EJGO European Journal of Gynaecological Oncology

HPV16 infection up-regulates Piwil2, which affects cell proliferation and invasion in cervical cancer by regulating MMP-9 via the MAPK pathway

W. Ling[1], H. Zhigang[2], H. Tian[3], Z. Bin[3], X. Xiaolin[3], Z. Hongxiu[3]

[1] Department of Gynaecology and Obstetrics, General Hospital of Lanzhou Military Region, Lanzhou
[2] Division of Medical Administration, General Hospital of Lanzhou Military Region, Lanzhou
[3] Department of Gynaecology and Obstetrics, Southwest Hospital of the Third Military Medical University, Chongqing (China)

Summary

Purpose of investigation: The present study aimed to investigate the effect of Piwil2 on proliferation and invasion of cervical cancer cells. *Materials and Methods:* Thirty-two HPV-positive or negative cervical cancer tissues and corresponding normal adjacent cervical tissues were obtained from General Hospital of Lanzhou Military Region. Piwil2 expression in these tissue samples, as well as two cervical cell lines were evaluated by quantitative real-time polymerase chain reaction (qRT-PCR) and immunohistochemical. A specific short hairpin RNA (shRNA) was used to knockdown the Piwil2 gene in SiHa cells. CCK-8 assay and flow cytometry (FCM) was used to evaluate cell proliferation. Cell invasion was detected by transwell chambers assays. Immunoblotting was used to assess the effect on relevant proteins. *Result:* In the early stage (I A1 – I B1) of curvival, 84.4% (27/32) tumor tissues have a more predominant expression of Piwil2 than the normal adjacent samples. Piwil2 overexpression was correlated with HPV16 infection ($p < 0.05$). Knockdown of Piwil2 gene in SiHa cells inhibited cell growth and invasion, and downregulated matrix metalloproteinase-9 (MMP-9) compared to scrambled shRNA transfected cells. Further analysis revealed that downregulation of Piwil2 gene induced inhibition of the MAPK signaling pathway activity. *Conclusion:* Piwil2, which stimulated by HPV16 infection, plays an important role in regulating proliferation and invasion of cervical cells by regulating MMP-9 expression via alternation of the MAPK signaling pathway.

Key words: Cervical cancer; Piwil2; Cell growth; Invasion; MMP-9.

Introduction

Cervical cancer, as the third most common cancer and the fourth that leads to death in women worldwide, is the most frequent cancer of females in developing countries. There are about 529,828 new cases and 275,125 of them die of cervical cancer every year [1, 2]. Although developments and progresses have been made in treatment with early diagnosis, many patients are threatened by tumor recurrence [3]. It is known that human papilloma virus (HPV) infection is a high risk factor in the progression and development of cervical cancer [4]. Infection of those high risk HPVs, such as HPV16, HPV18, causing cell immortalization, and when followed by further mutations, leads to cervical cancer and other anogenital tumors. [5-7]. Many clinical studies have revealed that HPV infection is related to prognosis and recurrence of carcinomas. In addition, the recurrence-free survival of those who remained HPV-positive after treatment was significantly lower than those who turned negative [8-10]. Piwi-like RNA-mediated gene silencing 2 (Piwil2) was essential in the initial phase of spermatogenesis: regulating RNA silencing and transcription, relating to germline stem cells self-renewal, involved in early prophase differentiation of the testis and embryo tissues [11, 12]. Furthermore, Piwil2 was also found as the candidate oncogene in recent studies. With a high-level expression in the gastric, breast, colorectal, and thyroid cancer, Piwil2 was considered to be a potential biomarkers for these cancers. [13-17]. Additionally, Piwil2 overexpression also cause anti-apoptosis and cell proliferation [17, 18], suggesting that Piwil2 may be related to the prognosis of tumor [19, 20]. Nevertheless, the potential mechanism of Piwil2 in cervical cancer still remains unknown.

A few of reports linked Piwil2 overexpression to cervical cancer [14, 21], but none of them mentioned the relationship between HPV infection and Piwil2, nor how Piwil2 worked in cell invasion and migration. The present authors are interested in the co-occurrence of HPV infection and Piwil2 upregulated in some cervical tumors in their prophase, and the poor prognosis that they represented. Therefore they conducted this comprehensive study to reveal the role that Piwil2 plays in cervical, and its potential correlation with HPV16 infection.

Revised manuscript accepted for publication April 7, 2014

Materials and Methods

Tumor specimens and cell lines

Thirty-two patients with cervical cancer in Stage I A1 – I B1 underwent radical trachelectomy from May 2011 to November 2012 in General Hospital of Lanzhou Military Region. Carcinomatous and adjacent tissues were confirmed by two experienced pathologists according to the 2009 Edition's WHO classification guideline of cervical cancer. HPV16 infected was determined by E7 gene expression or not [22]. Informed consent was signed by all patients and the study was approved by ethics committee of General Hospital of Lanzhou Military Region, Lanzhou, China. Human cervical cancer cell line SiHa and normal human keratinocyte line HaCaT were obtained from American Type Culture Collection and were cultured in RPMI-1640 complete medium supplemented with 10% fetal bovine serum (FBS) containing 100 U/ml of penicillin and 100 μg/ml of streptomycin.

SiHa cell transfection with shRNA

Following the manufacturer's protocol, the green fluorescent protein (GFP) tagged lentiviral vector integrated with Piwil2-specific shRNA (sequence: 5'-AAAGCCAGAGGAACCAAGCAC-3', named as lv-Piwil2-KD) or integrated with the scrambled shRNA (sequence: 5'-GTACCGCACGTCATTCGTATC-3', named as lv-Piwil2-scr) was transfected into SiHa cells in the 24-well plate. Then the serum-free medium in the 24-well plate was replaced with the complete medium after six hours. The GFP positive cells were harvested through the flow cell cytometry (FCM) on the next day. SiHa cells transfected with lv-Piwil2-KD or lv-Piwil2-scr were referred to as the Piwil2-shRNA group and the Mock group, respectively.

Quantitative real-time polymerase chain reaction (qRT-PCR) and immunoblotting assay

Total RNA was extracted from tissues or adhere cells using RNAiso plus and then was reversely transcribed to cDNA using TaKaRa RNA-PCR Kit. The qRT-PCR was performed using the Maxima SYBR Green qPCR Master Mix Kit. The expression level of concerned genes was calculated based on the formula: $2^{-\Delta\Delta Ct}$, carried on by CFX96 Touch Real-Time PCR Detection System. The β-actin gene was applied as a balanced control. The sequences of primers using in the qRT-PCR were as follow: Piwil2 (forward: 5'-TCTATGGGGCCATCAAGAAG-3', reverse: 5'-CCATCCCGATCACCATTAAC-3'); β-actin (forward: 5'-CACCCAGCACAATGAAGAT-3', reverse: 5'-CAAATAAAGCCATGCCAAT-3') and TP53(forward: 5'-AGAAAACCTACCAGGGCAGC-3' reverse: 5'-ACCATCGCTATCTGAGCAGC-3').

Immunoblotting was performed according to the protocol as below. Protein extraction of adhere cells was performed as described by Gierke *et al.* [23]. Briefly, cells were cultured in free-serum starvation for 24 hours to detect the level of protein phosphorylation and the total protein concentration was determined in accordance with the Pierce BCA Protein Assay Kit protocol. Thirty micrograms of protein were loaded into each lane, and separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After separated proteins were electrophoretically transferred onto polyvinylidene difluoride (PVDF) membrane, blocked with 5% skimmed milk for 30 minutes, and then incubated with the primary antibodies against Piwil2 (1:400), β-actin (1:1500), Erk1/2 (1:800), JNK (1:1000), p38 (1:800), MMP-9 (1:500 dilution), phospho-Erk1/2 (Thr202/Tyr204) (1:1500), phospho-JNK (Thr183/Tyr185) (1:1000), phospho-p38 (1:1000) overnight at 4 °C. Followed by incubation for two hours at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti-mouse or anti-rabbit for the host species of primary antibody). Immunoreactive protein bands were detected with enhanced chemiluminescence (ECL) substrate.

CCK-8 assay and flow cytometry (FCM) for cell proliferation

Cell growth assay was performed according to the manufacturer document of Cell Counting Kit-8. Briefly, 2×10^3 cells were seeded in every well of 96-well plate. The medium were removed on the next day, 200 ul of fresh medium containing 10% CCK-8 solution were added, and incubated for two hours. Last, plate was measured at 450 nm on a PT-3502PC microplate reader. The growth curve was generated with the GraphPad Prism 4 software. FCM was performed to detect the proportion of cells in different cell cycle phases. In detail, 1×10^5 adherent cells in the logarithmic phase were trypsinized, collected by centrifugation, washed twice with ice-cold PBS, and added 70% ethanol overnight at 4°C. These fixed cells were collected by centrifugation again, and 500 ul of PBS buffer contained 50 ug/ml propidium iodide (PI), 100ug/ml RNAase and 0.2% Triton X-100 were added to the eppendorf tube for 30 minutes' incubation at 4°C. Cell cycles were detected by FCM and analyzed with the CellQuest Pro Analysis software.

Migration and invasion assay

Transwell chambers were used for migration and invasion assays *in vitro*. Two thousand cells from the Piwil2-shRNA group and the Mock in the serum-free medium were seeded into the upper chamber, and the complete medium was added to the lower chamber as a chemoattractant. For invasion assay, the upper side of the filter was pre-coated with RPMI-1640 diluted BD matrigel. For migration assay, no matrigel was coated in the upper side. After 24 hours incubation for invasion and 12 hours for migration, respectively, cells from the upper side were removed using cotton swab carefully, and the cells adhered on the undersurface membrane were fixed in 4% formaldehyde and stained with crystal violet for 15 minutes. The cell number of each group was counted in the five random fields under a high power field (HPF, ×200). All assays were performed in triplicate.

Statistical analysis

All data were analyzed by SPSS 15.0. The data are shown as mean ± SEM. The chi-square test was used to evaluate the relationship between Piwil2 expression and clinicopathological parameter, and Independent-Samples T-test was used for comparison between the Piwil2-shRNA group and the Mock. A $p < 0.05$ was considered as statistically significant.

Results

Piwil2 expression differed in cervical cancer tissue and cancer cells

The expression of Piwil2 and TP53 mRNA was detected by qRT-PCR. Notably, the result showed that 27 of 32 patients who underwent radical trachelectomy had a higher level expression of Piwil2 than corresponding adjacent tissues (Figure 1A). Furthermore, Piwil2 in HPV16-positive patients (9/32) had an over-expression compared with that in HPV16-negative patients (23/32) (Figure 1A, $p < 0.05$). Interestingly, there was a significant decrease in TP53 expression following the overexpression of *Piwil2 i*n mRNA level (Figure 1 B).

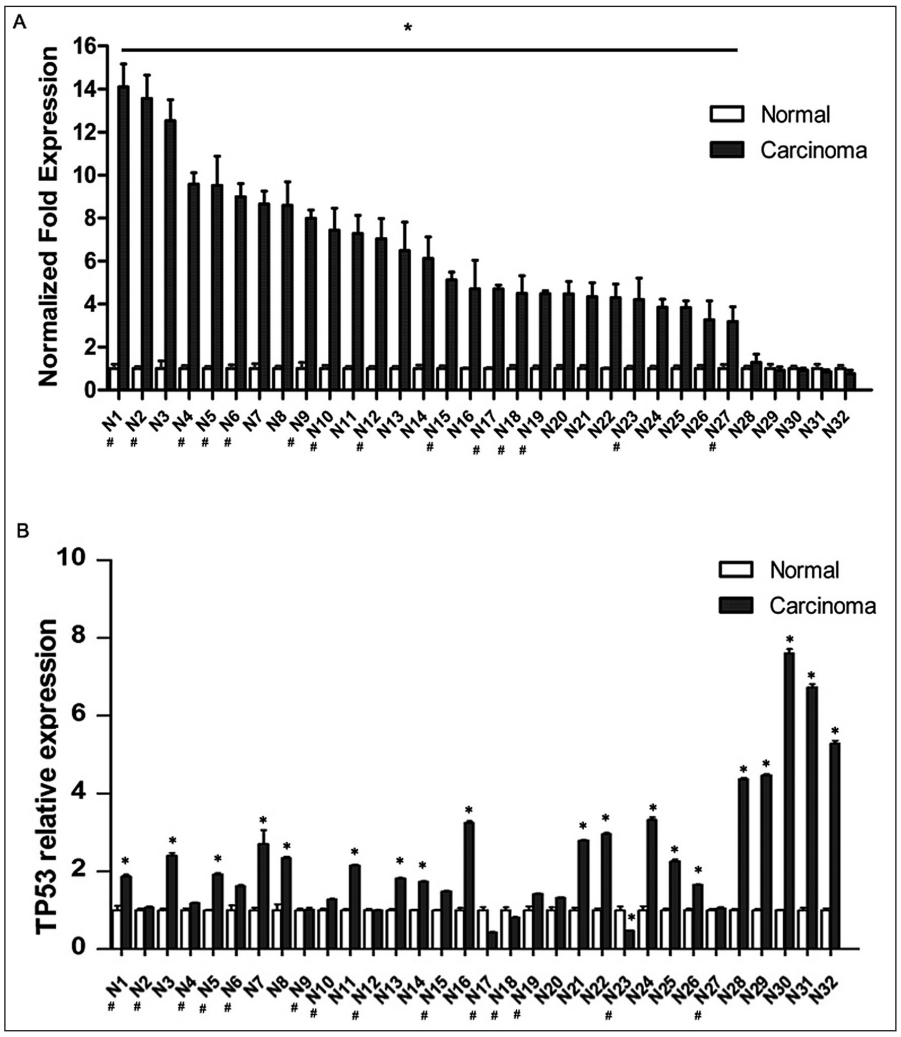

Figure 1. — Piwil2 gene overexpression in cervical cancer tissue. (A) In contrast to corresponding normal adjacent tissues, significantly high level expression of Piwil2 gene in 27 cases of 32 cervical cancer patients. (B) Expression of TP53 gene was lower in the HPV16-positive specimen than that of HPV16-negative. * indicates $p < 0.05$. # signifies HPV-positive.

SiHa are HPV16-positive human cervical carcinoma cell line, while HaCaT cells are HPV16-negative. qRT-PCR and Immunoblotting results revealed that SiHa cells highly expressed Piwil2, with a low level of TP53, while the immortal human keratinocyte HaCaT had a low expression of Piwil2 (Figure 2) and high level of TP53. Knockdown of Piwil2 in SiHa cells increased TP53 level (Figure 3A). The silencing efficient of Piwil2 gene was also evaluated in SiHa cell by qRT-PCR assay and Immunoblotting (Figure 3A and B). No difference of Piwil2 expression with Mock and untransfected SiHa cells, while siRNA of Piwil2 mediated down-regulation of Piwil2.

Piwil2 knockdown significantly suppressed SiHa cells growth via trapping cells in G0/G1 phase.

Piwil2 knockdown significantly suppressed the growth of SiHa cells. Compared with the mock group, the number of cells in G0/G1 phase significantly increased in sh-Piwil2 group ($p < 0.05$), while G2/M and S phase cells were significantly reduced ($p < 0.05$) (Table 1). The result indicated that downregulation of Piwil2 expression made SiHa cells stasis in G_0/G_1 phase. The CCK-8 assay was applied to evaluate the proliferation of transfected SiHa cells. The inhibition rate of cell proliferation was 29% at fourth day, 32% at fifth day, 40% at sixth day, and 41% at seventh day (Figure 4C).

Piwil2 knockdown inhibited SiHa cells migration and invasion.

The present authors assessed whether Piwil2 had an effect on cell migration and invasion. As shown in Figure 5A and B, comparing to Mock, downregulation of Piwil2 inhibited SiHa cells migration and invasion. It indicated that silencing Piwil2 gene reduced the ability of cell migration and invasion in the SiHa cell.

Piwil2 downregulated MMP-9 via regulating the MAPK signaling pathway in SiHa cells

Matrix metalloproteinases (MMPs), which degrade the extracellular matrix [24] having an essential role in inva-

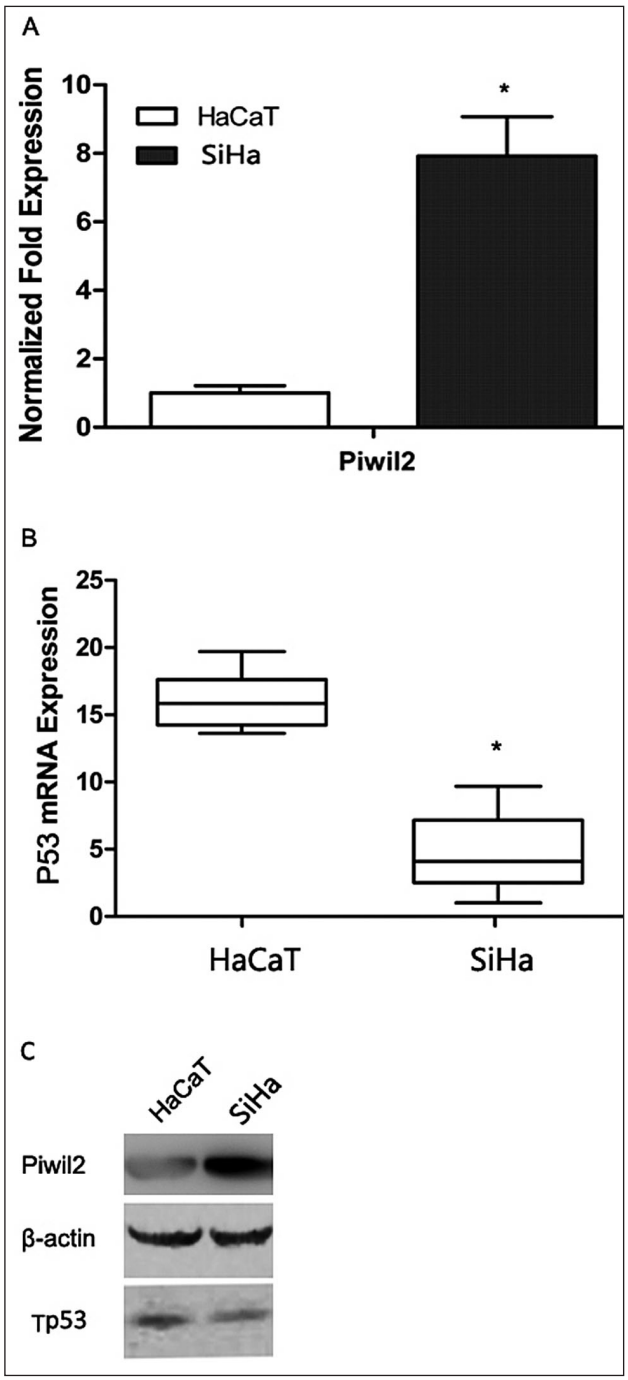

Figure 2. — Piwil2 expression in cervical cancer cell line. Compared with the immortal human keratinocyte HaCaT cells, Piwil2 mRNA (A) and protein (C) in HiSa (HPV16-positive) was in a higher level, flowing with lower TP53 level (B).

Table 1. — *Piwil2 knockdown significantly suppressed the growth of SiHa cells.*

Group	G0/G1	G2+M	S
SiHa -scrambled	40.32 ±1.23	6.31 ±1.03	53.36 ±1.23
SiHa -Sh-Piwil2	68.37 ±1.33[a]	6.45 ±0.43	26.18 ±0.76[a]

a: $p < 0.05$, compared mock group was a statistically significant difference.

sion and metastasis of tumor. The aforementioned results also demonstrated that Piwil2 downregulation effectively inhibited migration and invasion in SiHa cells. Therefore, the present authors evaluated the expression of MMPs by qRT-PCR assay and Immunoblotting (Figures 4C and D). Knockdown Piwil2 by shRNA rapidly decreased MMP-9 expression

MAPK signaling pathway was involved in a variety of functions in cell migration, invasion, and metastasis, and the stress-activated c-Jun NH2-terminal kinase (JNK), the p38 kinase (p38), and the extracellular signal-regulated kinase1/2 (ERK1/2) are the most important members of MAPK pathway. To the present authors' knowledge, activation of MAPK pathway could promote the transcription of MMPs. Therefore they investigated the expression of JNK, p38, ERK1/2 phosphorylation by immunoblotting after Piwil2 knockdown. Western-blot demonstrated that downregulating Piwil2 expression significantly inhibited protein phosphorylation of ERK1/2 and JNK (p-ERK1/2, p-JNK),while phosphorylation of p38 (p-p38) had no change (Figure 3A). p-JNK specific inhibitor SP600125 and p-ERK1/2 specific inhibitor PD98059 were applied to cervical cancer cell and resulted in down-regulation of MMP-9 protein (Figures 3B and C).

Additionally, in sh-Piwil2 cells, the present authors detected a lower expression of cyclin D1 and cyclin E which were downstream effectors of the ERK1/2 signaling pathway. By using p-ERK1/2 specific inhibitor PD98059 in SiHa cells, the expression of cyclin D1 and cyclin E remarkably decreased (Figure 3C). Bizari *et al.* studies also revealed that the high level expression of Cyclin D1 and cyclin E could prevent cell cycle arrested in the G_0/G_1 phase and accelerate cell out through S phase [25]. According to the above results, Piwil2 gene probably regulated cell proliferation via the ERK1/2 pathway.

Discussion

Piwi-like RNA-mediated gene silencing 2 (Piwil2) belongs to Ago/Piwi family proteins, which are comprised of Piwil1/Hiwi, Piwil2/Hili, Piwil3 and Piwil4/Hiwi2. Piwil2 serves as the candidate oncogene and was involved in genital tumors, such as ovarian and endometrial cancer [26]. Increasing evidences show overexpression of Piwil2 in several type of cancers, and correlation with tumorigenicity [13-17]. He *et al.* found that Piwil2 gene was expressed in various stages of cervical neoplasia, and was an essential supplement to screening for HPV16-infected patients with cervical cancer [14], which was consistent with the present study. In this study, the authors investigated that Piwil2 overexpressed in cervical cancer tissue (27/32) as compared to adjacent normal tissue, weakly expressed in human keratinocyte HaCaT in contrast to cervical cancer cell line SiHa cells.

The mRNA expression level of Piwil2 was significant correlated with HPV16 infection. Consistently, there is in-

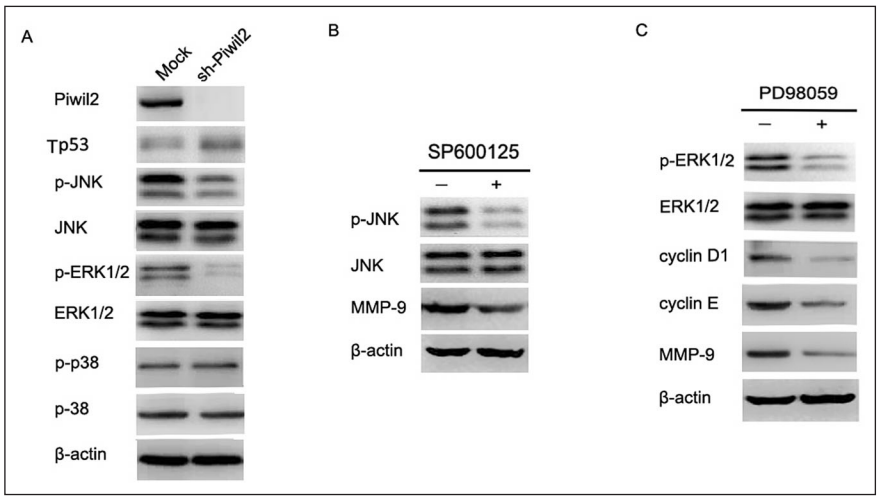

Figure 3. — Piwil2 gene downregulated MMP-9 transcription activities via regulating the MAPK signaling pathway in the SiHa cell. (A) Deleting Piwil2 gene significantly decreased p-ERK1/2, p-JNK protein in sh-Piwil2 group compared with mock group, but no significant alternation in p-p38. (B) p-JNK specific inhibitor SP600125 resulted in downregulation of MMP-9 protein in cervical cancer cell. (C) p-ERK1/2 specific inhibitor PD98059 induced downregulation of cyclin D1, cyclin E and MMP-9 protein in cervical cancer cell. * indicates $p < 0.05$.

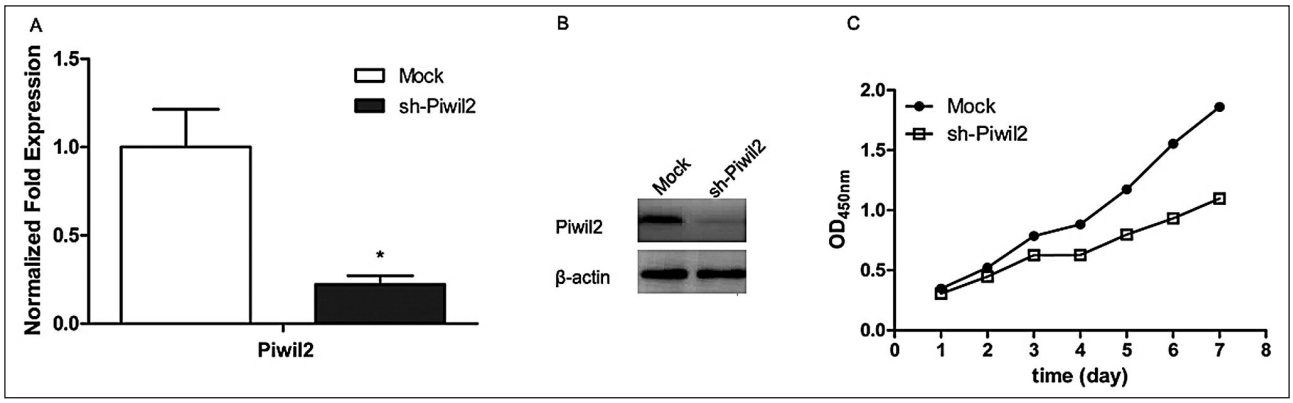

Figure 4. — Specific shRNA knockdown Piwil2 expression in the cervical cancer cell line. (A, B) Compared with scrambled shRNA group (Mock), Piwil2-targeted shRNA group (sh-Piwil2) significant downexpression of Piwil2 mRNA and protein in SiHa cells, silencing efficient was detected by qRT-PCR and immunoblotting assay. (C) The CCK-8 assay was performed to evaluate the proliferation of in Mock and sh-Piwil2 group. The sh-Piwil2 significantly inhibited cell proliferation in SiHa cells. * indicates $p < 0.05$.

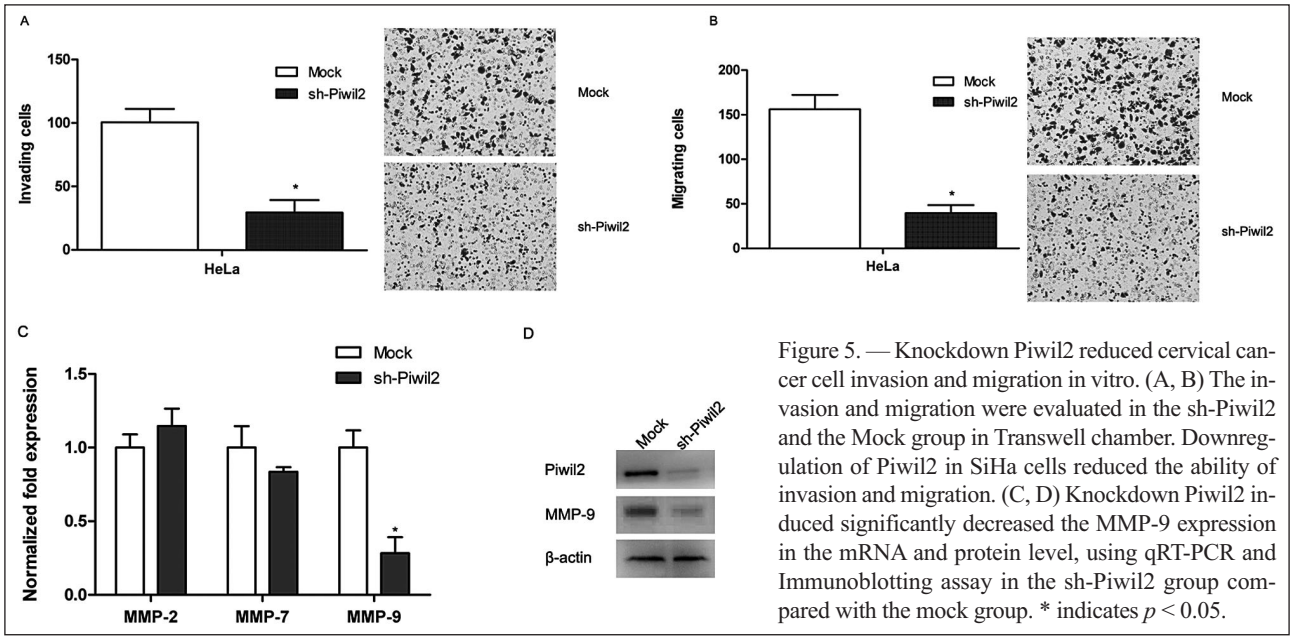

Figure 5. — Knockdown Piwil2 reduced cervical cancer cell invasion and migration in vitro. (A, B) The invasion and migration were evaluated in the sh-Piwil2 and the Mock group in Transwell chamber. Downregulation of Piwil2 in SiHa cells reduced the ability of invasion and migration. (C, D) Knockdown Piwil2 induced significantly decreased the MMP-9 expression in the mRNA and protein level, using qRT-PCR and Immunoblotting assay in the sh-Piwil2 group compared with the mock group. * indicates $p < 0.05$.

creasing evidence demonstrated that HPV is a risk factor in cervical cancer; about 70.9% of cervical cancers are attributed to HPV types 16 and/or 18 worldwide [4, 27, 28]. However, how HPV16 and Piwil2 interact mutually in the cervical cancer remains unclear. Notably, the present result showed that HPV-positive tumor tissues get a higher level of Piwil2 than negative ones. It has been proved HPV16 infected cells lost TP53 activation gradually, which may be responsible for the anti-apoptosis and immortalization of cells [29, 30]. Interestingly, the present authors investigated TP53 level of HPV-positive and negative tumor tissues, finding that high level of Piwil2 always accompany with a low level of TP53, however, a low level of Piwil2 did not signify a high level of TP53. Similar pattern was shown in HPV16-positive SiHa cells but not in HPV16-negative HaCaT cells. Since Piwil2 was reported to suppress TP53 by regulating STAT3 signaling pathway and plays a role in anti-apoptosis in tumor cells [31]. It was reasonable to believe that HPV-positive may be responsible for increasing Piwil2, which downregulated TP53. However, further studies will be needed for a comprehensive understanding.

Invasion and metastasis are included in biological behavior of malignancies, which are also important factors related to prognosis and survival rate of patients with the result of interaction of multiple factors, and also involved in many biological events, including cell adhesion, angiogenesis, and growth [32]. The present authors found that Piwil2 expression was closely related to tumor migration and invasion, and making Piwil2 gene silence in SiHa cells, significantly inhibited the ability of cell migration and invasion. Piwil2 was also capable in regulating the expression of MMP-9. In SiHa cells, both mRNA and protein expression of MMP-9 rapidly decreased in the sh-Piwil2 group when compared with the mock group. MMP family members are required for increasing motility of the epithelial cancer cells [33]. Overexpression of MMP-9 plays an important role in malignant tumors progression and development [34-36]. It has been reported in several studies that upregulation of MMP-2 and MMP-9 in cervical neoplasias were significantly correlated with poor prognosis [37, 38]. Moreover, it is the MAPK pathway that plays crucial role in tumorigenesis and development [39-44]. JNK, p38, and ERK1/2 are three most important members in the MAPK signaling pathway, which are involved in the regulation of cell growth, differentiation, migration, invasion, metastasis, inflammation, and cell apoptosis process [45, 46]. When JNK signaling pathway is activated by upstream signals, translocation of JNK from the cytoplasm to the nucleus induces the nuclear transcription factor c-Jun N-terminal phosphorylation of 63 and 73 serine residue [47]. The transcription factor c-Jun is phosphorylated and the c-Fos and activator protein-1 (AP-1) complex is formed, which regulates downstream genes [48]. ERK1/2 inhibitor PD098059 integrated with liposomes and significantly reduced invasive ability of oral cancer, which was associated with a decreased ERK1/2 activity and MMP-9 downregulation [49]. These reports are consistent with the present study, in which downregulation of piwil2 expression inhibited phosphorylation of ERK 1/2 and JNK, and when p-JNK specific inhibitor SP600125 and p-ERK1/2 specific inhibitor PD98059 were respectively applied to cervical cancer cell, and MMP-9 protein expression was significantly downregulated. The above result suggested that Piwil2 could regulate the MMP-9 activity via ERK1/2 and JNK pathway, but not p38. Furthermore, cyclin D1 and cyclin E were also involved in Piwil2-induced cell proliferation. It has been reported that CDK2 and CDK4 are two important cyclin-dependent kinases, which can interact with cyclin D1 and cyclin E to form a complex leading to uncontrolled cell cycle regulation and cell proliferation [50].

In conclusion, Piwil2 gene was identified to be highly expressed in cervical cancer tissues and SiHa cells. HPV-positive tissues with a low level of TP53 while highly expressed Piwil2, indicating HPV-16 infection may be responsible for the increase of Piwil2, which downregulated TP53, in HPV-positive tumors. In addition, Piwil2 was involved in regulating cell proliferation via regulating cyclin D1 and cyclin E, and it also increases invasion of cervical cancer cell by regulating MMP-9 via MAPK pathway. Finally, the present study provides evidence that Piwil2 was an essential supplement to screening for HPV16-infected patients with cervical cancer and should be considered as a potential target for therapy.

Acknowledgments

This study was supported by the Science and Technology Planning Project of Gansu Province (No. 1204FKCA174).

References

[1] Siegel R., DeSantis C., Virgo K., Stein K., Mariotto A., Smith T., *et al.*, "Cancer treatment and survivorship statistics, 2012". *CA Cancer J. Clin.*, 2012, *62*, 220.

[2] Ferlay J., Shin H.R., Bray F., Forman D., Mathers C., Parkin D.M.: "Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008". *Int. J. Cancer*, 2010, *127*, 2893.

[3] Scatchard K., Forrest J.L., Flubacher M., Cornes P., Williams C.: "Chemotherapy for metastatic and recurrent cervical cancer". *Cochrane Database Syst. Rev.*, 2012, *10*, CD006469.

[4] Bosch, F.X., M.M. Manos, N. Munoz, M. Sherman, A.M. Jansen, J. Peto, et al., "Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. International biological study on cervical cancer (IBSCC) Study Group". *J. Natl., Cancer Inst.*, 1995, *87*, 796.

[5] de Vet H.C., van Leeuwen F.E.: "Dietary guidelines for cancer prevention: the etiology of a confused debate". *Nutr. Cancer*, 1986, *8*, 223.

[6] Tindle R.W.: "Immune evasion in human papillomavirus-associated cervical cancer". *Nat. Rev. Cancer*, 2002, *2*, 59.

[7] Syrjanen K.J.: "Spontaneous evolution of intraepithelial lesions according to the grade and type of the implicated human papillomavirus (HPV)". *Eur. J. Obstet. Gynecol. Reprod. Biol.*, 1996, *65*, 45.

[8] Burger R.A., Monk B.J., Kurosaki T., Anton-Culver H., Vasilev S.A., Berman M.L., Wilczynski S.P.: "Human papillomavirus type 18: association with poor prognosis in early stage cervical cancer". *J. Natl. Cancer Inst.*, 1996, *88*, 1361.

[9] Soderlund-Strand A., Kjellberg L., Dillner J.: "Human papillomavirus type-specific persistence and recurrence after treatment for cervical dysplasia". *J. Med. Virol.*, 2014, *447*, 300.

[10] Inaba K., Nagasaka K., Kawana K., Arimoto T., Matsumoto Y., Tsuruga T., *et al*.: "High-risk human papillomavirus correlates with recurrence after laser ablation for treatment of patients with cervical intraepithelial neoplasia 3: A long-term follow-up retrospective study". *J. Obstet. Gynaecol. Res.*, 2014, *40*, 554.

[11] Sasaki T., Shiohama A., Minoshima S., Shimizu N.: "Identification of eight members of the Argonaute family in the human genome small star, filled". *Genomics*, 2003, *82*, 323.

[12] Kuramochi-Miyagawa S,. Kimura T., Ijiri T.W., Isobe T., Asada N., Fujita Y., *et al.*: "Mili, a mammalian member of piwi family gene, is essential for spermatogenesis". *Development*, 2004, *131*, 839.

[13] Liu X., Sun Y., Guo J., Ma H., Li J., Dong B., *et al.*: "Expression of hiwi gene in human gastric cancer was associated with proliferation of cancer cells". *Int. J. Cancer*, 2006, *118*, 1922.

[14] He G., Chen L., Ye Y., Xiao Y., Hua K., Jarjoura D., *et al.*: "Piwil2 expressed in various stages of cervical neoplasia is a potential complementary marker for p16". *Am. J. Transl. Res.*, 2010, *2*, 156.

[15] Liu J.J., Shen R., Chen L., Ye Y., He G., Hua K., *et al.*: "Piwil2 is expressed in various stages of breast cancers and has the potential to be used as a novel biomarker". *Int. J. Clin. Exp. Pathol.*, 2010, *3*, 328.

[16] Yin D.T., Li H.Q., Wang Y.F., Cao S.L., Zhou Y.B., Zheng L.Y., *et al.*: "Expression of Piwil2 and its relationship with tumor invasion and metastasis in papillary thyroid carcinoma". *Zhonghua Er Bi Yan Hou Tou Jing Wai Ke Za Zhi*, 2011, *46*, 237.

[17] Li D., Sun X., Yan D., Huang J., Luo Q., Tang H., Peng Z.: "Piwil2 modulates the proliferation and metastasis of colon cancer via regulation of matrix metallopeptidase 9 transcriptional activity". *Exp. Biol. Med. (Maywood)*, 2012, *237*, 1231.

[18] Lee J.H., Jung C., Javadian-Elyaderani P., Schweyer S., Schutte D., Shoukier M., *et al.*: "Pathways of proliferation and antiapoptosis driven in breast cancer stem cells by stem cell protein piwil2". *Cancer Res.*, 2010, *70*, 4569.

[19] Oh S.J., Kim S.M., Kim Y.O., Chang H.K.: "Clinicopathologic Implications of PIWIL2 Expression in Colorectal Cancer". *Korean J. Pathol.*, 2012, *46*, 318.

[20] Greither T., Koser F., Kappler M., Bache M., Lautenschlager C., Gobel S., et al.: "Expression of human Piwi-like genes is associated with prognosis for soft tissue sarcoma patients". *BMC Cancer*, 2012, *12*, 272.

[21] Feng D., Peng C., Li C., Zhou Y., Li M., Ling B., *et al.*: "Identification and characterization of cancer stem-like cells from primary carcinoma of the cervix uteri". *Oncol. Rep.*, 2009, *22*, 1129.

[22] Choo K.B., Cheung W.F., Liew L.N., Lee H.H., Han S.H.: "Presence of catenated human papillomavirus type 16 episomes in a cervical carcinoma cell line". *J. Virol.*, 1989, *63*, 782.

[23] Gierke P., Zhao C., Brackmann M., Linke B., Heinemann U., Braunewell K.H.: "Expression analysis of members of the neuronal calcium sensor protein family: combining bioinformatics and Western blot analysis". *Biochem. Biophys. Res. Commun.*, 2004, *323*, 38.

[24] Maddirela D.R., Kesanakurti D. , Gujrati M., Rao J.S.: "MMP-2 suppression abrogates irradiation-induced microtubule formation in endothelial cells by inhibiting alphavbeta3-mediated SDF-1/CXCR4 signaling". *Int. J. Oncol.*, 2013, *42*, 1279.

[25] Bizari, L., Borim A.A., Leite K.R., Goncalves Fde T., Cury P.M., Tajara E.H., Silva A.E.: "Alterations of the CCND1 and HER-2/neu (ERBB2) proteins in esophageal and gastric cancers". *Cancer Genet. Cytogenet.*, 2006, *165*, 41.

[26] Lee J.H., Schutte D., Wulf G., Fuzesi L., Radzun H.J., Schweyer S., *et al.*: "Stem-cell protein Piwil2 is widely expressed in tumors and inhibits apoptosis through activation of Stat3/Bcl-XL pathway". *Hum. Mol. Genet.*, 2006, *15*, 201.

[27] Baussano I., Elfstrom K.M., Lazzarato F., Gillio-Tos A., De Marco L., Carozzi F., *et al.*: "Type-specific human papillomavirus biological features: validated model-based estimates". *PLoS One*, 2013, *29*, 81171.

[28] de Sanjose S., Serrano B., Castellsague X., Brotons M., Muñoz J., Bruni L., Bosch F.X.: "Human papillomavirus (HPV) and related cancers in the Global Alliance for Vaccines and Immunization (GAVI) countries. A WHO/ICO HPV Information Centre Report". *Vaccine*, 2012, *30*, D1.

[29] Lorenz L.D., Rivera Cardona J., Lambert P.F.: "Inactivation of TP53 rescues the maintenance of high risk HPV DNA genomes deficient in expression of E6". *PLoS Pathog.*, 2013, *9*, e1003717.

[30] Geiger T., Levitzki A.: "Loss of robustness and addiction to IGF1 during early keratinocyte transformation by human papilloma virus 16". *PLoS One*, 200,m *2*, e605.

[31] Lu Y., Zhang K., Li C., Yao Y., Tao D., Liu Y., *et al.*: "Piwil2 suppresses TP53 by inducing phosphorylation of signal transducer and activator of transcription 3 in tumor cells". *PLoS One*, 2012, *7*, e30999.

[32] Tarin D., Matsumura Y.: "Recent advances in the study of tumour invasion and metastasis". *J. Clin. Pathol.*, 1994, *47*, 385.

[33] Pilcher B.K., Dumin J.A., Sudbeck B.D., Krane S.M., Welgus H.G., Parks W.C.: "The activity of collagenase-1 is required for keratinocyte migration on a type I collagen matrix". *J. Cell. Biol.*, 1997, *137*, 1445.

[34] Westermarck J., Kahari V.M.: "Regulation of matrix metalloproteinase expression in tumor invasion". *FASEB J.*, 1999, *13*, 781.

[35] Coussens L.M., Tinkle C.L., Hanahan D., Werb Z.: "MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis". *Cell*, 2000, *103*, 481.

[36] Bergers G., Brekken R., McMahon G., Vu T.H., Itoh T., Tamaki K., *et al.*: "Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis". *Nat. Cell. Biol.*, 2000, *2*, 737-44.

[37] Nuovo G.J., MacConnell P.B., Simsir A., Valea F., French D.L.: "Correlation of the in situ detection of polymerase chain reaction-amplified metalloproteinase complementary DNAs and their inhibitors with prognosis in cervical carcinoma". *Cancer Res.*, 1995, *55*, 267.

[38] Chebassier N., Leroy S., Tenaud I., Knol A.C., Dreno B.: "Overexpression of MMP-2 and MMP-9 in squamous cell carcinomas of immunosuppressed patients". *Arch. Dermatol. Res.*, 2002, *294*, 124.

[39] Liang B., Wang S., Zhu X.G., Yu Y.X., Cui Z.R., Yu Y.Z.: "Increased expression of mitogen-activated protein kinase and its upstream regulating signal in human gastric cancer". *World J. Gastroenterol.*, 2005, *11*, 623.

[40] Guo X., Ma N., Wang J., Song J., Bu X., Cheng Y., *et al.*: "Increased p38-MAPK is responsible for chemotherapy resistance in human gastric cancer cells". *BMC Cancer*, 2008, *8*, 375.

[41] Whyte J., Bergin O., Bianchi A., McNally S., Martin F.: "Key signalling nodes in mammary gland development and cancer. Mitogen-activated protein kinase signalling in experimental models of breast cancer progression and in mammary gland development". *Breast Cancer Res.*, 2009, *11*, 209.

[42] Schubbert S., Shannon K., Bollag G.: "Hyperactive Ras in developmental disorders and cancer". *Nat. Rev. Cancer*, 2007, *7*, 295.

[43] Zhang C., Zhu H., Yang X., Lou J., Zhu D., Lu W., et al.: "TP53 and p38 MAPK pathways are involved in MONCPT-induced cell cycle G2/M arrest in human non-small cell lung cancer A549". *J. Cancer Res. Clin. Oncol.*, 2010, *136*, 437.

[44] Uzgare A.R., Kaplan P.J., Greenberg N.M.: "Differential expression and/or activation of P38MAPK, erk1/2, and jnk during the initiation and progression of prostate cancer". *Prostate*, 2003, *55*, 128.

[45] Reddy K.B., Nabha S.M., Atanaskova N.: "Role of MAP kinase in tumor progression and invasion". *Cancer Metastasis Rev.*, 2003, *22*, 395.

[46] Wagner E.F., Nebreda A.R.: "Signal integration by JNK and p38 MAPK pathways in cancer development". *Nat. Rev. Cancer*, 2009, *9*, 537.

[47] Minden A., Lin A., Claret F.X., Abo A., Karin M.: "Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs". *Cell*, 1995, *81*, 1147.

[48] Oktay M., Wary K.K., Dans M., Birge R.B., Giancotti F.G.: "Integrin-mediated activation of focal adhesion kinase is required for signaling to Jun NH2-terminal kinase and progression through the G1 phase of the cell cycle". *J. Cell. Biol.*, 1999, *145*, 1461.

[49] Simon C., Hicks M.J., Nemechek A.J., Mehta R., O'Malley B.W. Jr., Goepfert H., *et al.*: "PD 098059, an inhibitor of ERK1 activation, attenuates the in vivo invasiveness of head and neck squamous cell carcinoma". *Br. J. Cancer*, 1999, *80*, 1412.

[50] Hubalek M.M., Widschwendter A., Erdel M., Gschwendtner A., Fiegl H.M., Muller H.M., *et al.*: "Cyclin E dysregulation and chromosomal instability in endometrial cancer". *Oncogene*, 2004, *23*, 4187.

Address reprint requests to:
L. WANG, M.D.
Department of Gynaecology and Obstetrics
General Hospital of Lanzhou Military Region
98 Xiaoxihu West Street
Lanzhou 730050 (China)
e-mail: linowang@163.com