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Shikonin Inhibites Migration and Invasion of Thyroid Cancer Cells by Downregulating *DNMT1*

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Background: Shikonin is a component of Chinese herbal medicine. The aim of this study was to investigate the effects of shikonin on cell migration of papillary thyroid cancer cells of the TPC-1 cell line *in vitro* and expression levels of the phosphate and tensin homolog deleted on chromosome 10 (*PTEN*) and DNA methyltransferase 1 (*DNMT1*) genes.

Material/Methods: The Cell Counting Kit-8 (CCK-8) assay was performed to evaluate the proliferation of TPC-1 papillary thyroid cancer cells, and the normal thyroid cells, HTori-3, *in vitro*. A transwell motility assay was used to analyze the migration of TPC-1 cells. Western blot was performed to determine the expression levels of *PTEN* and *DNMT1* genes. A methylation-specific polymerase chain reaction (PCR) (MSP) assay was used to evaluate the methylation of *PTEN*.

Results: Following treatment with shikonin, the cell survival rate of TPC-1 cells decreased in a dose-dependent manner; the inhibitory effects on HTori-3 cells were less marked. Shikonin inhibited TPC-1 cell migration and invasion in a dose-dependent manner. The methylation of PTEN was suppressed by shikonin, which also reduced the expression of *DNMT1* in a dose-dependent manner, and increased the expression of *PTEN*. Overexpression of *DNMT1* promoted the migration of TPC-1 cells and the methylation of *PTEN*. Levels of protein expression of PTEN in TPC-1 cells treated with shikonin decreased, and were increased by *DNMT1* knockdown.

Conclusions: Shikonin suppressed the expression of *DNMT1*, reduced *PTEN* gene methylation, and increased PTEN protein expression, leading to the inhibition of TPC-1 cell migration.

MeSH Keywords: Methylation • Parathyroid Neoplasms • PTEN Phosphohydrolase

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Background

Worldwide, thyroid cancer is one of the most common endocrine malignancies [1–3]. Clinically, there are four main types of thyroid cancer; papillary (the most common type), follicular, medullary, and anaplastic, and the characteristics and prognoses of each subtype differ [4]. The current treatment options for thyroid cancer include surgical resection, radioactive iodine therapy, usually following surgery, and inhibition of thyroid stimulating hormone (TSH) [5,6]. However, current treatments for thyroid cancer are often not curative [7], and alternative treatment options remain limited [8], with many patients having thyroid cancer that is refractory to radioactive iodine therapy. Therefore, new therapeutic strategies for patients with thyroid cancer are still required.

Finegersh et al. first showed that hypermethylation of DNA was associated with the potentiation of gene expression in head and neck squamous-cell carcinoma (HNSCC) [9]. Traditional studies of DNA methylation have focused on CpG islands as promoters of the majority of genes, which are hypermethylated to suppress gene expression [10]. However, the majority of CpGs occur outside these promoters, and have inverse effects on expression, as hypermethylation usually promotes expression [11]. Because DNA methyltransferase 1 (*DNMT1*) is a main regulator of methylation, this study explored the correlation between DNA methylation by the *DNMT1* gene and thyroid cancer.

The phosphate and tensin homolog deleted on chromosome 10 (*PTEN*) gene spans 105 kb and contains nine exons on chromosome 10q23.31. *PTEN* is a well-characterized tumor suppressor gene, which antagonizes the phosphoinositol-3-kinase/protein kinase B (PKB or Akt) signaling pathway [12]. The *PTEN* gene acts as a tumor suppressor gene in multiple cancers, including breast cancer [13,14], nasopharyngeal carcinoma [15], renal cell carcinoma [16], non-small-cell lung cancer [17], hepatocellular carcinoma [18], human NK/T-cell lymphoma [19], and osteosarcoma [20]. The loss of function of *PTEN* in tumor cells results in the accumulation of critical cell messengers, which increases Akt phosphorylation and activity and leads to decreased apoptosis and/or increased mitogenic signaling [21,22]. Epigenetic alterations play an important role in cancer progression through hypermethylation and the silencing of tumor suppressor genes, and somatic *PTEN* hypermethylation has been recognized as a means of *PTEN* downregulation in a subset of malignancies, including prostate cancer, colon cancer, and endometrial cancer [23–25]. It has been reported that loss of *PTEN* expression can occur through promoter hypermethylation and is associated with tumorigenesis and that this process of methylation is mediated by the *DNMT1* gene [26].

Shikonin is a plant derivative and a major component of Zi Cao, or purple gromwell, the dried root of *Lithospermum erythrorhizon* [27–29]. Shikonin is a Chinese herbal medicine that has been reported to have biological activities that include the inhibition of bacterial growth, cell replication, and platelet aggregation [27–29]. Previously published studies have shown that shikonin and its analogs induce cell cycle arrest and apoptosis, and inhibit human colorectal cancer cell growth *in vitro* and *in vivo* [30], leukemia cells *in vitro* [31,32], breast cancer [33] and hepatocellular cancer cells *in vitro* [34] through varied molecular mechanisms. These previous and mainly *in vitro* studies have supported the potential role for shikonin as an antitumor agent. A study published in 2006 by Nigorikawa et al. showed that shikonin inhibited the expression of the *PTEN* gene [35]. Recently, the study of shikonin as an antitumor agent has attracted attention. The study of Yang et al. demonstrated that shikonin inhibited thyroid cancer and apoptosis without significant hepatotoxicity [7], which supports the view that shikonin may have potential as a targeted antitumor agent for thyroid cancer.

The aim of this study was to investigate the effects of shikonin on cell migration of papillary thyroid cancer (PTC) cells of the TPC-1 cell line *in vitro* and expression levels of the *PTEN* and *DNMT1* genes.

Material and Methods

Cell lines

The human papillary thyroid cancer (PTC) cell line, TPC-1 (BNCC, Beijing, China), and the normal human thyroid cell line, HTori-3 (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin. The cells were incubated in a 5% CO_2 incubator at 37°C. When the cells reached 70–80% confluence, they were passaged, in accordance with standard procedures.

The Cell Counting Kit 8 (CCK8) cytotoxicity assay

Using a Cell Counting Kit 8 (CCK-8) assay, (Beyotime, Beijing, China), the TPC-1 cell viabilities were assayed after exposure to increasing concentrations of shikonin (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China) (0.1, 0.25, 0.5, 1.0, 1.5, 2.0, and 2.5 μg/mL), which was dissolved in phosphate-buffered saline (PBS). TPC-1 cells were seeded in 96-well plates (100 μL, containing 2,000 cells each well), treated with increasing concentrations of shikonin, and 10 μL of CCK-8 solution was added to each well. After incubation for 4 hrs, the optical density at 450 nm was measured by using an ultraviolet spectrophotometer (Bio-Rad, Hercules, CA, USA).

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DNMT1 gene knockdown and overexpression

A *DNMT1*-specific siRNA (primer sequences: 5'-AGGACAA GCTCATGTACTT-3' (forward) and 5'-AAGTACATGAGCTTGTCCT-3' (reverse)) and a negative control siRNA (primer sequences: 5'-CTTATCGTGCAGCATGTAA-3' (forward) and 5'-TTACATGCTGCACGATAAG-3' (reverse)) were obtained from GenePharma (Shanghai, China). The cDNA encoding *DNMT1* was amplified by polymerase chain reaction (PCR), which was for 35 cycles of amplification at 94°C for 60 s, 56°C for 180 s, and 72°C for 1 min, followed by 10 min at 72°C. Then, the PCR product was sub-cloned into the pcDNA 3.0 vector. The empty pcDNA 3.0 vector was used as the negative control. Transfections were performed using the Lipofectamine 2000 reagent (Invitrogen, CA, USA) in accordance with the manufacturer's instructions.

Transwell cell migration and invasion assay

For the transwell migration assay, 1×10^5 TPC-1 papillary thyroid carcinoma cells in 200 ml of DMEM without FBS were seeded into the upper part of each transwell assay chamber (pore size: 8 μm) (Corning, New York, NY, USA) containing a non-coated membrane. Cell invasion was examined by using transwell chambers (Corning, New York, NY, USA) coated with Matrigel (BD Biosciences, CA, USA) on the upper surface of the membrane, and 1×10^5 TPC-1 cells were suspended in serum-free medium and re-seeded into the upper chamber of each insert. The culture medium containing 10% fetal bovine serum was added to the lower chamber. After overnight incubation at 37°C, cells were treated for additional 24 hrs with DNMT1 (Boster, Wuhan, China), shikonin alone, or a combination of both. Then, cells in each chambers were fixed with 4% paraformaldehyde, and stained with 0.1% crystal violet solution and placed on a glass slide. Cells adhering to the bottom of the membrane were counted in five random visual fields under a light microscope at 200× magnification. All assays were performed in triplicate.

DNA extraction and preparation for the methylation-specific polymerase chain reaction (PCR) (MSP) assay

Total DNA was extracted from transfected cells in a column-based centrifugation procedure, according to the manufacturer's protocol of the GeneAll Nucleic Acid Purification Kit (GeneAll Biotechnology, Seoul, Korea). The DNA pool was treated with bisulfite using the EpiTect Bisulfite Kit (Qiagen, Hilden, Germany) and prepared for methylation-specific polymerase chain reaction (PCR) (MSP) assay. After treated with bisulfite, the non-methylated cytosine bases were converted to uracil, while 5-methylcytosine bases remained intact. Molecular biology grade glycogen was added as a co-precipitant to counteract the decrease in DNA concentration, for reducing the loss of DNA after modification.

Methylation-specific PCR (MSP) assay

TPC-1 cells were seeded into 6-well plates at 2×10^4 cells/well and cultured overnight. After 24 hours of treatment with shikonin at increasing concentrations, the cells were harvested for the MSP assay, which was a feasible and low-cost method to evaluate DNA methylation patterns. Non-methylated and methylated regions of DNA were amplified after treatment with sodium bisulfite. Amplification was conducted with a thermocycler using the following conditions: 94°C for 2 min, 36 cycles composed of denaturation (94°C for 30 s), annealing (54°C for 30 s), and elongation (72°C for 45 s), followed by an extension at 7 °C for 7 min.

The *PTEN* gene methylated primer sequences used were as follows: 5'-GGCGGCGGTCGCGGTTC-3' (forward) and 5'-GACTCC CCGAAAACGCTAC-3' (reverse), amplified fragment length of 71 bp.

The *PTEN* gene non-methylated primer sequences used were as follows: 5'-GTGTTGGTGGAGGTAGTTGTTT-3' (forward) and 5'-ACCACTTAACTCTAAACCACAACCA-3' (reverse), the length of the amplified fragment was 162bp.

Amplification of the template using M and U primers, respectively represented the methylated and non-methylated CpG islands, while the amplification of both M and U primers indicated that target sequences were partially methylated. The CpG methyltransferase (M. SssI) (New England Biolabs, Ipswich, MA, USA) and the untreated genomic DNA was set up as positive and negative controls using this approach. Eventually, the products of PCR were detected by 1.5% agarose gel electrophoresis.

Western blotting

Western blotting was performed to determine the expression of the DNMT1 and PTEN proteins. Whole cell proteins were separated in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with blocking buffer at room temperature for 1h, followed by incubation at 4°C overnight with a mouse monoclonal antibody against DNMT1 or PTEN (Santa Cruz Biotechnology, CA, USA) diluted at 1: 500. The membranes were then washed and incubated at room temperature for 2h with anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology, CA, USA) diluted at 1: 5000. Enhanced chemiluminescence was used for assessing protein expression. Lab Works Image Acquisition and Analysis Software (UVP Inc., Bioimaging Systems, Cambridge, U.K.) were used to quantitate band intensities. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control.

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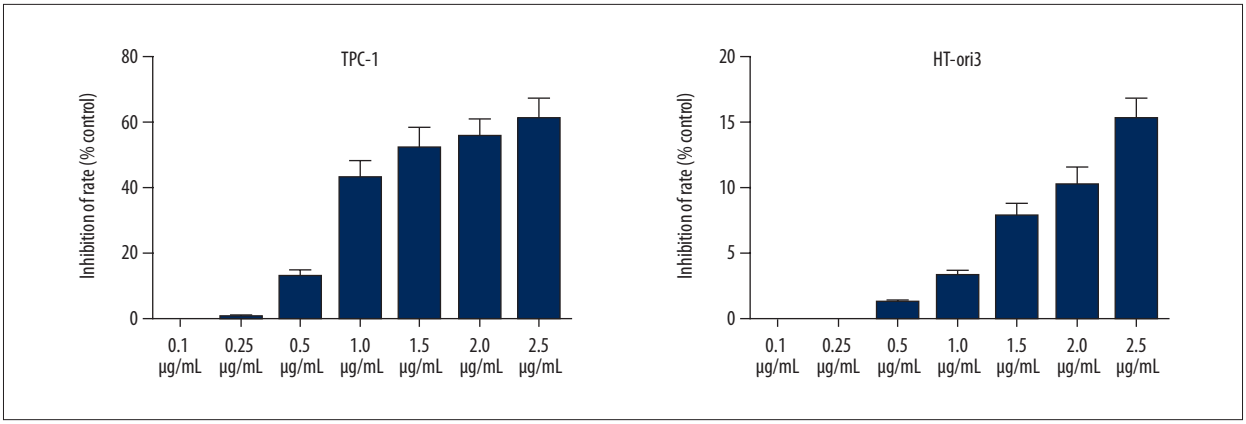

Figure 1. Shikonin decreased the proliferative capacities of TPC-1 and HTori-3 cells. The Cell Counting Kit-8 (CCK-8) assay was performed to determine the rates of inhibition of the proliferation of TPC-1 and HTori-3 cells after treatment with the indicated concentrations of shikonin for 48 hrs.

Statistical analysis

The results are expressed as the mean ± standard deviation (SD). Statistical significance was evaluated by using SPSS software version.19.0 (SPSS Inc., Chicago, Ill, USA). Comparison between two groups was made using Student's t-test or one-way analysis of variance (ANOVA), with a P-value <0.05 considered to be statistically significant.

Results

Shikonin treatment resulted in dose-dependent inhibition of TPC-1 papillary thyroid carcinoma (PTC) cell proliferation *in vitro*

Using the Cell Counting Kit-8 (CCK-8) assay to examine the inhibitory effects of different concentrations of shikonin (0.1, 0.25, 0.5, 1.0, 1.5, 2.0, and 2.5 μg/mL) on TPC-1 papillary thyroid cancer (PTC) cells and HTori-3 normal thyroid epithelial cells. As shown in Figure 1, shikonin treatment significantly inhibited cell proliferation in a dose-dependent manner, with IC_{50} values of 0.744 μg/mL for TPC-1 and 11.52 μg/mL for HTori-3, indicating inhibition of TPC-1 cell proliferation.

Shikonin treatment inhibited the migration and invasion of TPC-1 papillary thyroid carcinoma (PTC) cells in a dose-dependent manner

The inhibitory effect of shikonin on cell migration and invasion were determined by the transwell assay. As shown in Figure 2A, compared with the control HTori-3 cells, the number of TPC-1 cells that migrated per field was inhibited in the cells that were treated with shikonin. This inhibition occurred in a dose-dependent manner, and the number of cells that passed through the Matrigel-coated membrane into the lower chamber were significantly lower in cells treated with the increasing concentrations of shikonin, compared with the control HTori-3 cells (Figure 2B), which indicated that shikonin inhibited the invasive potential of TPC-1 cells, in a dose-dependent manner.

Shikonin upregulated the expression of *PTEN* through inhibition of *PTEN* methylation

As shown in Figure 3A, the results of the methylation-specific polymerase chain reaction (PCR) (MSP) assay showed a clear band for the methylated form of *PTEN*, with a dark band for the non-methylated form in the control HTori-3 cells, and a clear band for the non-methylated form; a dark band was found for the methylated form, in the presence of increasing concentrations of shikonin. A clear band was present for both the methylated and non-methylated forms of *PTEN* in the presence of low or medium concentrations of shikonin, which indicated that shikonin exerted a dose-dependent inhibition of *PTEN* methylation. Based on these results, shikonin inhibited the biological activity of TPC-1 through the demethylation of *PTEN*.

The effect of shikonin on *PTEN* demethylation in TPC-1 cells was subjected to further testing, with a Western blotting assay, which supported these findings. As shown in Figure 3B, shikonin significantly inhibited the expression of *DNMT1* and upregulated the expression of *PTEN* in a dose-dependent manner. Figure 3C shows bar graphs of these results.

Amplification of *DNMT1* was responsible for the survival TPC-1 papillary thyroid carcinoma (PTC) cells treated with shikonin

In the previous experiment, the inhibitory effects of shikonin on *DNMT1* expression were confirmed (Figure 3B). The Western blot assay (Figure 4C) demonstrated that the presence of

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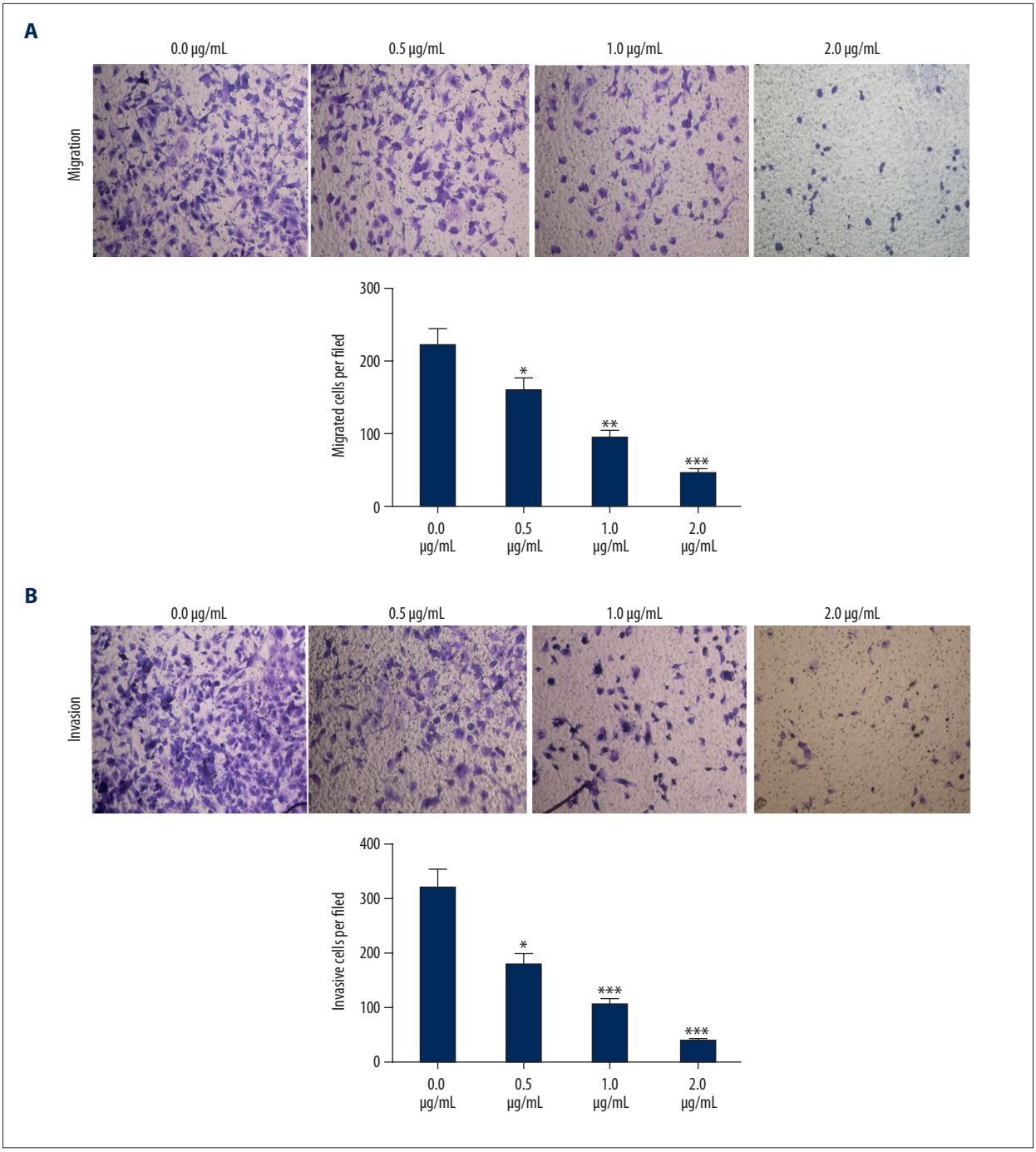

Figure 2. Inhibition of TPC-1 thyroid cancer cell migration by shikonin. (**A**) The transwell migration assay was performed to evaluate the effect of shikonin on cell migration. The cells were treated with the indicated concentrations of shikonin. Representative images of cell migration of TPC-1 cells in the migration assay are shown in the left panel. (Magnification ×200). (**B**) Transwell motility assays were performed to evaluate the effect of shikonin on cell invasion. The cells were treated with the indicated concentrations of shikonin for 36 hrs. Representative images of cell invasion of TPC-1 cells are shown in the left panel. (Magnification ×200). * P<0.05; ** P<0.01; *** P<0.001

DNMT1 expression inhibited the expression of *PTEN* in TPC-1 papillary thyroid cancer (PTC) cells compared with the HTori-3 control cells. A transwell assay was performed to test the effect of *DNMT1* on cell migration and invasion. As shown in Figure 4A, compared with the HTori-3 control cells, the number of migrated cells per field markedly increased in TPC-1 papillary thyroid

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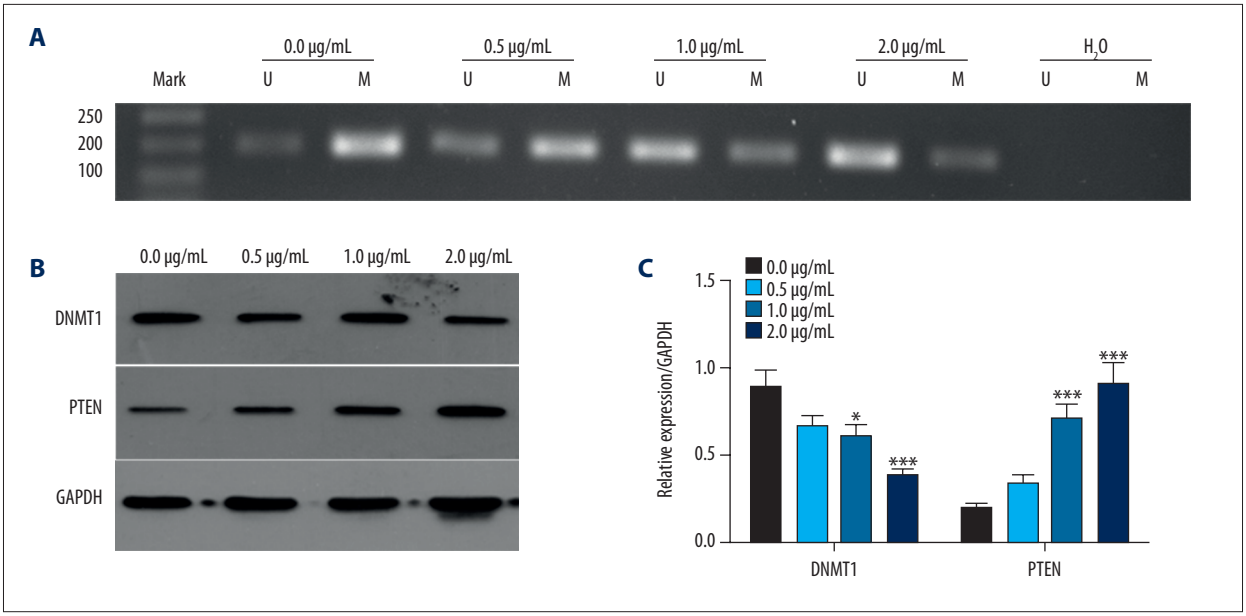

Figure 3. Effect of shikonin on the expression of *DNMT1* and *PTEN*, and the methylation of PTEN in TPC-1 papillary thyroid cancer cells. (**A**) A methylation-specific polymerase chain reaction (PCR) (MSP) assay was performed to evaluate the correlation between the *PTEN* methylation status and the concentration of shikonin. 5' CpG island methylation status of the *PTEN* gene promoter induced by different concentration of shikonin determined via the MSP assay. U – non-methylated product; M – methylated product. (**B**) Western blotting assays show the effect of shikonin on the expression of *DNMT1* and *PTEN*. The cells were treated with the indicated concentrations of shikonin. Representative images of the outcome of the Western blotting assay. (**C**) The bar graphs of the relative expression of *DNMT1* and *PTEN*. * P<0.05; ** P<0.01; *** P<0.001

cancer (PTC) cells expressing *DNMT1* and treated with shikonin, which implied that *DNMT1* promoted TPC-1 cell migration.

Also, the Matrigel assays showed that the number of cells that passed through the Matrigel-coated membrane into the lower chamber were significantly increased in TPC-1 papillary thyroid cancer (PTC) cells expressing *DNMT1* and treated with shikonin compared with the HTori-3 control cells, indicating that *DNMT1* also promoted the invasive potential of TPC-1 cells.

As shown in Figure 4B, the MSP products were exclusively observed in the methylated forms of the promoter region, which indicated that the occurrence of promoter DNA methylation of *PTEN* in TPC-1 cells expressing *DNMT1* and treated with shikonin resulted in reduced expression of *PTEN* in the TPC-1 cells.

DNMT1 knockdown was a key event in the effect of shikonin on TPC-1 papillary thyroid carcinoma (PTC) cells *in vitro*

To verify the role of *DNMT1* in the effect of shikonin treatment of papillary thyroid cancer (PTC) cells of the TPC-1 cell line *in vitro* from a different perspective, a Western blot assay was performed (Figure 5C), and showed that the absence of *DNMT1* could promote the expression of *PTEN*. Therefore, there was increased expression of *PTEN* in TPC-1 cells treated with *DNMT1* siRNA compared with the HTori-3 cell controls.

A transwell assay was used to test the effect of *DNMT1* knockdown on cell migration and invasion. As shown in Figure 5A, compared with HTori-3 cell controls, the number of migrated cells per field was markedly inhibited in the TPC-1 cells treated with *DNMT1* siRNA, indicating that the loss of *DNMT1* inhibited TPC-1 papillary thyroid cancer (PTC) cell migration. Also, the Matrigel assays showed that the number of cells that passed through the Matrigel-coated membrane into the lower chamber were significantly less in cells treated with *DNMT1* siRNA compared with the HTori-3 control cells, which indicated that the absence of *DNMT1* inhibited the invasive potential of TPC-1 cells *in vitro*.

As shown in Figure 5B, the MSP products were exclusively observed in the non-methylated forms of the promoter region, which indicated an absence of promoter DNA methylation of *PTEN* in TPC-1 cells treated with *DNMT1* siRNA and indicated that the absence of *DNMT1* resulted in increased expression of *PTEN* in the TPC-1 papillary thyroid cancer (PTC) cells *in vitro*.

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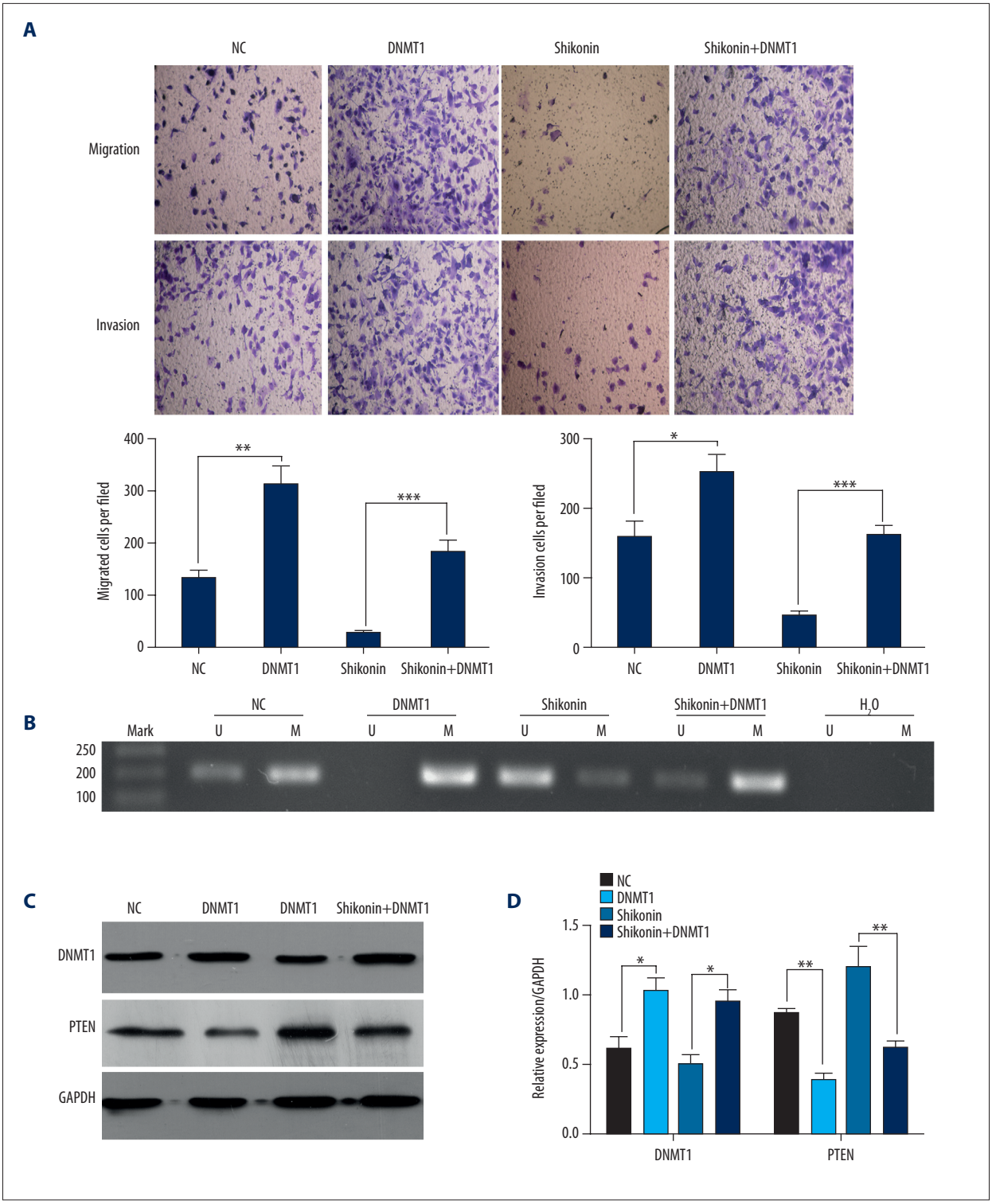

Figure 4. The effects of *DNMT1* and shikonin on TPC-1 papillary thyroid cancer cells. (**A**) The transwell migration and motility assays were performed to evaluate the effects of shikonin and *DNMT1* on cell migration and invasion. (Magnification ×200). (**B**) The methylation-specific polymerase chain reaction (PCR) (MSP) assay was performed to evaluate the correlation between the PTEN methylation status. U – non-methylated product; M – methylated product. (**C**) The Western blot assays were performed to evaluate the expression of *DNMT1* and *PTEN*. (**D**) The bar graphs of the relative expression of *DNMT1* and *PTEN*. * P<0.05; ** P<0.01; *** P<0.001

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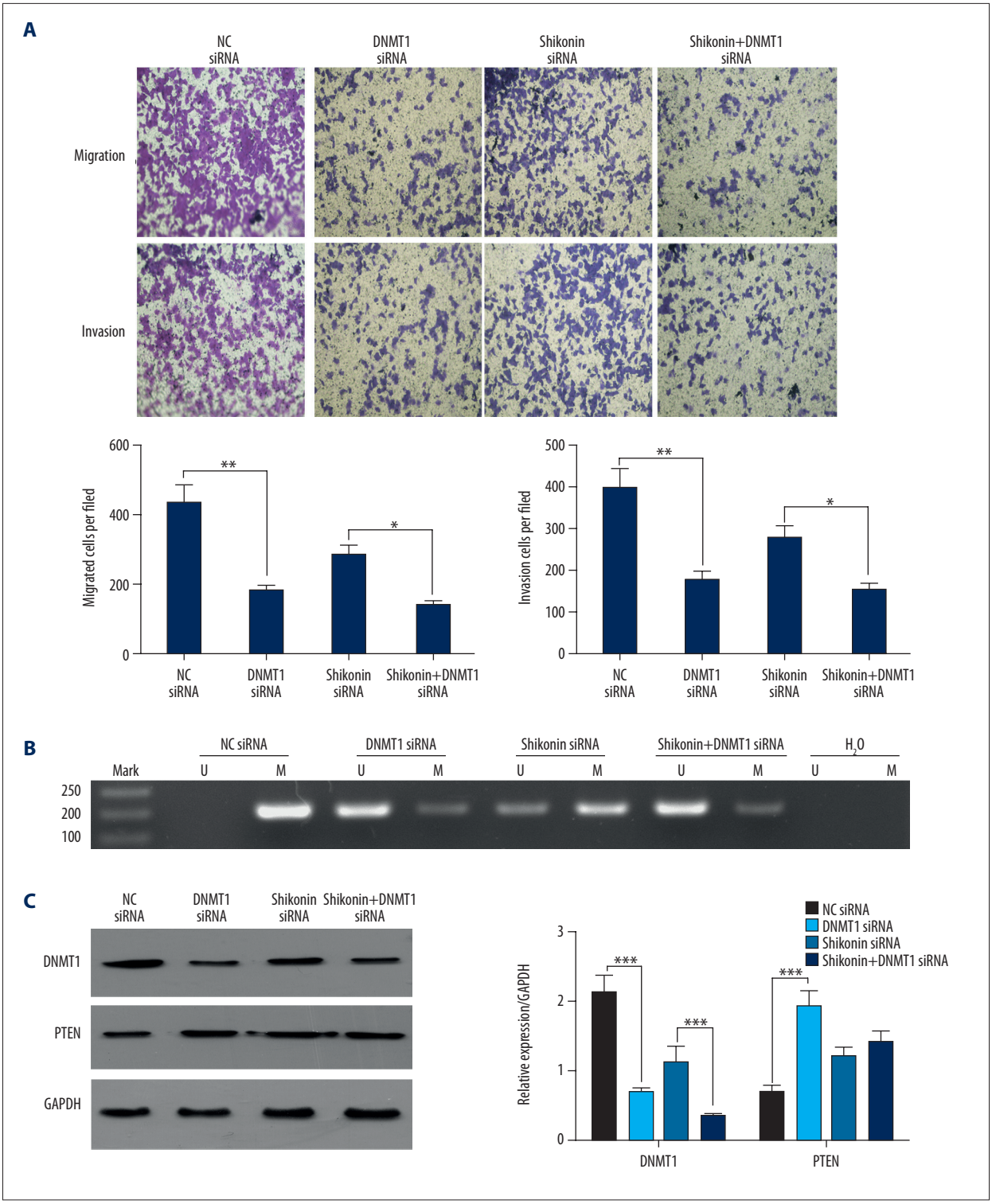

Figure 5. The effects of *DNMT1* small interfering RNA (siRNA) and shikonin on TPC-1 papillary thyroid cancer cells. (**A**) The transwell migration and motility assays were performed to evaluate the effects of shikonin and *DNMT1* siRNA on cell migration and invasion. (Magnification ×200). (**B**) The methylation-specific polymerase chain reaction (PCR) (MSP) assay was performed to evaluate the correlation between the *PTEN* methylation status. U – non-methylated product; M – methylated product. (**C**) The Western blotting assay was performed to evaluate the expression of *DNMT1* and *PTEN*. (**D**) The bar graphs of relative expression of *DNMT1* and *PTEN*. * P<0.05; ** P<0.01; *** P<0.001.

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Discussion

As a negative regulator of the PI3K/AKT signaling pathway, the phosphate and tensin homolog deleted on chromosome 10 (PTEN) is recognized as a tumor suppressor protein [36]. PTEN protein acts as a lipid phosphatase that dephosphorylates phosphatidylinositol (3,4,5)-trisphosphate (PIP3) to phosphatidylinositol (4,5)-bisphosphate (PIP2) [37–39]. Also, reduction in PTEN protein expression has previously been reported to be associated with abnormal promoter methylation [40]. Given that the CpG-rich region of tumor suppressors is regulated by the DNA methyltransferase 1 (*DNMT1*) gene, the *PTEN* gene has been reported to be one of the downstream genes of *DNMT1* in tumor cells [26–41]. The aim of this study was to explore the correlation between *PTEN* methylation induced by *DNMT1* and the proliferation, migration, and invasion of the TPC-1 papillary thyroid cancer (PTC) cells *in vitro*.

Shikonin is a plant-derived natural product that has been used safely for treatment of a variety of inflammatory diseases and has been demonstrated the antitumor activity [40,43] However, the antitumor effects of shikonin in thyroid cancer have not previously been studied. In this study, shikonin was shown to inhibit the bioactivity, cell proliferation, migration and invasion of TPC-1 cells *in vitro*. Also, the antitumor mechanism of action of shikonin was studied, and it was shown to inhibit *PTEN* methylation through the suppression of *DNMT1* expression.

The Cell Counting Kit-8 (CCK-8) assay was performed to evaluate the proliferation of TPC-1 papillary thyroid cancer (PTC) cells, and the normal thyroid cells, HTori-3, *in vitro*. The results showed that shikonin significantly inhibited the proliferation of TPC-1 cells in a dose-dependent manner. Transwell assays were performed to demonstrate its inhibitory effect on the migration and invasion of TPC-1 cells. However, in this study, the molecular mechanisms of the effects of treatment with shikonin were not determined. However, to investigate the molecular mechanism responsible for the inhibition of TPC-1 cell proliferation, the effect of *DNMT1* expression was examined on cell growth, and the findings were that the DNMT1 protein mediated the inhibitory effect on tumor cells treated with shikonin, indicating that *DNMT1* was a key element of the shikonin-induced tumor inhibition.

Also, the expression levels of *PTEN* and *DNMT1* were detected using Western blotting. The data showed that the methylation of *PTEN* in TPC-1 cells was significantly inhibited after treatment with shikonin, and this might be associated the suppression of *DNMT1* expression. Previously published studies have shown that *DNMT1* promoted DNA methylation and plays an important role in suppression of the expression of several genes involved in cell growth and differentiation. The results of the current study were consistent with those of previous studies. In the present study, the findings from the Western blotting and methylation-specific polymerase chain reaction (PCR) (MSP) assay, showed that *DNMT1* expression promoted *PTEN* methylation, which might lead to reduced expression of *PTEN*. Furthermore, the results of the transwell assay showed that overexpression of *DNMT1* inhibited the migration and invasion of TPC-1 cells. Based on these studies, it might be reasonable to propose that shikonin treatment of TPC-1 papillary thyroid cancer (PTC) cells suppressed the methylation of *PTEN* by causing a reduction in the expression of *DNMT1*, which might then inhibit the migration and invasion of TPC-1 cells *in vitro*.

Conclusions

The aim of this study was to investigate the effects of shikonin, a component of Chinese herbal medicine, on cell migration of papillary thyroid cancer (PTC) cells of the TPC-1 cell line *in vitro* and expression levels of the phosphate and tensin homolog deleted on chromosome 10 (*PTEN*) and DNA methyltransferase 1 (*DNMT1*) genes. Shikonin suppressed the expression of *DNMT1*, reduced *PTEN* gene methylation, and increased PTEN protein expression, leading to the inhibition of TPC-1 cell migration. Further studies are required to determine whether shikonin might have similar functions in other primary histological types of thyroid cancer. Future large-scale, multi-center, controlled clinical studies are required to determine whether shikonin may have a role in the treatment of papillary thyroid cancer *in vivo*.

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