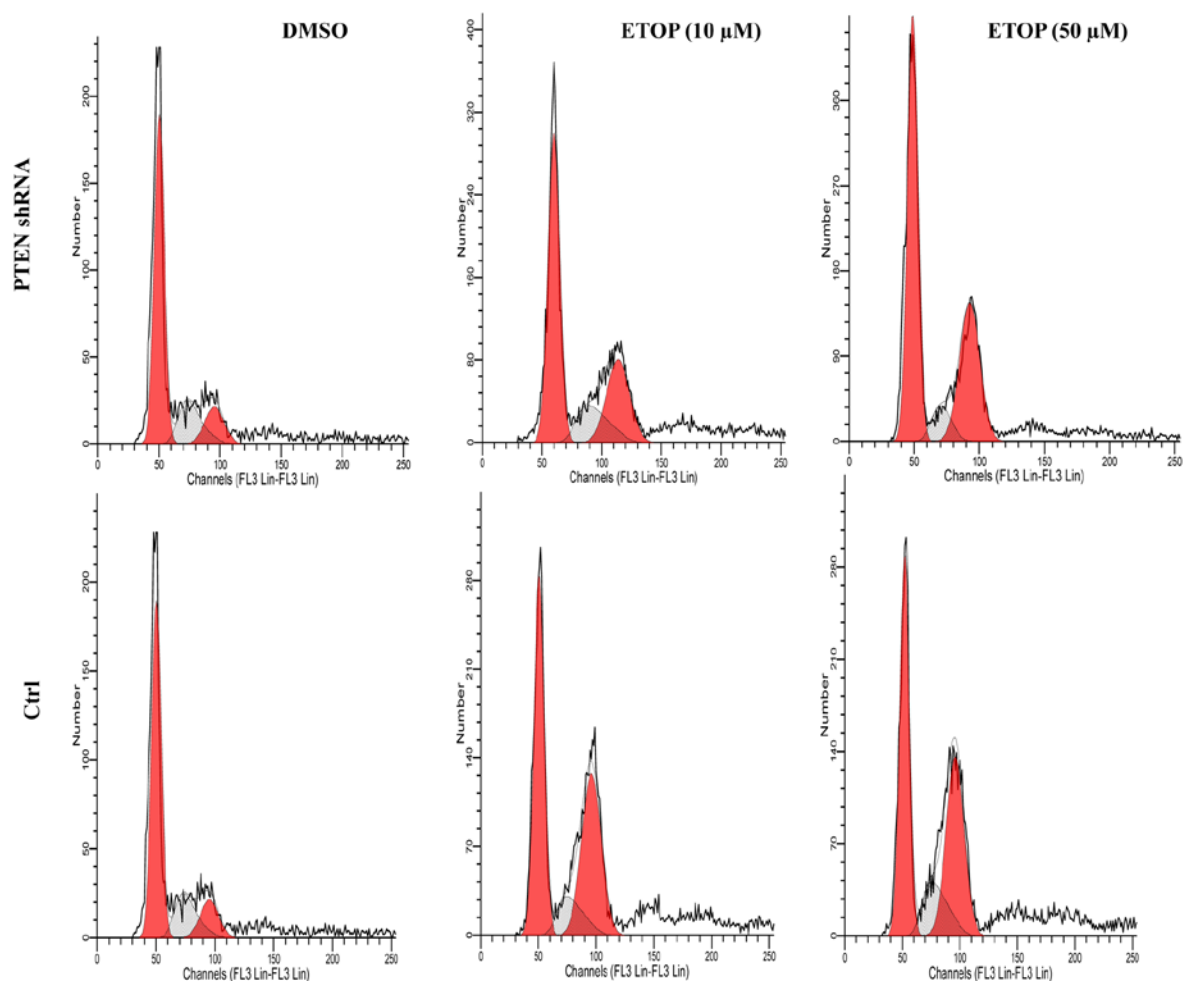


Figure 7. Knockdown of *PTEN* reduces the etoposide-induced G2/M arrest in MCF7 cells. Control and *PTEN*-knockdown MCF-7 cells were treated with DMSO or different doses of etoposide for 24 h, followed by the determination of cell cycle progression using flow cytometry.

Table I. Knockdown of *PTEN* reduces the etoposide-induced G2/M arrest in MCF-7 cells.

Cell cycle phase	DMSO		ETOP (10 μ M)		ETOP (50 μ M)	
	Control	<i>PTEN</i> shRNA	Control	<i>PTEN</i> shRNA	Control	<i>PTEN</i> shRNA
G1	63.7 \pm 5.3	61.5 \pm 5.2	36.7 \pm 3.2	47.4 \pm 3.9 ^a	24.8 \pm 4.3	42.8 \pm 1.3 ^a
S	14.3 \pm 5.7	21.5 \pm 4.4	9.0 \pm 3.0	17.2 \pm 2.4 ^a	6.2 \pm 1.9	11.2 \pm 1.6 ^a
G2/M	18.7 \pm 9.3	17.0 \pm 1.1	54.4 \pm 1.4	35.4 \pm 3.4 ^a	69.0 \pm 6.2	48.1 \pm 2.1 ^a

Cell cycle distributions are presented as mean \pm SE (n=3), *p<0.05 in comparison to the respective cell cycle distributions of the control group. PTEN, phosphatase and tensin homolog; DMSO, dimethyl sulfoxide; ETOP, etoposide.

G2M arrest, we performed flow cytometry to analyze the cell cycle profile in the *PTEN*-knockdown cells. As shown in Fig. 7 and Table I, etoposide induced G2/M arrest in a dose-dependent manner in both the control and *PTEN*-knockdown cells. However, when *PTEN* was knocked down in the etoposide-treated cells, the rate of G2/M arrest was significantly reduced. These data, together with other data shown above, suggest that *PTEN* may enhance etoposide-induced G2/M arrest through activation of the ATM-p53/Chk2-CDC2C pathway.

Discussion

PTEN is one of the most commonly mutated tumor suppressors in human cancers (12,14,19). During tumor progression, mutations of *PTEN* can result in increased cell proliferation and decreased cell death. *PTEN* is involved in the regulation of cell proliferation, at a normal rate. *PTEN* has been one of the targets for drug candidates such as the oncomiR, miR-21 (12,43-45). It is widely recognized that *PTEN* dephos-

phorylates phosphatidylinositol (3,4,5)-trisphosphate [PtdIns (3,4,5) P_3 or PIP $_3$] (46), which leads to the inhibition of the AKT signaling pathway. It was reported that PTEN interacts with p53 and PTK2 and modulates the progression of various cancers (23,47).

Over the last decades, it has been commonly accepted that DNA damage response (DDR) is one of the most important factors to maintain genomic integrity. In fact, DDR is not only essential to DNA repair, but is also critical to distinct cellular events, such as cell proliferation, apoptosis and gene transcription. The AKT signaling pathway has been demonstrated to function in these cellular processes while PTEN was also reported to be partially involved in progression of DDR (48-50). The contributions of molecular factors or pathways to DDR are variable and cell type-specific. Although some mechanisms underlying DDR are well understood, more factors associated with DDR, such as PTEN, remain to be further explored and elucidated. PTEN has certain impacts on some DNA repair pathways, for example the AKT pathway (50-53). To date, the effects of PTEN on the ATM pathway, particularly on etoposide-induced ATM-Chk2-CDC2 activation, have not yet been systematically investigated. Indeed, PTEN can be an important player in DDR given that it can functionally interact with a number of key important factors such as p53.

Etoposide is a medicine commonly used as a chemotherapy agent to treat cancers, such as Kaposi's sarcoma, Ewing's sarcoma, lung and testicular cancer, lymphoma, non-lymphocytic leukemia and glioblastoma multiforme. It is also used in a conditioning regimen prior to bone marrow or blood stem cell transplant (54). The cancer cell killing activity of etoposide is indeed dependent on the activation of the ATM pathway followed by G2/M arrest (31,55,56). Considering that the ATM/p53 pathway is critical in DDR, scientists have made efforts to investigate this pathway. Recently, BMI1, ERK1 and ERK2, have been reported to affect etoposide-induced ATM activation, indicating that they are also required for etoposide-induced G2/M arrest (22,57).

In the present study, we provide evidence that PTEN facilitates etoposide-induced ATM activation, and also contributes to etoposide-induced changes of other factors in the ATM pathway, including activation of Chk2 and inactivation of CDC25C, eventually leading to G2/M arrest. Our data demonstrated that PTEN is required for proper activation of the ATM pathway in response to etoposide treatment. It is not clear yet how PTEN functions in activation of the ATM pathway and ATM pathway-mediated G2/M arrest. It is possible that PTEN facilitates the etoposide-induced activation of the ATM pathway through interacting with ATM directly or other proteins such as AKT, p53 and BRCA1 indirectly. Further studies are required to clarify these questions.

Taken together, our finding that PTEN regulates DNA damage through activation of the ATM-Chk2 pathway provides a new perspective with which to understand the relationship between PTEN and initiation/progression of cancer cells; it also provides a new potential therapeutic target for cancer treatment. In addition, the fact that PTEN affects etoposide-induced G2/M arrest in cancer cells provides us with important information when patients are treated with etoposide, which is critical for precision medicine.

Acknowledgements

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References

1. Spryszyńska S, Smok-Pieniążek A, Ferlińska M, Roszak J, Nocuń M and Stępnik M: The influence of ATM, ATR, DNA-PK inhibitors on the cytotoxic and genotoxic effects of dibenzo[def,p]chrysene on human hepatocellular cancer cell line HepG2. *Mutat Res Genet Toxicol Environ Mutagen* 791: 12-24, 2015.
2. Zhang P, Wei Y, Wang L, Debeb BG, Yuan Y, Zhang J, Yuan J, Wang M, Chen D, Sun Y, *et al*: ATM-mediated stabilization of ZEB1 promotes DNA damage response and radioresistance through CHK1. *Nat Cell Biol* 16: 864-875, 2014.
3. Benada J and Macurek L: Targeting the checkpoint to kill cancer cells. *Biomolecules* 5: 1912-1937, 2015.
4. Reddy V, Wu M, Ciavattone N, McKenty N, Menon M, Barrack ER, Reddy GP and Kim SH: ATM inhibition potentiates death of androgen receptor-inactivated prostate cancer cells with telomere dysfunction. *J Biol Chem* 290: 25522-25533, 2015.
5. Seol HJ, Yoo HY, Jin J, Joo KM, Kong DS, Yoon SJ, Yang H, Kang W, Lim DH, Park K, *et al*: Prognostic implications of the DNA damage response pathway in glioblastoma. *Oncol Rep* 26: 423-430, 2011.
6. Neumann J, Yang Y, Köhler R, Giaisi M, Witzens-Harig M, Liu D, Krammer PH, Lin W and Li-Weber M: Mangrove dolabrane-type of diterpenes tagalsins suppresses tumor growth via ROS-mediated apoptosis and ATM/ATR-Chk1/Chk2-regulated cell cycle arrest. *Int J Cancer* 137: 2739-2748, 2015.
7. Kwok M, Davies N, Agathangelou A, Smith E, Petermann E, Yates E, Brown J, Lau A and Stankovic T: Synthetic lethality in chronic lymphocytic leukaemia with DNA damage response defects by targeting the ATR pathway. *Lancet* 385 (Suppl 1): S58, 2015.
8. Andrs M, Korabecny J, Nepovimova E, Jun D, Hodny Z, Moravcova S, Hanzlikova H and Kuca K: The development of ataxia telangiectasia mutated kinase inhibitors. *Mini Rev Med Chem* 14: 805-811, 2014.
9. Penedos A, Johnson AL, Strong E, Goldman AS, Carballo JA and Cha RS: Essential and checkpoint functions of budding yeast ATM and ATR during meiotic prophase are facilitated by differential phosphorylation of a meiotic adaptor protein, Hop1. *PLoS One* 10: e0134297, 2015.
10. Zhang XP, Liu F and Wang W: Two-phase dynamics of p53 in the DNA damage response. *Proc Natl Acad Sci USA* 108: 8990-8995, 2011.
11. Palmittelli M, de Campos-Nebel M and González-Cid M: Progression of chromosomal damage induced by etoposide in G2 phase in a DNA-PKcs-deficient context. *Chromosome Res* 23: 719-732, 2015.
12. Ming M and He YY: PTEN in DNA damage repair. *Cancer Lett* 319: 125-129, 2012.
13. Ming M, Feng L, Shea CR, Soltani K, Zhao B, Han W, Smart RC, Trempus CS and He YY: PTEN positively regulates UVB-induced DNA damage repair. *Cancer Res* 71: 5287-5295, 2011.
14. Shen WH, Balajee AS, Wang J, Wu H, Eng C, Pandolfi PP and Yin Y: Essential role for nuclear PTEN in maintaining chromosomal integrity. *Cell* 128: 157-170, 2007.
15. Rosser CJ, Tanaka M, Pisters LL, Tanaka N, Levy LB, Hoover DC, Grossman HB, McDonnell TJ, Kuban DA and Meyn RE: Adenoviral-mediated PTEN transgene expression sensitizes Bcl-2-expressing prostate cancer cells to radiation. *Cancer Gene Ther* 11: 273-279, 2004.
16. Pappas G, Zumstein LA, Munshi A, Hobbs M and Meyn RE: Adenoviral-mediated PTEN expression radiosensitizes non-small cell lung cancer cells by suppressing DNA repair capacity. *Cancer Gene Ther* 14: 543-549, 2007.

17. Gupta A, Yang Q, Pandita RK, Hunt CR, Xiang T, Misri S, Zeng S, Pagan J, Jeffery J, Puc J, *et al*: Cell cycle checkpoint defects contribute to genomic instability in PTEN deficient cells independent of DNA DSB repair. *Cell Cycle* 8: 2198-2210, 2009.
18. Paez J and Sellers WR: PI3K/PTEN/AKT pathway. A critical mediator of oncogenic signaling. *Cancer Treat Res* 115: 145-167, 2003.
19. Lee C, Kim JS and Waldman T: *PTEN* gene targeting reveals a radiation-induced size checkpoint in human cancer cells. *Cancer Res* 64: 6906-6914, 2004.
20. Puc J, Keniry M, Li HS, Pandita TK, Choudhury AD, Memeo L, Mansukhani M, Murty VV, Gaciong Z, Meek SE, *et al*: Lack of PTEN sequesters CHK1 and initiates genetic instability. *Cancer Cell* 7: 193-204, 2005.
21. Ali IU: Gatekeeper for endometrium: The PTEN tumor suppressor gene. *J Natl Cancer Inst* 92: 861-863, 2000.
22. Wei F, Xie Y, Tao L and Tang D: Both ERK1 and ERK2 kinases promote G2/M arrest in etoposide-treated MCF7 cells by facilitating ATM activation. *Cell Signal* 22: 1783-1789, 2010.
23. Nakanishi A, Kitagishi Y, Ogura Y and Matsuda S: The tumor suppressor PTEN interacts with p53 in hereditary cancer (Review). *Int J Oncol* 44: 1813-1819, 2014.
24. Conde-Perez A, Gros G, Longvert C, Pedersen M, Petit V, Aktary Z, Viros A, Gesbert F, Delmas V, Rambow F, *et al*: A caveolin-dependent and PI3K/AKT-independent role of PTEN in β -catenin transcriptional activity. *Nat Commun* 6: 8093, 2015.
25. Puzio-Kuter AM, Laddha SV, Castillo-Martin M, Sun Y, Cordon-Cardo C, Chan CS and Levine AJ: Involvement of tumor suppressors PTEN and p53 in the formation of multiple subtypes of liposarcoma. *Cell Death Differ* 22: 1785-1791, 2015.
26. Ushio Y, Tada K, Shiraishi S, Kamiryo T, Shinojima N, Kochi M and Saya H: Correlation of molecular genetic analysis of p53, MDM2, p16, PTEN, and EGFR and survival of patients with anaplastic astrocytoma and glioblastoma. *Front Biosci* 8: e281-e288, 2003.
27. Zhou M, Gu L, Findley HW, Jiang R and Woods WG: PTEN reverses MDM2-mediated chemotherapy resistance by interacting with p53 in acute lymphoblastic leukemia cells. *Cancer Res* 63: 6357-6362, 2003.
28. Bradbury JM and Jackson SP: ATM and ATR. *Curr Biol* 13: R468, 2003.
29. Yang J, Xu ZP, Huang Y, Hamrick HE, Duerksen-Hughes PJ and Yu YN: ATM and ATR: Sensing DNA damage. *World J Gastroenterol* 10: 155-160, 2004.
30. Shiloh Y: ATM and ATR: Networking cellular responses to DNA damage. *Curr Opin Genet Dev* 11: 71-77, 2001.
31. Nam C, Doi K and Nakayama H: Etoposide induces G2/M arrest and apoptosis in neural progenitor cells via DNA damage and an ATM/p53-related pathway. *Histol Histopathol* 25: 485-493, 2010.
32. Kim KY, Cho YJ, Jeon GA, Ryu PD and Myeong JN: Membrane-bound alkaline phosphatase gene induces antitumor effect by G2/M arrest in etoposide phosphate-treated cancer cells. *Mol Cell Biochem* 252: 213-221, 2003.
33. Redon C, Pilch D, Rogakou E, Sedelnikova O, Newrock K and Bonner W: Histone H2A variants H2AX and H2AZ. *Curr Opin Genet Dev* 12: 162-169, 2002.
34. Sánchez-Flores M, Páscar E, Bonassi S, Laffon B and Valdiglesias V: γ H2AX assay as DNA damage biomarker for human population studies: Defining experimental conditions. *Toxicol Sci* 144: 406-413, 2015.
35. Rao VA, Conti C, Guirouilh-Barbat J, Nakamura A, Miao ZH, Davies SL, Saccà B, Hickson ID, Bensimon A and Pommier Y: Endogenous gamma-H2AX-ATM-Chk2 checkpoint activation in Bloom's syndrome helicase deficient cells is related to DNA replication arrested forks. *Mol Cancer Res* 5: 713-724, 2007.
36. Savic V, Yin B, Maas NL, Bredemeyer AL, Carpenter AC, Helmink BA, Yang-Iott KS, Sleckman BP and Bassing CH: Formation of dynamic gamma-H2AX domains along broken DNA strands is distinctly regulated by ATM and MDC1 and dependent upon H2AX densities in chromatin. *Mol Cell* 34: 298-310, 2009.
37. Riballo E, Kühne M, Rief N, Doherty A, Smith GC, Recio MJ, Reis C, Dahm K, Fricke A, Krempler A, *et al*: A pathway of double-strand break rejoining dependent upon ATM, Artemis, and proteins locating to gamma-H2AX foci. *Mol Cell* 16: 715-724, 2004.
38. Mah LJ, El-Osta A and Karagiannis TC: gammaH2AX: A sensitive molecular marker of DNA damage and repair. *Leukemia* 24: 679-686, 2010.
39. Agarwal C, Tyagi A and Agarwal R: Gallic acid causes inactivating phosphorylation of cdc25A/cdc25C-cdc2 via ATM-Chk2 activation, leading to cell cycle arrest, and induces apoptosis in human prostate carcinoma DU145 cells. *Mol Cancer Ther* 5: 3294-3302, 2006.
40. Ahn J and Prives C: Checkpoint kinase 2 (Chk2) monomers or dimers phosphorylate Cdc25C after DNA damage regardless of threonine 68 phosphorylation. *J Biol Chem* 277: 48418-48426, 2002.
41. Huang H, Hu M, Zhao R, Li P and Li M: Dihydromyricetin suppresses the proliferation of hepatocellular carcinoma cells by inducing G2/M arrest through the Chk1/Chk2/Cdc25C pathway. *Oncol Rep* 30: 2467-2475, 2013.
42. Bahassi M, Myer DL, McKenney RJ, Hennigan RF and Stambrook PJ: Priming phosphorylation of Chk2 by polo-like kinase 3 (Plk3) mediates its full activation by ATM and a downstream checkpoint in response to DNA damage. *Mutat Res* 596: 166-176, 2006.
43. Amir S, Ma AH, Shi XB, Xue L, Kung HJ and Devere White RW: Oncomir *miR-125b* suppresses p14^{ARF} to modulate p53-dependent and p53-independent apoptosis in prostate cancer. *PLoS One* 8: e61064, 2013.
44. Mavridis K, Gueugnon F, Petit-Courty A, Courty Y, Barascu A, Guyetant S and Scorilas A: The oncomir *miR-197* is a novel prognostic indicator for non-small cell lung cancer patients. *Br J Cancer* 112: 1527-1535, 2015.
45. Fletcher CE, Dart DA, Sita-Lumsden A, Cheng H, Rennie PS and Bevan CL: Androgen-regulated processing of the oncomir *miR-27a*, which targets Prohibitin in prostate cancer. *Hum Mol Genet* 21: 3112-3127, 2012.
46. Vazquez F and Devreotes P: Regulation of PTEN function as a PIP3 gatekeeper through membrane interaction. *Cell Cycle* 5: 1523-1527, 2006.
47. Lu Y, Yu Q, Liu JH, Zhang J, Wang H, Koul D, McMurray JS, Fang X, Yung WK, Siminovich KA, *et al*: Src family protein-tyrosine kinases alter the function of PTEN to regulate phosphatidylinositol 3-kinase/AKT cascades. *J Biol Chem* 278: 40057-40066, 2003.
48. Shehata M, Schnabl S, Demirtas D, Hilgarth M, Hubmann R, Ponath E, Badrnya S, Lehner C, Hoelbl A, Duechler M, *et al*: Reconstitution of PTEN activity by CK2 inhibitors and interference with the PI3-K/Akt cascade counteract the antiapoptotic effect of human stromal cells in chronic lymphocytic leukemia. *Blood* 116: 2513-2521, 2010.
49. Stocker H, Andjelkovic M, Oldham S, Laffargue M, Wymann MP, Hemmings BA and Hafen E: Living with lethal PIP3 levels: Viability of flies lacking PTEN restored by a PH domain mutation in Akt/PKB. *Science* 295: 2088-2091, 2002.
50. Yu J, Zhang SS, Saito K, Williams S, Arimura Y, Ma Y, Ke Y, Baron V, Mercola D, Feng GS, *et al*: PTEN regulation by Akt-EGR1-ARF-PTEN axis. *EMBO J* 28: 21-33, 2009.
51. Alyasiri NS, Mehdi SJ, Alam MS, Ali A, Mandal AK, Gupta S, Singh I and Rizvi MM: *PTEN*-mediated AKT activation contributes to the reduced apoptosis among Indian oral squamous cell carcinoma patients. *J Cancer Res Clin Oncol* 138: 103-109, 2012.
52. Abbott RT, Tripp S, Perkins SL, Elenitoba-Johnson KS and Lim MS: Analysis of the PI-3-Kinase-PTEN-AKT pathway in human lymphoma and leukemia using a cell line microarray. *Mod Pathol* 16: 607-612, 2003.
53. Wen YG, Wang Q, Zhou CZ, Qiu GQ, Peng ZH and Tang HM: Mutation analysis of tumor suppressor gene *PTEN* in patients with gastric carcinomas and its impact on PI3K/AKT pathway. *Oncol Rep* 24: 89-95, 2010.
54. Hande KR: Etoposide: Four decades of development of a topoisomerase II inhibitor. *Eur J Cancer* 34: 1514-1521, 1998.
55. Théard D, Coisy M, Ducommun B, Concannon P and Darbon JM: Etoposide and adriamycin but not genistein can activate the checkpoint kinase Chk2 independently of ATM/ATR. *Biochem Biophys Res Commun* 289: 1199-1204, 2001.
56. Fu X, Wan S, Lyu YL, Liu LF and Qi H: Etoposide induces ATM-dependent mitochondrial biogenesis through AMPK activation. *PLoS One* 3: e2009, 2008.
57. Wei F, Ojo D, Lin X, Wong N, He L, Yan J, Xu S, Major P and Tang D: BMI1 attenuates etoposide-induced G2/M checkpoints via reducing ATM activation. *Oncogene* 34: 3063-3075, 2015.