Azidothymidine inhibits cell growth and telomerase activity and induces DNA damage in human esophageal cancer

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Abstract. Esophageal cancer is one of the most common type of malignancies. Telomerase activity, which is absent or weakly detected in the majority of human somatic cells, is elevated in esophageal cancer. Although azidothymidine (AZT), a reverse transcriptase inhibitor, has been utilized as a treatment for tumors, its role in treating esophageal cancer has not been confirmed. The aim of the present study was to determine the effect of AZT on telomerase activity and the proliferation of the human esophageal cancer cell line TE-11. A telomeric repeat amplification assay was utilized to detect telomerase activity following treatment of TE-11 cells with AZT. The effect of AZT on TE-11 cell cycle distribution was determined by flow cytometry. Cellular DNA damage was evaluated by a comet assay and an MTT assay demonstrated that AZT significantly inhibited the viability of TE-11 cells, in a time-and dose-dependent manner. In addition, TE-11 cells treated with various concentrations of AZT exhibited a significant reduction in telomerase activity and percentage of cells in the G1/G0 phase, and an increase in the percentage of cells in the S phase. High doses of AZT caused DNA damage, and enhanced the expression levels of γ-H2A histone family member X and phosphorylated checkpoint kinase 2 in TE-11 cells. These results demonstrated that AZT effectively inhibits proliferation of the TE-11 human esophageal cancer cell line *in vitro*. The growth inhibitory effects were associated with a reduction in telomerase activity, S and G2/M phase cell cycle arrest, and enhanced DNA damage, suggesting that AZT may be utilized in the clinic for the treatment of esophageal cancer.

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Introduction

Telomerase is an RNA nuclear protease that is composed of human telomerase reverse transcriptase (hTERT), human telomerase RNA and a number of associated proteins (1). Telomerase RNA has a short template element that directs the synthesis of telemetric repeats at the end of chromosomes, maintains chromosomal stability, stabilizes telomere length and may promote cancer progression and cell immortality (2). Telomerase activity, which is absent or weakly detected in the majority of human somatic cells, is elevated in immortalized cell lines, stem and germ cells and in ~85% of human cancers, including esophageal cancer (3-5). Esophageal cancer is the eighth most common type of malignancy and is the sixth leading cause of cancer-associated mortality worldwide (6). Incidence rates of esophageal squamous cell carcinoma have been increasing in certain Asian countries. In particular, areas of China contain the highest incidence rates of esophageal cancer in the world (7). Esophageal cancer is the fourth most frequently diagnosed cancer and is the fourth most common cause of cancer-associated mortality in China (8). Despite numerous advances in diagnosis and treatment, the 5-year survival rate for patients diagnosed with esophageal cancer ranges between 15 and 20% due to the aggressive nature of this type of malignancy (9). Therefore, telomerase activity may represent a useful diagnostic marker for human esophageal cancer and may be a potential target for pharmacological intervention.

Azidothymidine (AZT) is a thymidine analog used in the treatment of acquired immune deficiency syndrome (AIDS). It is phosphorylated to AZT-triphosphate (AZT-TP) by a thymidine kinase enzyme, and in this form, it is incorporated into viral DNA where it acts as a false substitute for viral reverse transcription (RT) and blocks chain elongation. AZT-TP has a high affinity for RT and a low affinity for DNA polymerases α, β and γ. The identification that the hTERT component of telomerase is a functional catalytic RT, prompted studies to inhibit telomerase with RT viral inhibitors, including AZT (10). AZT was demonstrated to inhibit the activity of telomerase and cell growth in various tumor cells *in vitro*, including those derived from human cancers of the bladder, colon, ovarian, parathyroid, breast and liver (11-16). In the present study, TE-11 cells were treated with AZT and the effect on telomerase activity, cell proliferation, cell cycle progression and DNA damage were investigated. The results suggested that AZT may be a possible clinical therapy for esophageal cancer.

Materials and methods

Cell culture and treatments. TE-11 cells, a cell line derived from a patient with human esophageal cancer, were purchased from the American Type Culture Collection and were maintained at 37˚C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum and 100 U/ml penicillin/streptomycin. AZT was purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany) and dissolved in PBS.

MTT assay. A total of $1x10^4$ cells/well were seeded in a 96-well plate. Cells were treated with 2, 20, 100 and 200 μM AZT for 24, 48, 72 and 96 h. The wells were subsequently replaced with 10 μl/well MTT solution (5 mg/ml) and incubated at 37˚C for 4 h. The supernatant was removed and 100 μl/well dimethyl sulfoxide was added for 15 min. The spectrometric absorbance at a wavelength of 490 nm was measured on a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). As a control, TE-11 cells were additionally treated with DMEM alone.

Measurement of telomerase activity. TE-11 cells ($2x10^6$) were cultured in 6-well plates and treated with 2, 20, 100 and 200 μM AZT for 48 h at 37˚C. Lysates were prepared by treating cells for 30 min on ice with lysis buffer [10 mM Tris-HCl (pH 7.5), 1.5 mM $MgCl_2$, 1 mM EGTA, 1% 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate, 10% glycerol, 5 mM β-mercaptoethanol and 0.1 mM phenylmethane sulfonyl fluoride]. Lysates were centrifuged at 15,000 x g for 20 min at 4˚C. Supernatants were collected and the protein concentrations were determined using a Bicinchoninic Assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Lysates were subsequently diluted to 10 mg/ml. Telomerase activity was measured by polymerase chain reaction (PCR) and the telomeric repeat amplification (TRAP) assay. The PCR reaction mixture was prepared using TRAP reaction buffer [20 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 63 mM KCl, 0.005% Tween-20 and 1 mM EGTA]. The PCR reaction mixture was comprised of 312.5 μM dNTP, 0.625 μM telomerase substrate (TS) primer, the reverse primer for amplification (CX) and 1 U hot-start Taq DNA polymerase, in a total volume of 50 μl. In addition, each reaction mixture contained 0.625 μM internal control primer (NT) and 0.01 aM internal control template (TSNT) for amplification of a 36 bp internal standard. A total of 1 μl lysate was subsequently added to this mixture, which was placed in a thermal cycler. Primers were as follows: 5'-AATCCGTCG AGCAGAGTT-3' for TS; 5'-CCCTTACCCTTACCCTTACCC TAA-3' for CX; 5'-ATCGCTTCTCGGCCTTTT-3' for NT and 5'-AATCCGTCGAGCAGAGTTAAAAGGCCGAGA AGCGAT-3' for TSNT. Cycling conditions were as follows: An initial telomerase extension step at 30˚C for 30 min, followed by 35 cycles of denaturation at 95˚C for 30 sec, annealing at 50˚C for 30 sec and extension at 72˚C for 1 min. For each sample, a total 10 μl PCR product was loaded onto 10% polyacrylamide gels and subjected to electrophoresis at 180-200 V for ~1 h in Tris/Borate/EDTA buffer. The gel was stained with GelRed™ (Biotium, Inc., Hayward, CA, USA) and visualized under an ultraviolet (UV) illuminator to determine telomerase activity. An internal control was included and was evident by a 36 bp PCR product. TE-11 cells cultured in DMEM medium alone were utilized as a control, and the band intensities were quantified with ImageJ software (version 1.47; National Institutes of Health, Bethesda, MD, USA).

Cell cycle analysis. Following treatment with 2, 20, 100 and 200 μM AZT for 48 h, $2x10^6$ cells were washed twice with PBS. Following an overnight fixation in 70% ethanol at 4˚C, cells were harvested by centrifugation at 200 x g for 10 min at room temperature and washed twice with PBS. Cells were subsequently stained with propidium iodide and analyzed on the BD FACScan™ system using BD Accuri C6 Software version 1.0.264.21 (BD Biosciences, Franklin Lakes, CA, USA).

Comet assay. TE-11 cells were cultured for 48 h in a 6 well plate and treated with 20, 100 and 200 μM AZT for 48 h. Additionally, cells treated with DMEM alone served as an untreated control, and cells treated with UV served as a positive control. The Comet assay was performed under alkaline conditions. Cells were resuspended in DMEM at a concentration of $1x10^5$/ml and were combined with molten LMAgarose (Trevigen, Gaithersburg, MD, USA) (at 37˚C) at a ratio of 1:10, prior to pipetting 75 μl onto CometSlides™ (Trevigen). Slides were stored in the dark at 4˚C for 30 min and immersed in alkaline solution (0.25 M NaOH containing 0.1 μM EDTA; pH 12.6) at 4˚C for 2 h. Slides were gently removed from the lysis buffer and rinsed with distilled H_2O. Slides were placed in freshly prepared alkaline solution at pH>13 for 30 min at room temperature. Gel electrophoresis was performed at 1 V/cm for 30 min. Subsequently, slides were washed in 70% ethanol, stained with GelRed™ (Biotium, Inc.) and analyzed under a fluorescence microscope at x400 magnification.

Western blot analysis. TE-11 cells were lysed with radioimmunoprecipitation assay buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% TritonX-100, 1% sodium deoxycholate, 0.1% SDS, 2 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 1 mM EDTA, 1 mM Na_3VO_4) following treatment with 20, 100 and 200 μM AZT for 48 h. Protein concentrations were determined using the Bicinchoninic Assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Heat-denatured protein samples (20 mg/lane) were loaded onto 10% gels and subjected to electrophoresis prior to transfer onto polyvinylidene fluoride membranes. The membranes were incubated with 5% bovine serum albumin (Sigma-Aldrich; Merck KGaA) for 1 h at room temperature. The membranes were then incubated at 4˚C overnight with the following primary antibodies: Rabbit anti-phosphorylated-checkpoint kinase 2 [pChk2 (Thr68); cat. no. ab85743; 1:1,000; Abcam, Cambridge, MA, USA], mouse anti-γ-H2A histone family member X (γ-H2AX; cat. no. ab180651; 1:1,000; Abcam) and mouse anti-β-actin (cat. no. sc-8432; 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Subsequently, membranes were probed with goat anti-rabbit-horseradish peroxidase (-HRP; cat. no. sc2004; 1:5,000; Santa Cruz Biotechnology, Inc.) or goat-anti-mouse-HRP (cat. no. sc2005; 1:5,000; Santa Cruz Biotechnology, Inc.) secondary antibodies for 1 h at room temperature. Proteins were detected using the Enhanced Chemiluminescence Detection reagent (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol.

Table I. Percentage inhibition of AZT on the growth of TE-11 cells.

	AZT concentration, μM			
Time, h	2	20	100	200
24	4.80	-2.48	0.08	16.98[a]
48	-4.86	40.03[b]	52.20[b]	57.97[b]
72	-5.58	42.21[b]	56.37[b]	65.87[b]
96	-8.38	45.99[b]	67.54[b]	76.64[b]

[a]P<0.05, [b]P<0.01 vs. The control. AZT, azidothymidine.

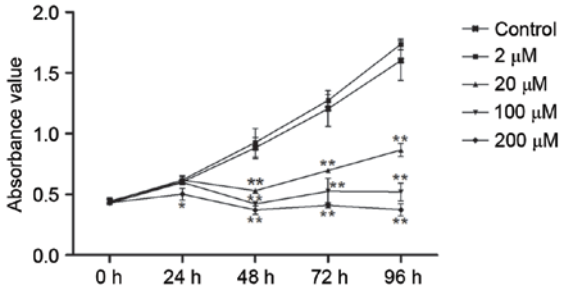

Figure 1. Effect of AZT on TE-11 cell viability. An MTT assay was utilized to measure cell viability in the TE-11 cell line following treatment with 2, 20, 100 or 200 μM AZT for 24, 48, 72 and 96 h. Data are expressed the mean ± standard deviation of three independent experiments. *P<0.05 and **P<0.01 vs. Control. AZT, azidothymidine.

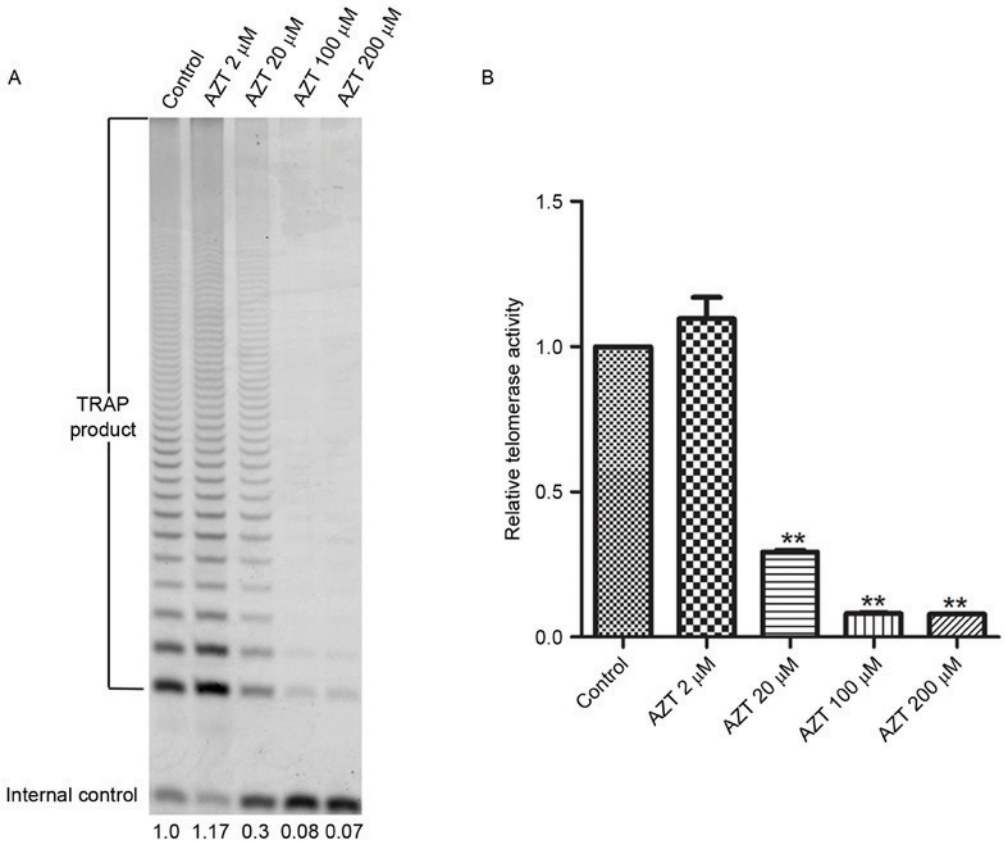

Figure 2. AZT inhibits telomerase activity of TE-11 cells. TE-11 cells were treated with 2, 20, 100 or 200 μM AZT for 48 h and were subjected to a TRAP assay for measurement of telomerase activity. (A) Gel electrophoresis of PCR products. An internal control was included and was evident by a 36 bp PCR product. (B) Telomerase activity was determined by normalizing the TRAP product to the internal control, which was assigned a value of 1. The values represent the mean ± standard deviation of three independent experiments. **P<0.01 vs. Control. AZT, azidothymidine. PCR, polymerase chain reaction; TRAP, telomeric repeat amplification.

Statistical analysis. Data are expressed as the mean ± standard deviation of three experiments. Statistical differences were analyzed using one-way analysis of variance followed by Tukey's post-hoc test via GraphPad Prism software version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) or Microsoft Excel 2007 software (Microsoft Corporation, Redmond, WA, USA).

Results

Inhibition of TE-11 cell proliferation by AZT. TE-11 cell proliferation was inhibited following treatment with 20, 100 and 200 μM AZT for 0, 24, 48, 72 and 96 h, as determined by an MTT assay (Fig. 1). However, treatment with 2 μM of AZT did not have an inhibitory effect on TE-11 cells. Therefore, the inhibitory effect of AZT was time- and dose-dependent (Fig. 1; Table I).

Inhibition of telomerase activity in TE-11 cells by AZT. The effect of AZT on telomerase activity in TE-11 cells was determined by a TRAP assay. Treatment with 20, 100 and 200 μM AZT for 48 h resulted in a dose-dependent decrease in telomerase activity (Fig. 2A and B) compared with the control.

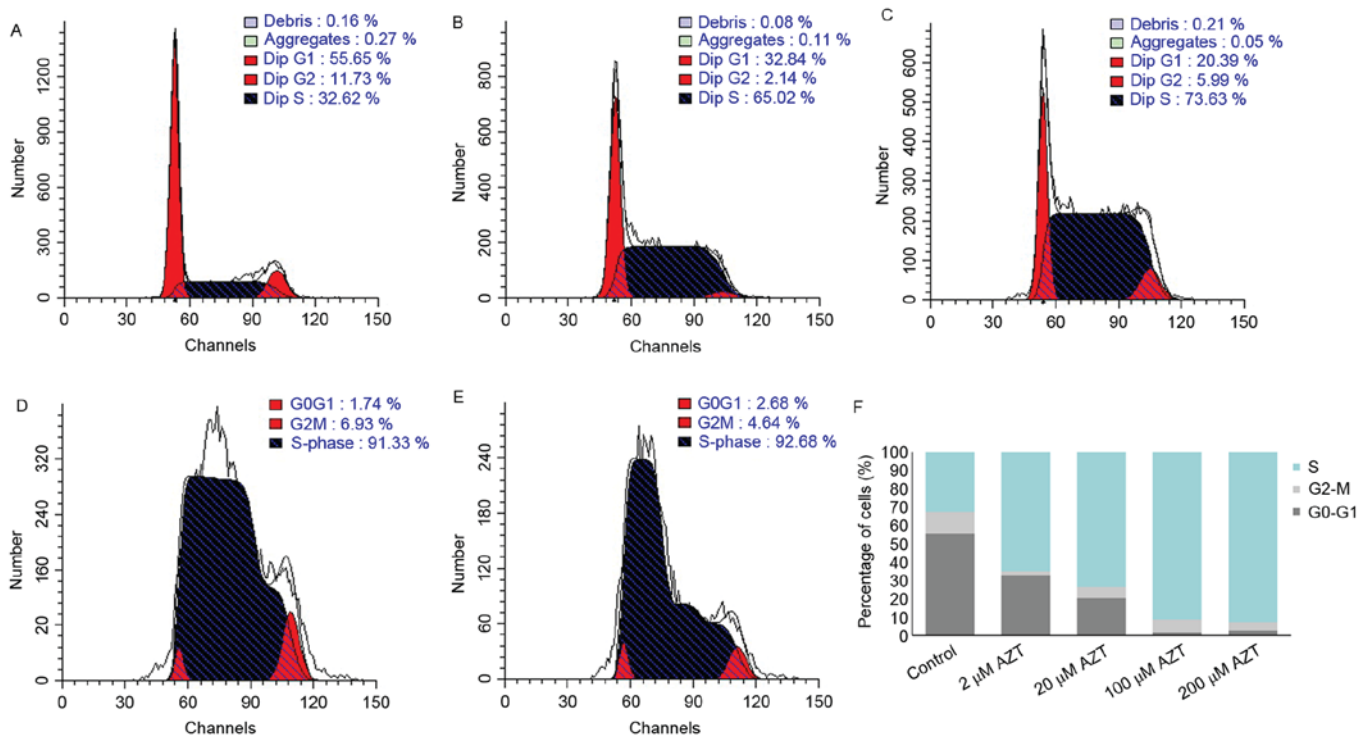

Figure 3. Analysis of cell cycle distribution by flow cytometry. AZT altered the cell cycle of TE-11 cells. Plots demonstrating the cell cycle phases of (A) Control cells and (B) cells treated with 2 μM (C) 20 μM (D) 100 μM (E) 200 μM AZT for 48 h. (F) Cell cycle distribution. AZT, azidothymidine.

However, there was no significant difference in activity following treatment with 2 μM AZT.

Effect of AZT on cell cycle progression. The effect of AZT on cell cycle distribution was assessed by flow cytometric analysis. Representative cell cycle profiles of TE-11 cells treated with 2, 20, 100 and 200 μM AZT for 48 h are demonstrated in Fig. 3A-E. Treatment with AZT led to a marked dose-dependent decrease in the percentage of G1/G0 phase cells and a marked dose-dependent increase in S-phase cells, compared with the control (Fig. 3F).

Effect of AZT on DNA damage. Degradation of DNA is an irreversible event following apoptotic cascade events (17). To investigate whether treatment with AZT may induce DNA degradation, a comet assay was performed. As demonstrated in Fig. 4A, treatment of TE-11 cells with increasing concentrations of AZT for 48 h resulted in significant DNA damage compared with control cells; the comet tails of the treated cells demonstrate DNA migration out of the nucleus due to DNA breakage and loss of structure. Consistent with comet assay results, western blot analysis revealed that treatment with increasing concentrations of AZT resulted in enhanced expression levels of γ-H2AX and pChk2 (Fig. 4B), which are markers of the DNA damage response (DDR) pathway. In conclusion, AZT may induce DNA damage in TE-11 cells.

Discussion

The majority of cancer cells have been reported to exhibit enhanced telomerase activity, whereas healthy somatic cells generally exhibit a low level of telomerase activity. Previously, telomerase activity has been identified in tumor initiating cells (3,18). Telomerase has been reported to be expressed in 86.2% esophageal carcinoma tissues; however, healthy esophageal tissue did not express it (19). Therefore, this suggests that there may be a window for telomerase inhibition-based treatment. Repressing telomerase activity may limit cell growth and induce apoptosis. Therefore, telomerase is an attractive target for cancer therapy and various strategies that target telomerase have been applied in clinical practice (20,21).

Telomerase serves a role in abnormal proliferation of tumor cells. A reverse transcriptase inhibitor, AZT, has been utilized in the treatment for AIDS-associated Kaposi sarcoma, Kaposi sarcoma-associated primary effusion lymphoma, Epstein-Barr-associated lymphoma, primary central nervous system lymphoma and adult T cell leukemia (22). AZT has been reported to regress tumors in phase I and II clinical trials, as a single agent or in combination with other drugs for gastrointestinal cancers, pancreatic cancer and various advanced malignancies (23-27). By interacting with hTERT, AZT causes a series of events, including telomere shortening, cell cycle blockade, termination of cellular replication and inhibition of cell growth (10). Chemotherapy is a conventional treatments for esophageal cancer, and is considered to be an essential therapeutic strategy (28). Compared with chemotherapy, AZT may serve as a promising drug as it directly inhibits telomerase activity and causes little injury to healthy cells, and therefore may be less toxic. In the present study, the effects of AZT on TE-11 cells were investigated at various concentrations and time points.

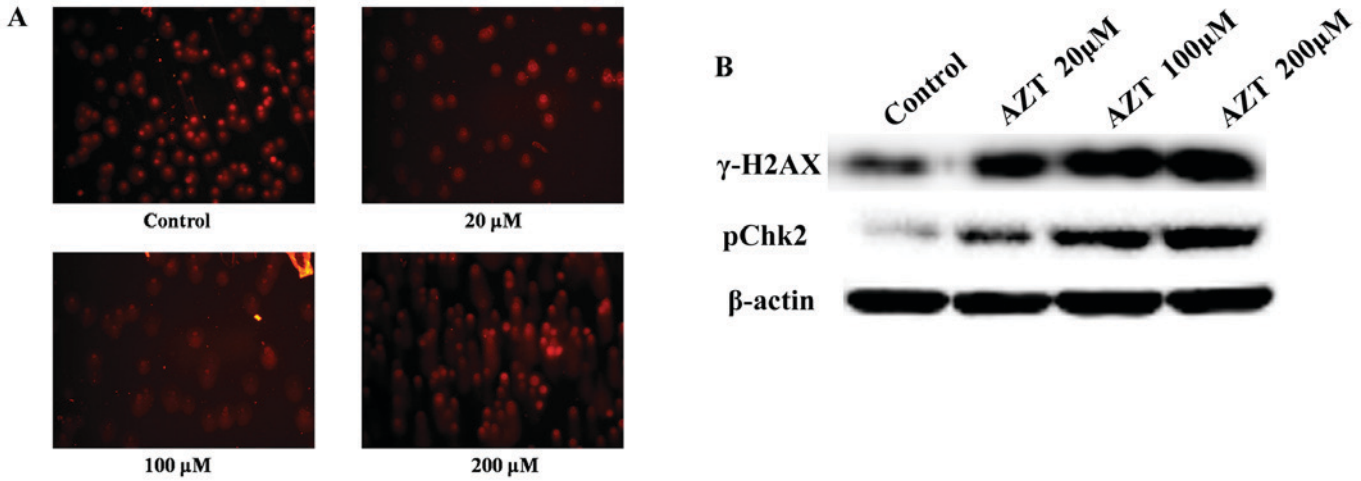

Figure 4. DNA damage by AZT in TE-11 cells was determined by (A) the comet assay and (B) western blotting of γ-H2AX and pChk2 in Control cells or cells treated with 20, 100 and 200 μM AZT for 48 h. β-actin was included as a loading control. AZT, azidothymidine; γ-H2AX, γ-H2A histone family member X; pChk2, phosphorylated checkpoint kinase 2.

Results demonstrated that AZT inhibited telomerase activity and proliferation, delayed cell cycle progression and induced apoptosis of human esophageal cancer TE-11 cells *in vitro*. The results of the MTT assay revealed that AZT inhibits the growth of cancer cells, which provides an experimental foundation for esophageal tumor therapy. The inhibitory effect was time- and dose-dependent, which suggested that these were important parameters for the treatment of esophageal cancer cells. Treatment of TE-11 cells with AZT resulted in a dose-dependent decrease in telomerase activity and cells in the G1/G0 phase of the cell cycle, and a dose-dependent increase in cells in S-phase. These findings were consistent with previous reports, which demonstrated that AZT arrested NIH3T3 fibroblasts and SGC-7901 gastric cancer cells in S and G2/M phase (29,30).

Telomerase has been implicated in DNA double strand break (DSB) repair (31,32). To investigate whether AZT affects DNA damage in TE-11 cells, a comet assay was performed following treatment with AZT at various concentrations. DNA damage was enhanced with increasing concentrations of AZT. The expression levels of γ-H2AX and pChk2, which are markers of the DDR pathway (33), were enhanced following treatment with AZT. Treatment with AZT leads to a reduction in telomerase activity, shortening of telomeres, end-to-end fusions and chromosome instability. In addition, a reduction in telomerase activity interferes with correct rejoining of DSB ends, causes a deficiency in DNA repair and induces apoptosis (31). Telomerase activity is directly associated with protection against cell death, and therefore the inhibition of telomerase in cancer cells leads to apoptosis (12,14,15).

Telomerase as target for cancer treatment has great potential (34). It has been reported that AZT synergistically interacts with other treatment modalities, including chemotherapy agents (35,36). Therefore, AZT may be a novel strategy for the treatment of cancer, including those derived from the esophagus. However, the mechanism by which AZT inhibits cell growth and arrests cell cycle progression in esophageal cancer requires further investigation.

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